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THE EMBO JOURNAL

Goliath family E3 ligases regulate the recycling endosome pathway via VAMP3 ubiquitylation

Yasuo Yamazaki[1], Christina Schönherr[1,3], Gaurav K Varshney[2,3], Murat Dogru[1], Bengt Hallberg[1] and Ruth H Palmer[1,*]

[1]Department of Molecular Biology, Umeå University, Umeå, Sweden and [2]Developmental Genomics Section, Genome Technology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

Diverse cellular processes depend on endocytosis, intracellular vesicle trafficking, sorting and exocytosis, processes regulated post-transcriptionally by modifications such as phosphorylation and ubiquitylation. In addition to sorting to the lysosome, cargo is recycled to the plasma membrane via recycling endosomes. Here, we describe a role of the *goliath* gene family of protease-associated (PA) domain E3 ligases in regulating recycling endosome trafficking. The two *Drosophila* members of this family—Goliath and Godzilla[CG10277]—are located on endosomes, and both ectopic expression and loss-of-function lead to the accumulation of Rab5-positive giant endosomes. Furthermore, the human homologue RNF167 exhibits similar behaviour. We show that the soluble N-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) protein VAMP3 is a target of these ubiquitin ligases, and that recycling endosome trafficking is abrogated in response to their activity. Furthermore, mutation of the Godzilla ubiquitylation target lysines on VAMP3 abrogates the formation of enlarged endosomes induced by either Godzilla or RNF167. Thus, Goliath ubiquitin ligases play a novel role in regulating recycling endosome trafficking via ubiquitylation of the VAMP3 SNARE protein.

Introduction

Many important cellular processes such as cell migration (Marin *et al*, 2010) and neurotransmitter release depend on intracellular vesicle trafficking, sorting and exocytosis (Sudhof and Rothman, 2009). While endocytosis provides a switching-off mechanism for cellular signalling by removing activated receptors from the cell surface, more recent studies suggest signalling from endocytosed receptors is still active and may even be amplified in the endocytic pathway (von Zastrow and Sorkin, 2007; Mazot *et al*, 2011). Endocytic vesicles are can be roughly divided into early endosomes, late endosomes and recycling endosomes (Platta and Stenmark, 2011) and trafficking is regulated post-transcriptionally by modifications such as phosphorylation and ubiquitylation (Piper and Lehner, 2011; Haglund and Dikic, 2012). In addition to sorting to the lysosome, an estimated two thirds of internalized receptors are recycled back to the plasma membrane via recycling endosomes from early endosome (Steinman *et al*, 1983; Schmid *et al*, 1988; Mayor *et al*, 1993). Generally, vesicles are transported along actin filaments or microtubules prior to docking and fusion with an acceptor membrane. The latter step is largely regulated by soluble N-ethylmaleimide-sensitive fusion attachment protein receptors (SNARES) (Proux-Gillardeaux *et al*, 2005; Tayeb *et al*, 2005; Veale *et al*, 2010; Zylbersztejn and Galli, 2011).

SNARE proteins are characterized by a conserved stretch of 60–70 amino acids known as the SNARE motif, and most have a carboxyl-terminal transmembrane domain connected by a short linker sequence (Jahn and Scheller, 2006). SNAREs form a complex with two other specific partner SNAREs, leading to specific regulation of membrane fusion. When appropriate sets of SNAREs combined, they form a stable complex consisting of coiled-coil four-helix bundle. While this complex formation is known to be sufficient to drive membrane fusion *in vitro*, post-transcriptional modifications such as phosphorylation and palmitoylation have also shown to regulate the function and localization of SNAREs (Snyder *et al*, 2006). A large number of SNARE mammalian proteins have been characterized, including the endosomal SNARE VAMP3/Cellubrevin, which is implicated in recycling of membrane receptors from endosome to the plasma membrane (McMahon *et al*, 1993; Galli *et al*, 1994). In *Drosophila*, two VAMP3/Cellubrevin homologous SNAREs are encoded in the genome; n-Syb is neuronally specific and associated primarily with synaptic vesicles (DiAntonio *et al*, 1993), while the closely related Syb/dVAMP is more generally expressed (Chin *et al*, 1993; DiAntonio *et al*, 1993). These two proteins appear to be functionally interchangeable not only with one another, but also with mammalian VAMP3/ cellubrevin (Bhattacharya *et al*, 2002).

Many studies have established the importance of ubiquitylation in controlling receptor endocytosis and endosomal sorting, and a number of molecular mechanisms and components regulating these processes have been described (Piper and Lehner, 2011; Haglund and Dikic, 2012). Ubiquitylation is considered to be an important signal in regulation of intracellular traficking, offering advantages such as the ability to regulate the modification by removal deubiquitinating enzymes, and the creation of binding sites for ubiquitin binding domain containing proteins. An estimated 300 human genes encode RING domain proteins in keeping with the already comprehensive repertoire of tasks that are undertaken by this class of proteins in the cell

*Corresponding author. RH Palmer, Department of Molecular Biology, Umeå University, Umeå 90187, Sweden. Tel.: +46 90 785 6798; Fax: +46 90 778 007; E-mail: ruth.palmer@ucmp.umu.se
[3]Joint second authors.
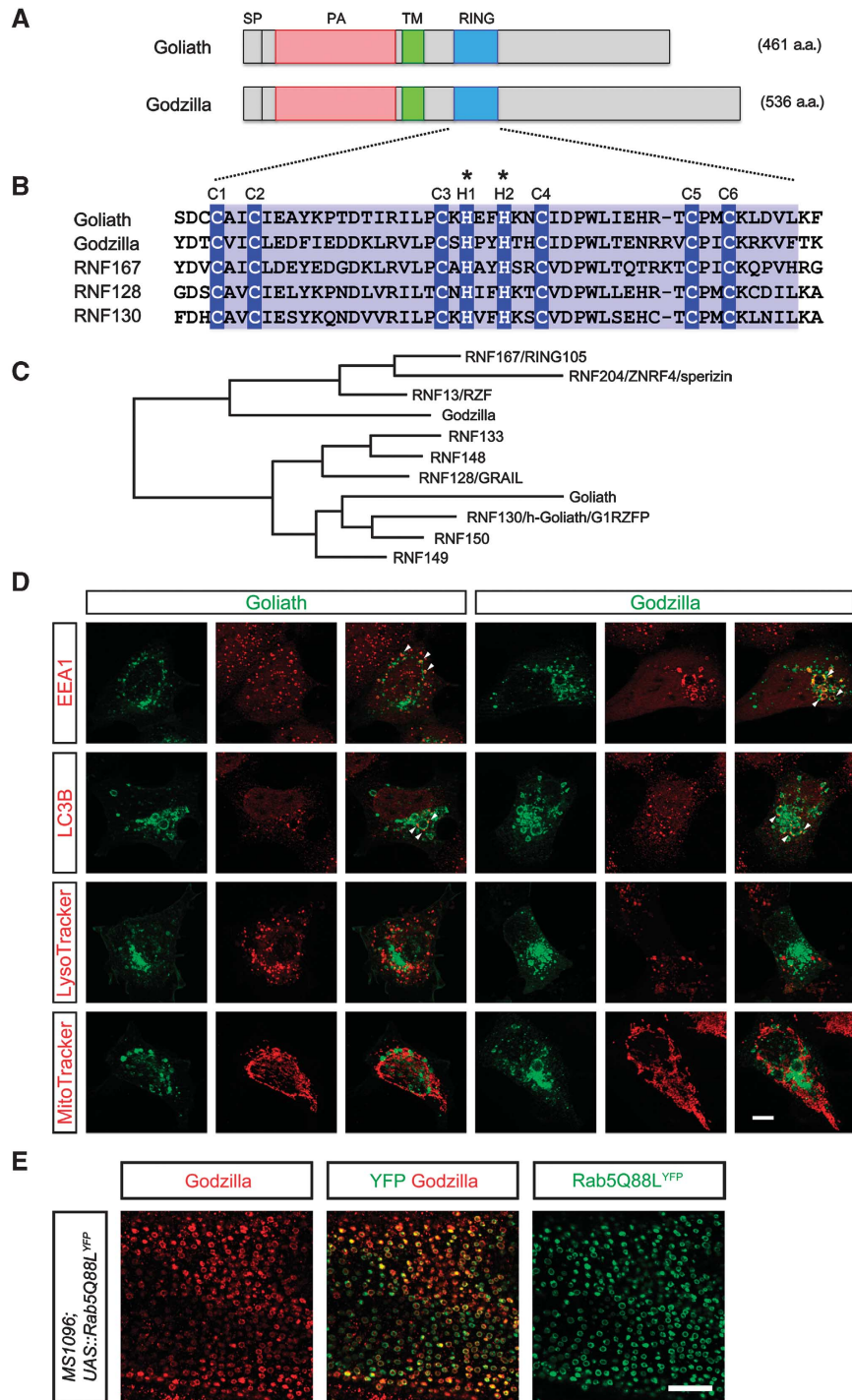
Figure 1 Goliath and Godzilla encode endosomally localized PA-TM-RING E3 ligases. (**A**) Schematic domain structure of *Drosophila* Goliath and Godzilla. SP, signal peptide; PA, protease-associated domain; TM, transmembrane, and RING domain. (**B**) Sequence alignment of related RING domains. RNF167, RNF128 (also known as GRAIL) and RNF130 (also known as hGoliath) are human sequences. The RING domain (*blue*) contains six conserved Cys (C1–C6) and two His (H1 and H2) residues, which coordinate Zn^{2+} and are important for ligase activity. *For ligase-dead mutants, two His residues were substituted to Arg, corresponding to His^{323} and His^{326} in Goliath and His^{255} and His^{258} in Godzilla. (**C**) Phylogenic relationship of PA-TM-RING E3-ligase genes. (**D**) Subcellular localization of Goliath and Godzilla. Goliath-C-GFP (*green*, left) or Godzilla-C-GFP (*green*, right) transfected HEK293 cells were co-stained with cell organelle markers (*red*). EEA1, early endosome; LC3B, autophagosome; LysoTracker, lysosome; MitoTracker, mitochondria. Goliath-C-GFP and Godzilla-C-GFP-induced enlarged vesicle-like structures colocalize with EEA1 (*arrowheads*), and partially with LC3B (*arrowheads*); indicating that Goliath and Godzilla localize on an enlarged endosome membranes, but not on lysosomes or mitochondria. Bar 10 μm. The results shown here are typical images from at least four independent experiments. (**E**) Endogenous *Drosophila* protein is localized on endosomes *in vivo*. Endosomes were enlarged by expression of constitutively active Rab5 (Rab5Q88L^{YFP}) in the *Drosophila* wing disc with *MS1096-Gal4*. Endogenous Godzilla is accumulated on the resultant enlarged Rab5-positive endosomes. The results shown here are typical images from at least four independent experiments.

(Li *et al*, 2008). The *Drosophila melanogaster* Goliath PA domain E3 ligase has been described as expressed in the mesoderm (Bouchard and Cote, 1993), although further analysis of Goliath function in the fruitfly has not been reported. In fact, in *Drosophila* the *goliath* gene family comprises two members—*goliath* and *godzilla*[CG10277]. *Xenopus* GREUL1 (Goliath-related E3 ubiquitin ligase1) has been reported to function in the development of the anterior ectoderm (Borchers *et al*, 2002). The mouse orthologue G1RP (G1-related protein) was isolated from 32Dcl3 myeloblastic cells and has shown to be induced in apoptosis in response to IL-3 depletion (Baker and Reddy, 2000). Although a human homologue of Goliath, h-Goliath, has been described as expressed in normal and pathological haematopoietic cells, its function remains unknown (Guais *et al*, 2006). Most functional information comes from studies of mammalian GRAIL/RNF128, which plays a role in immune tolerance (Mueller, 2004).

In this work, we describe the *goliath* gene family of PA domain E3 ligases as key regulators of the recycling endosome pathway. We show that the two *Drosophila* members of this family—Goliath and Godzilla[CG10277]—exhibit an endosomal location, and that both gain- and loss-of-function lead to the accumulation of Rab5-positive giant endosomes, implying a regulatory role in endosomal processes. Furthermore, expression of RNF167, a human member of the Goliath E3-ligase family, exhibits similar behaviour. Here, we identify the SNARE protein, VAMP3, as a prominent ubiquitylation target of these ubiquitin ligases. In agreement with VAMP3 being a target for Goliath family ubiquitin ligases, we show that recycling endosome trafficking is abrogated in response to their activity. Furthermore, loss of VAMP3 reverses both the Godzilla and RNF167 induced Rab5-positive giant endosome phenotype. In conclusion, our data suggest a novel function for the Goliath ubiquitin ligases in regulating recycling endosome trafficking *via* ubiquitylation of the VAMP3 SNARE protein.

Results

Goliath and Godzilla encode endosomally localized PA-TM-RING domain ubiquitin ligases.

In *Drosophila melanogaster*, the *goliath* gene family comprises two members—*goliath* and *godzilla*[CG10277]. *Goliath* is expressed in muscles (Artero *et al*, 2003), while the related *godzilla* exhibits a more general expression pattern. Together, they encode a family of ubiquitin ligases in the fly that exhibit a distinct domain architecture: a signal peptide (a.a. 1–25), a PA domain (a.a. 98–207 Goliath; a.a. 53–155 Godzilla) (Mahon and Bateman, 2000), a transmembrane domain (a.a. 235–257 Goliath; a.a. 173–195 Godzilla) and a RING

domain (a.a. 303–343 Goliath; a.a. 235–276 Godzilla) (Deshaies and Joazeiro, 2009; Figure 1A). The unusual presence of a transmembrane domain (a.a. 235–257 Goliath; a.a. 173–195 Godzilla) suggests that Goliath and Godzilla are membrane bound proteins. The PA domain is a highly conserved motif found in members of the protease superfamily and displays 32.3% identity between Goliath and Godzilla (Mahon and Bateman, 2000). Goliath also contains a C3H2C3-type RING domain, which is well-conserved among homologues (Figure 1B). The RING family of E3 ligases is the largest E3-ligase family, containing C3H2C3 or C3HC4 RING domains (Deshaies and Joazeiro, 2009). This domain contains four pairs of metal binding residues with a characteristic linear sequence of Cys-X2-Cys-X9-39-Cys-X1-3His-X2-3Cys/HisX2-Cys-X4-48-Cys-X2-Cys, where X can be any amino acid although there are distinct preferences for particular types of amino acid at a particular position (Lovering *et al*, 1993). The RING domain of Godzilla is 51% identical to that of Goliath suggesting an important and conserved functional role. This consensus is conserved across species in the larger Goliath family (Figure 1B). In contrast to two members in *Drosophila*, there are nine predicted homologues in humans (Figure 1C), including GRAIL/RNF128, which plays a role in immune tolerance (Mueller, 2004).

Drosophila goliath null mutants, in which all five coding exons were deleted, are viable and fertile. Given the restricted expression of *goliath* in embryonic muscles (Artero *et al*, 2003) we subsequently generated mutants in the locus of the more generally expressed related family member *godzilla*[CG10277] (Supplementary Figure S1). Mutants in this locus—*Δgodzilla*[CG10277]—are larval lethal. In an initial attempt to clarify the mechanism of function of the Goliath family ligases, we examined their subcellular localization. A significant amount of Godzilla clearly colocalizes with endosomal markers, such as EEA1, upon overexpression (Figure 1D; Supplementary Figure S2). While less extensive than that observed with Godzilla, overlap with EEA1 is also observed with the related Goliath (Figures 1D and 2A). In general, we noted that expression of Godzilla results in a stronger enlarged endosome phenotype than Goliath. This was also reflected in the more obvious loss of Mannose-6-Phosphate Receptor staining in cells expressing Godzilla, suggesting that Godzilla expression may lead to perturbations in the endosomal maturation process (Figure 2A). In agreement with these results, endogenous Godzilla protein in *Drosophila* tissues displays a vesicle localization overlapping with endosomes (Supplementary Figure S1E–G). To confirm the presence of endogenous Godzilla protein in the endosomal compartment, we expressed activated Rab5—Rab5Q88LYFP (Stenmark *et al*, 1994) to produce enlarged

Figure 2 Goliath and Godzilla ligase activity leads to the accumulation of Rab5-positive giant endosomes. (**A**) Goliath C-GFP, Godzilla-C-GFP or pCGFP was transfected into HEK293 cells, and stained with antibodies detecting early and late endosome marker proteins (EEA1 and M6PR, respectively). Expression of either Goliath or Godzilla resulted in significantly enlarged endosomes (>3 μm in diameter) as compared with control (∼0.4 μm). *Dotted box* shows an expanded view of each panel. (**B**) Rab5 colocalizes on giant vesicles induced by expression of Goliath and Godzilla. HEK293 cells were transfected with Goliath-C-GFP or Godzilla-C-GFP together with pEGFP-DsRed-Rab5. *Dotted box* shows an expanded view of each panel. (**C**) Overexpression of Godzilla leads to the formation of giant endosomes in *Drosophila*. GFP-tagged Godzilla was expressed with the *MS1096-Gal4* wing driver. Early endosomes were visualized by anti-Rab5 (*upper panel*) or by co-expression of mCherry::2xFYVE, an early endosome biomarker (*lower panel*). Godzilla::GFP (green) is localized on specific microdomains of enlarged mCherry::2xFYVE-positive endosomes. Membrane targeted GFP (GFP[CAAX]) was used as control. Enlarged endosomes are seen in Godzilla-overexpressing tissue (*arrowheads*). Bar 10 μm. The results shown here are typical images from at least four independent experiments.

endosomes in a number of tissues. Expression of activated Rab5–Rab5Q88LYFP in the wing disc leads to the accumulation of endogenous Godzilla protein on the resulting enlarged endosomes (Figure 1E). Taken together, these data suggest that *Goliath* family ubiquitin ligases function in endosomal trafficking processes.

Goliath and Godzilla ubiquitin ligase activity induces the formation of giant endosomes

In both *Drosophila* wing disc expression and vertebrate cell culture experiments, we noticed that the endosomes were abnormally large upon Godzilla expression (>3 μm, up to 15 μm, Figure 2; quantified in Figure 3C). This is not due to
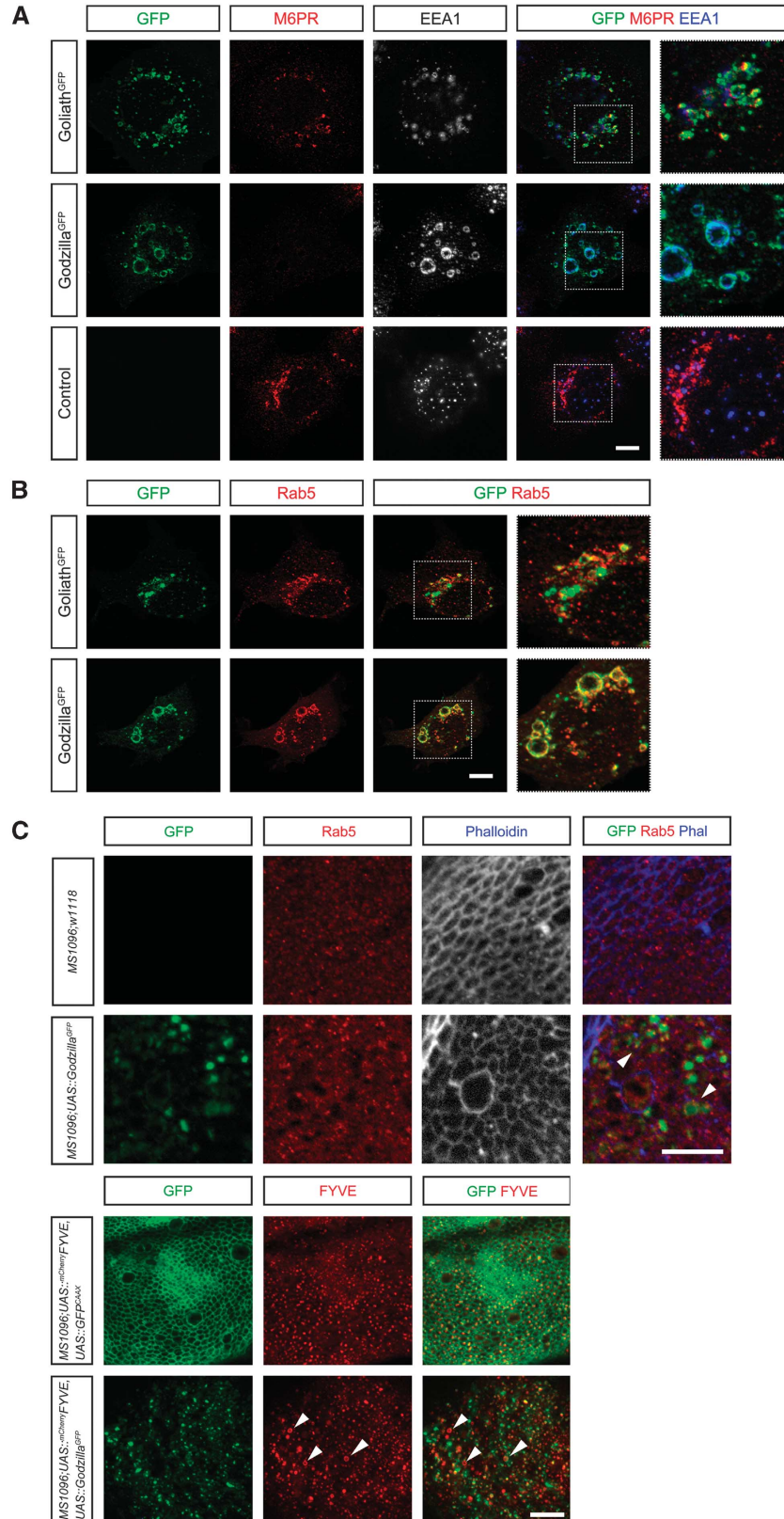
the addition of the carboxy-terminal GFP since HA-tagged versions of both Godzilla and Goliath display similar localization and activity as their GFP-tagged counterparts (Supplementary Figure S3). Consistently, we noted that


Godzilla was significantly more potent in the production of dramatically enlarged endosomes than Goliath. These enlarged endosomes were Rab5 and mCherry-FYVE positive in their nature (Figure 2B and C; Supplementary Figure S4), leading us to hypothesize that Godzilla/Goliath might play an important role in endosomal maturation perhaps *via* ubiquitylation of a key component of the endosomal machinery. In keeping with an important role for ubiquitin ligase activity, we were able to show that mutation of key His residues in the RING finger domain, corresponding to His[323] and His[326] in Goliath and His[255] and His[258] in Godzilla (Figure 1B; Su *et al*, 2009), abrogated the ability of either Godzilla or Goliath to generate large endosomes (Figure 3).

Godzilla function is required *in vivo* for integrity of endosome trafficking

In light of these results, we examined the effect of loss of function of these proteins on endosomes. To test this, we examined endosome accumulation in third instar larvae wing discs lacking Godzilla. RNAi knockdown of Godzilla resulted in a significant decrease in endogenous Godzilla protein (Supplementary Figure S1), and an accompanying robust accumulation of Rab5-positive endosomes (Figure 4A). This robust phenotype was also observed in $\Delta godzilla^{CG10277}$ mutant animals, where there is a dramatic buildup of Rab5-positive endosomes in third instar larva prior to lethality (Figure 4B and C). Importantly, this endosomal phenotype is rescued by *Godzilla* genomic rescue (Supplementary Figure S5).

Identification of VAMP3 as a target of Godzilla ubiquitin ligase activity

Taken together, our data strongly suggest that Godzilla and Goliath play an important role in endosomal processing, leading to perturbation of signalling processes *in vivo*. We rationalized that a key substrate for their ubiquitylation activity may be a component of the endosomal trafficking machinery, which may be regulated by this family of ligases. Indeed, expression of constitutively active Rab5 or knockdown of Rab7 has also been documented as generating large endosomes (Stenmark *et al*, 1994; Poteryaev *et al*, 2010), suggesting that Godzilla/Goliath may regulate Rab5 activity by some unknown mechanism. With this hypothesis in mind we set out to test a number of candidates, with focus on regulators of Rab5 and Rab7 activity. Those molecules tested included: Rab5, Rab7 (Chavrier *et al*, 1990), the Rab5-specific guanine nucleotide exchange factor (GEF) Rabex5 (Valsdottir *et al*, 2001; Penengo *et al*, 2006) and the Rab GDP dissociation inhibitor RabGDI (Ullrich *et al*, 1994; Poteryaev *et al*, 2010).
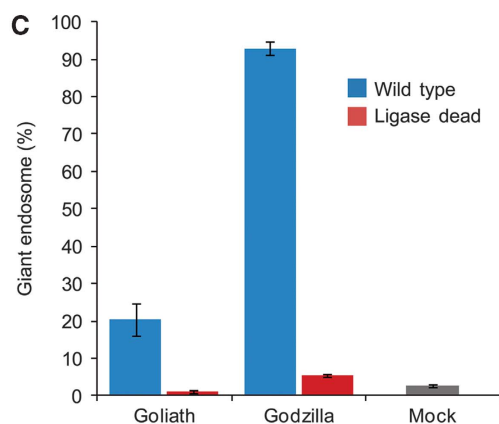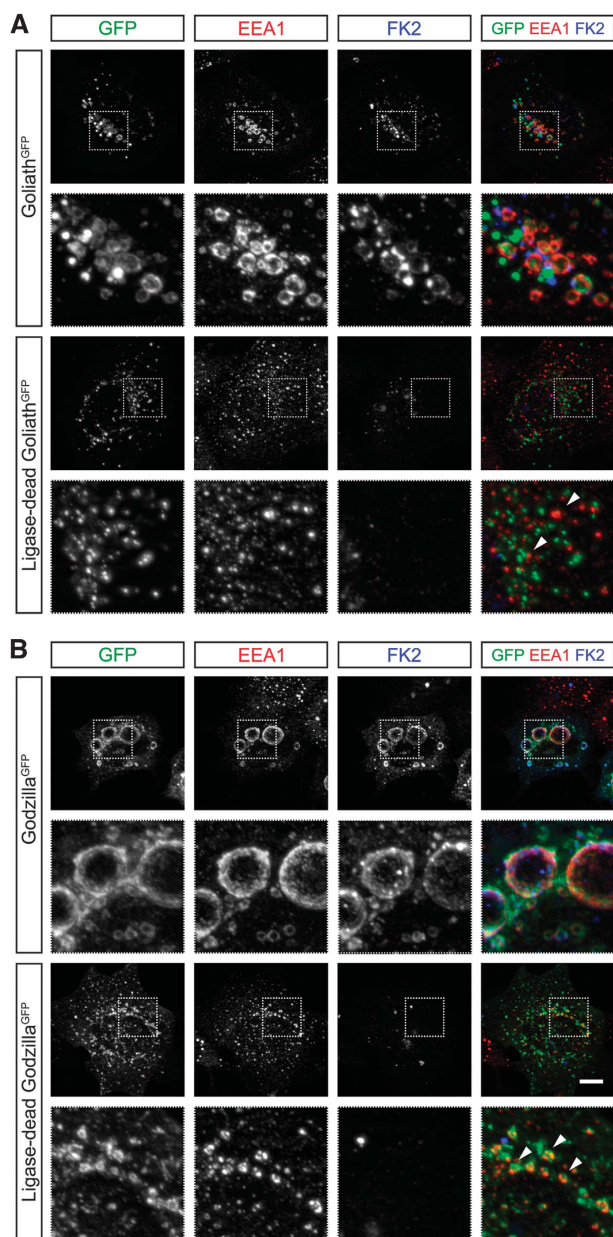
Figure 3 Goliath- or Godzilla-induced endosomal enlargement is dependent on their ubiquitin ligase activity. (**A**, **B**) HEK293 cells were transfected with Goliath-C-GFP, ligase-dead Goliath-C-GFP (**A**), Godzilla-C-GFP, or ligase-dead Godzilla-C-GFP (**B**), and stained for EEA1 and ubiquitylated proteins (FK2). Strong ubiquitylation was observed on enlarged endosomes upon expression of ligase-active forms of Goliath and Godzilla, but not ligase-dead forms. Lower panels show an expanded view of *dotted boxed* area of upper image. Bar 10 µm. (**C**) Quantification of induction of enlarged endosomes by Goliath and Godzilla. The number of transfection-positive (GFP-positive) HEK293 cells containing giant endosomes was quantified. Results are given as means ± s.d. of four independent experiments.
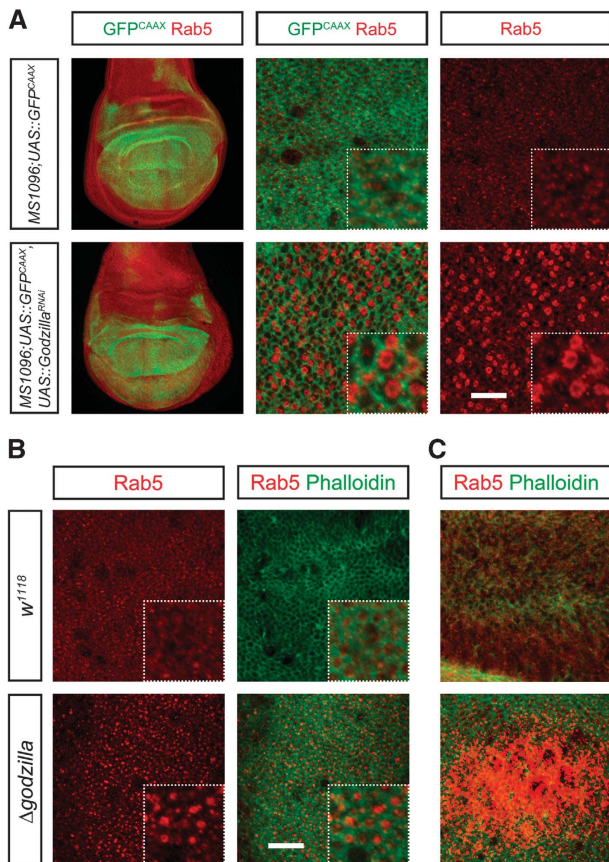
Figure 4 Loss of Godzilla function leads to a robust accumulation of Rab5 endosomes. (**A**) RNAi-mediated knockdown of Godzilla driven by *MS1096-Gal4* induces the formation of Rab5-positive giant endosomes in the *Drosophila* wing disc. Control is *MS1096-Gal4*-driven UAS-GFP^{CAAX}. Rab5 (*red*) and GFP (*green*) are shown. Left panels are lower magnification overview of wing discs. Right panels are close-up view of the centre of wing pouch. Enlarged Rab5-positive endosomes are observed with *godzilla RNAi* but not in control. (**B**) *Godzilla* mutant animals exhibit enlarged Rab5-positive endosomes. Wing discs of late third instar larvae were visualized with antibodies towards Rab5 (*red*) as well as phalloidin to visualize F-actin (*green*). *Dotted box* shows an expanded view of each panel. Rab5-positive endosomes are enlarged and accumulated in *godzilla* mutant wing discs, but not in control (*w^{1118}*). Bar 10 μm. (**C**) Rab5 endosomes accumulate to high levels in *godzilla* mutant wing discs. Both images were taken at identical exposure times. The results shown here are typical images from at least four independent experiments.

In spite of convincing evidence that expression of either Godzilla or Goliath resulted in a high level of ubiquitylation, as measured by anti-FK2 staining (Figure 3), we were unable to identify a cellular target by this candidate approach.

In order to proceed, we carried out a proteomics-based analysis (UbiSCAN), employing anti-GG antibodies to identify conserved cellular targets of *Drosophila* Godzilla (Xu *et al*, 2010). This analysis enables the identification, not only of substrate molecules, but also identification of those lysines targeted for ubiquitylation within the substrate. This approach led to the identification of the SNARE protein VAMP3, which is known to be involved in vesicle fusion and trafficking (McMahon *et al*, 1993) as a substrate for this family of ubiquitin ligases (Figure 5; Supplementary Figure S6; Supplementary Table S1). VAMP3 was heavily

ubiquitylated at Lys 66, Lys 68 and Lys 77 in samples containing active Godzilla, but not in Godzilla ligase-dead control lysates. Human VAMP3/Cellubrevin and the *Drosophila* orthologues Synaptobrevin (Syb, also called dVAMP) and neuronal-Synaptobrevin (n-Syb) show significant identity of 71 and 64%, respectively, and importantly these ubiquitylated Lys residues (Lys 66, 68 and 77) in VAMP3 are completely conserved in the *Drosophila* homologues (Figure 5B).

In order to confirm whether VAMP3 is a potential target of Godzilla, we investigated VAMP3 localization in Godzilla-transfected HEK293 cells. VAMP3 is known to localize on both early and recycling endosomes and is involved in endocytic recycling (McMahon *et al*, 1993; Galli *et al*, 1994). In both control and ligase-dead Godzilla-transfected cells, VAMP3 displays a similar intracellular distribution, partially overlapping with ligase-dead Godzilla; suggesting an early and recycling endosome distribution (Figure 5C). In contrast, in cells expressing wild-type Godzilla VAMP3 is found on enlarged endosomes, where it colocalizes with Godzilla (Figure 5C).

The Godzilla induced endosomal phenotype peturbs trafficking of both Rab5- and Rab11-positive endosomes and requires VAMP3

As VAMP3 has been shown to play a role in recycling endosome trafficking (Galli *et al*, 1994; Hong, 2005), we considered the hypothesis that Godzilla may regulate recycling endosome trafficking. In order to test this, we analysed transfected cells for the presence of Rab11-positive recycling endosomes (Ullrich *et al*, 1994). While cells transfected with ligase-dead Godzilla stain positive for Rab11 recycling endosomes compared with mock-transfected cells, we were unable to detect the presence of Rab11 recycling endosomes in the presence of wild-type ligase active Godzilla (Figure 5D). Interestingly, while the normal pattern of pericentriolar and dispersed Rab11-positive recycling endosomes was not seen, some residual Rab11 staining could still be observed in the vicinity of the Godzilla containing enlarged endosomes, suggesting an inability of Rab11 containing vesicles to be generated (Figure 5D, *upper panel*). In keeping with this, overexpression of Godzilla in the *Drosophila* with *MS1096-Gal4* also results in a loss of Rab11-positive vesicles in the wing disc (Supplementary Figure S7). To further investigate the significance of VAMP3 as a Godzilla target in the Godzilla-induced giant endosome phenotype, we transfected HEK293 cells either with scrambled control siRNA or with two independently employed siRNAs targeting VAMP3 (Supplementary Figure S8). Indeed, reduction of endogenous VAMP3 expression levels resulted in decreased levels of Rab11 recycling endosomes, while no impact on the presence or vesicle size of EEA1 early endosomes was seen (Supplementary Figure S8). Next, we investigated whether knockdown of VAMP3 was able to block the large endosome phenotype mediated by Godzilla overexpression (Figure 5E and F). Prior to transfection with wild-type Godzilla, endogenous VAMP3 expression levels were reduced by siRNAs targeting VAMP3. A strong reduction in the enlarged endosome phenotype was observed; leading us to conclude that loss of VAMP3 significantly blocks the Godzilla mediated enlarged endosome phenotype. These results suggest that ubiquitylation

does not result in a simple inhibition of VAMP3 function, such as inhibition of SNARE complex formation or complex assembly, but that the ubiquitylation of VAMP3 may work as a molecular switch for the recycling endosome pathway.

We then examined the effect of loss of Godzilla function *in vivo* returning to the *Drosophila* wing disc. *Drosophila* has two VAMP3 homologues, the neuronal n-Syb and a more generally expressed Syb (Chin *et al*, 1993; DiAntonio *et al*, 1993). Knockdown of *Drosophila* Syb reverses the *godzilla* mutant phenotype, resulting in a loss of giant Rab5 endosomes in *godzilla* mutant wing discs (Figure 5G). Furthermore, *Drosophila* Syb knockdown blocks the formation of FYVE-positive giant endosomes observed upon
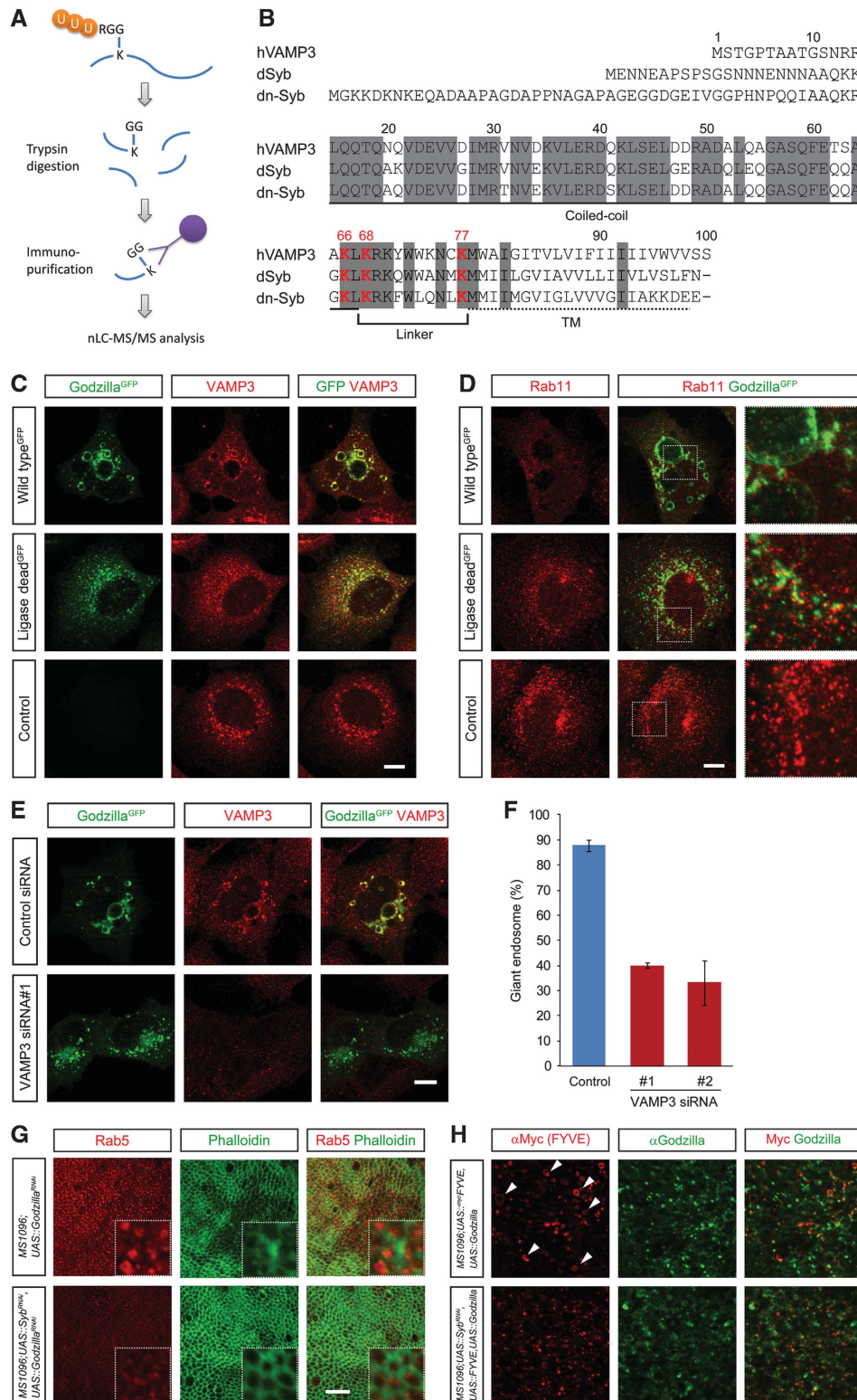
Godzilla overexpression in the *Drosophila* wing disc (Figure 5H). Together, these data provide convincing evidence that VAMP3/Syb is a relevant functional target of the Godzilla ligase *in vivo* in the fruitfly as well as in mammalian cells.

The related mammalian RNF167 PA-TM-RING domain ubiquitin ligase induces formation of giant endosomes

We then asked whether a role in endosomal trafficking may be conserved among the human members of the Goliath family. Consistent with this we found that expression of human RNF167 also led to the accumulation of giant endosomes that were positive for EEA1, FK2 and VAMP3, with a concomitant loss of Rab11-positive recycling endosomes (Figure 6A and B). Moreover, as with the Godzilla induced giant endosome phenotype, we were able to revert the RNF167 induced phenotype upon knockdown of VAMP3 (Figure 6C and D).

Mutation of Godzilla target lysines in VAMP3 abrogates the ability of Godzilla/RNF167 to induce giant endosomes

Having established VAMP3 as a target of Godzilla, we wished to examine the importance of ubiquitylation for VAMP3 by mutating the target Lysine residues in the VAMP3 molecule. It has been shown that the linker region, which contains Lysine 66, 68 and 77 targeted by Godzilla (Figure 7B), has a crucial role in SNARE-dependent fusion (McNew *et al*, 2000a, b; Knecht and Grubmuller, 2003). We generated three carboxyl-terminal HA-tagged VAMP3 mutants (VAMP3-WT^{HA}, VAMP3-K66,68,77R^{HA} and VAMP3-K0^{HA}; Figure 7B) in which target ubiquitylation lysine residues were mutated to arginine. These VAMP3 mutants, which are not targeted by the VAMP3 siRNA reagent employed, were introduced into HEK293 cells together with either Godzilla or RNF167 after knockdown of endogenous VAMP3 protein. First, mutation of the lysine residues in VAMP3 did not appear to affect localization of VAMP3 on endosomes upon cotransfection with Godzilla or RNF167. Second, we observed that the presence of all VAMP3 lysines are required for the formation of giant endosomes by either Godzilla or RNF167 (Figure 7A and C). These data suggest that ubiquiylation of VAMP3 is required to regulate its activity in the fusion process, in agreement with earlier observations of a critical role for the VAMP3 linker region in fusion (McNew *et al*, 2000a, b; Knecht and Grubmuller, 2003).

Induction of giant endosomes by Godzilla/RNF167 blocks Transferrin receptor recycling

Taken together, these results suggest a model in which Goliath family ubiquitin ligases such as Godzilla regulate trafficking through the recycling endosome pathway. To further support of this hypothesis, we decided to investigate the effect of Godzilla E3-ligase activity on Transferrin receptor trafficking (Figure 8). Transferrin internalization appears to be significantly delayed in Godzilla-transfected cells. While control cells expressing ligase-dead Godzilla displayed efficient trafficking of transferrin, active Godzilla expressing cells accumulated transferrin in enlarged endosomes (Figure 8A, *arrowheads*). A similar effect on transferrin receptor trafficking was observed with human RNF167 (Figure 8B). In order to investigate if incorporated transferrin is recycled in Godzilla of RNF167-transfected cells, we performed transferrin chasing for longer period up to 3 h (Figure 8C). While control cells displayed efficient trafficking of transferrin with transit of transferrin to endosomes clearly observed at 30 min and almost disappearing to 120 and 180 min, active Godzilla or RNF167-expressing cells accumulated transferrin which was present in enlarged endosomes even at 180 min after addition of transferrin (Figure 8C, *arrowheads*).

Discussion

Here, we report a number of novel findings concerning the poorly characterized Goliath family of PA-TM-RING domain E3 ligases. First, we show that they are localized at endosomes, where their activity leads to the ubiquitylation of target proteins. Second, manipulation of these ubiquitin ligases leads to a disruption of endosomal trafficking processes, both in cell culture and *in vivo* in *Drosophila* tissues. In particular, loss of the ubiquitously expressed Godzilla in the fly leads to a dramatic disruption of endosomal trafficking. Since mammalian endosomal VAMPs have been shown to mediate homotypic fusion of endosomes (Antonin *et al*, 2000), the robust accumulation of Rab5-positive endosomes we observe in the absence of Godzilla may be a result of enhanced homotypic fusion in the absence of recycling (Figure 8D). Third, our results identify the VAMP3 SNARE protein as a novel ubiquitylated target of these ligases, and show that these target lysines have functional relevance. Previous reports of the involvement of VAMP3 in the regulation of recycling endosomes (McMahon *et al*, 1993; Galli *et al*, 1994) are entirely consistent with the results presented here. Furthermore, since loss-of-function of the more generally

Figure 5 VAMP3, an endosomal SNARE protein, is ubiquitinated by Godzilla. (**A**) Schematic of identification of ubiquitiylated proteins by anti-GlyGly antibody purification (Xu *et al*, 2010). Cell extracts containing ubiquitylated proteins were digested by trypsin, resulting in generation of di-glycine conjugated peptides on the ε-amine group of lysine. Peptides were immuno-purified by anti-GlyGly antibody then subjected to nLC-MS/MS analysis. (**B**) Sequence alignment of VAMP3 and its *Drosophila* homologue, Synaptobrevin (Syb, dVAMP) and neuronal-Synaptobrevin (n-Syb). Identically conserved residues are highlighted in *grey* and the transmembrane domain and linker region are marked. Three ubiquitinated lysine residues (Lys^{66}, Lys^{68} and Lys^{77}) identified in VAMP3 are shown in *red*. These ubiquitinated lysine residues are completely conserved between human and *Drosophila* VAMP3 homologues. (**C**) Localization of VAMP3 on Godzilla-induced giant endosomes. Wild-type or ligase-dead Godzilla expressing HEK293 cells were stained with anti-VAMP3 antibody (*red*). (**D**) Rab11-mediated recycling is disrupted in wild-type Godzilla expressing cells, but not in ligase dead. (**E**) VAMP3 knockdown rescues the formation of giant endosomes induced by Godzilla, quantified in (**F**). Quantified results are given as mean ± s.d. for four independent experiments. Bar 10 μm. (**G**) Formation of Rab5-positive giant endosomes in *godzilla* loss-of-function wing discs is suppressed upon knockdown of the *Drosophila* VAMP3 homologue Synaptobrevin (Syb). Upper panel: late third instar larvae wing disc expressing *UAS-godzilla* RNAi driven by *MS1096-Gal4*; bottom panel: *MS1096-Gal4* driving both *UAS-godzilla* RNAi and *UAS-syb* RNAi wing disc. Wing discs were visualized with Rab5 (*red*) and phalloidin to visualize F-actin (*green*). *Dotted box* shows an expanded view. (**H**) Formation of FYVE-positive giant endosomes in Godzilla-overexpressing wing discs is suppressed upon knockdown of Syb. Giant endosomes observed upon Godzilla overexpression (upper panel, *arrowheads*) are suppressed in a Syb knockdown background (bottom panel).

expressed Syb/dVAMP protein is lethal in *Drosophila*, a general role in cellular trafficking has been predicted (Bhattacharya *et al*, 2002). This hypothesis is in keeping with the results presented here, which strongly suggest that Syb/dVAMP has a crucial role in endosome recycling in *Drosophila* as in mammals. Further, in agreement with our results, we note that several human members of the Goliath family E3 ligases, including RNF167, have been scored as positive hits in a genome-wide RNAi-based study of endocytosis by multiparametric image analysis (Collinet *et al*, 2010).

Vesicular-SNARE proteins form SNARE complexes with their partner proteins, consisting of a four-helix bundle held together by interacting layers. The current model of this process is that an initial *trans*-SNARE complex forms between pairing vesicles and further supports a directional zipping from the amino-terminal coiled-coil domain to the carboxyl-terminal transmembrane domain of the complex, resulting in pulling opposing membranes together and completing membrane fusion (Sutton *et al*, 1998; Stein *et al*, 2009). It has been shown that the linker region, which contains the Lysines 66, 68 and 77 targeted by Godzilla, has a crucial role in SNARE-dependent fusion (McNew *et al*, 2000a, b; Knecht and Grubmuller, 2003). Recently, studies of the VAMP2/syntaxin1A/SNAP-25 complex have examined the molecular mechanisms underlying the propagation of 'zipping' of the four-helix bundle to the transmembrane domain to complete the fusion (Hernandez *et al*, 2012). In this work, a VAMP2 mutant at residue Leu84, corresponding to Leu67 of VAMP3, was shown to be unable to fuse large liposomes. It has already been shown that post-transcriptional modifications of SNARE proteins such as phosphorylation and palmitoylation are known to regulate their functions (Snyder *et al*, 2006; Valdez-Taubas, 2005). However, although ubiquitylation has also been noted in several SNARE proteins including VAMP3 in large-scale proteomics analyses (Supplementary Figure S6; Hornbeck *et al*, 2012), its role or consequence is not understood. Here, we show that Godzilla/RNF167 regulates endosome recycling by the ubiquitylation of VAMP3 on Lys66, Lys68 and Lys77; namely, two adjacent Lys residues on the both sides of the critical interface of SNARE complex are ubiquitylated. Given the elegant studies on SNARE fusion dynamics (Hernandez *et al*, 2012) a significant effect of ubiquitylation at these sites on SNARE complex assembly can be envisioned. The endosome phenotypes observed in both loss-of-function and gain-of-function Godzilla suggest that the ubiquitylation of VAMP3 may be employed as a switch in the dynamic regulation endosome recycling, such that both correct spatial and temporal ubiquitylation and deubiquitylation of VAMP3 are important. While we observed ubiquitylation of VAMP3 by Godzilla, we are unable to describe the nature of this ubiquitination, be it mono-ubiquitin or extended ubiquitin chains. Novel tools to address the nature of ubiquitin modifications have recently been developed and may help to elucidate the nature of VAMP3 ubiquitylation in the future (van Wijk *et al*, 2012). It will also be of interest to examine the functional relevance of VAMP3 ubiquitylation in the fusion process at a structural level.

It follows that if ubiquitylation is employed as a switch in recycling endosome trafficking, that DUB(s) may also play an important function in the regulation of this process. In conclusion, the data presented here demonstrate a novel function for the Goliath PA-TM-RING domain ubiquitin ligase family in regulating recycling endosome trafficking through ubiquitylation of the VAMP3 SNARE protein (Figure 8D), with implications for intracellular recycling and signalling events in both physiological and pathological conditions.

Materials and methods

Recombinant DNA constructs
The following constructs were used for transfection in HEK293 cells: GFP-tagged wild type or ligase-dead Goliath or Godzilla constructs (Goliath WT-C-GFP, Goliath LD-C-GFP, Godzilla WT-C-GFP, Godzilla LD-C-GFP), HA-tagged wild Goliath or Godzilla (pIRES-hrGFP-2a-Goliath WT, pIRES-hrGFP-2a-Godzilla WT) and GFP-tagged human RNF167 (RNF167-C-GFP) (construction details available upon request). For the construction of ligase-dead mutants, two conserved His residues in the RING domain (His^{323} and His^{326} in Goliath, His^{255} and His^{258} in Godzilla), reported to be involved in the coordination of Zn^{2+} ions, were substituted to Arg residues (Figure 1B; Su *et al*, 2009). pEGFP-DsRed-Rab5 is from Addgene (Cambridge, MA). cDNAs for C-terminal HA-tagged VAMP3 mutants (WT^{HA}, K66,68,77K^{HA}, and K0^{HA}) were synthesized (GenScript, NJ) and cloned into pcDNA3 expression vector at *Bam*HI and *Eco*RI sites.

Cell culture and transfections
HEK293 cells were cultured in Dulbecco's modified Eagle's medium, containing 10% foetal bovine serum, penicillin and streptomycin. HEK293 cells were transfected with the above constructs as indicated using Lipofectamine (Invitrogen) according to manufacturer's protocol prior to fixation and immunostaining.

Transferrin uptake assays
For localization studies on early and recycling endosomes, transfected HEK293 cells were incubated for 2 h in serum-free cell culture medium containing 0.2% BSA at 37°C (Daro *et al*, 1996). Cells were subsequently incubated with 10 ng/μl Alexa FluoR 555 conjugated transferrin (Molecular Probes) in serum-free cell culture medium + 0.2% BSA for 45 min at 4°C, followed by three washes in medium + 0.2% BSA. Vesicle recycling was analysed after incubation at 37°C for the indicated times. For the transferrin chase analysis (Figure 8C), transfected HEK293 cells were incubated for 2 h in serum-free medium containing 0.2% BSA at 37°C. Cells were subsequently incubated with 10 ng/μl Alexa Fluor 555 conjugated transferrin for 45 min at 4°C, followed by three washes in 37°C pre-warmed serum containing complete medium. Vesicle recycling was analysed after incubation for up to 180 min at 37°C.

RNA interference
For VAMP3 knockdown, double-stranded RNA duplexes (siMax siRNA, Eurofins MWG Operon, Germany) targeting human VAMP3 were employed. The sequences were the following: siVAMP3 #1: 5′-UCAUUACCUUGUGUGUGAA-3′; siVAMP3 #2: 5′-UCGAAGACUUCAGCAGACA-3′. As a negative control, Stealth RNAi-negative control low CG duplex was used (Invitrogen). At 48 h post transfection, employing Lipofectamine 2000 (Invitrogen), cells were either lysed and knockdown of VAMP3 expression levels were analysed by immunoblotting as described previously (Yang *et al*, 2007) or fixed and stained for analysis by immunofluorescence. For endogenous VAMP3 depletion, HEK293 cells were transfected with siVAMP3 #1, targeting the 3′-UTR of VAMP3 mRNA on day 1, prior to transfection of Godzilla, RNF167 or VAMP3 on day 2. Cells were subjected to analysis on day 3. *Drosophila* transgenic RNAi lines to *Godzilla* (109001KK) and *dVAMP/Syb* (102922KK) were obtained from Vienna Drosophila Resource Center (Dietzl *et al*, 2007). Knockdown efficiency of *Godzilla*^{RNAi} was confirmed with immunofluorescence by anti-Godzilla antibody.

### Drosophila strains and genetics
Standard *Drosophila* husbandry procedures were followed. *Drosophila* strains were maintained on standard potato-meal med-
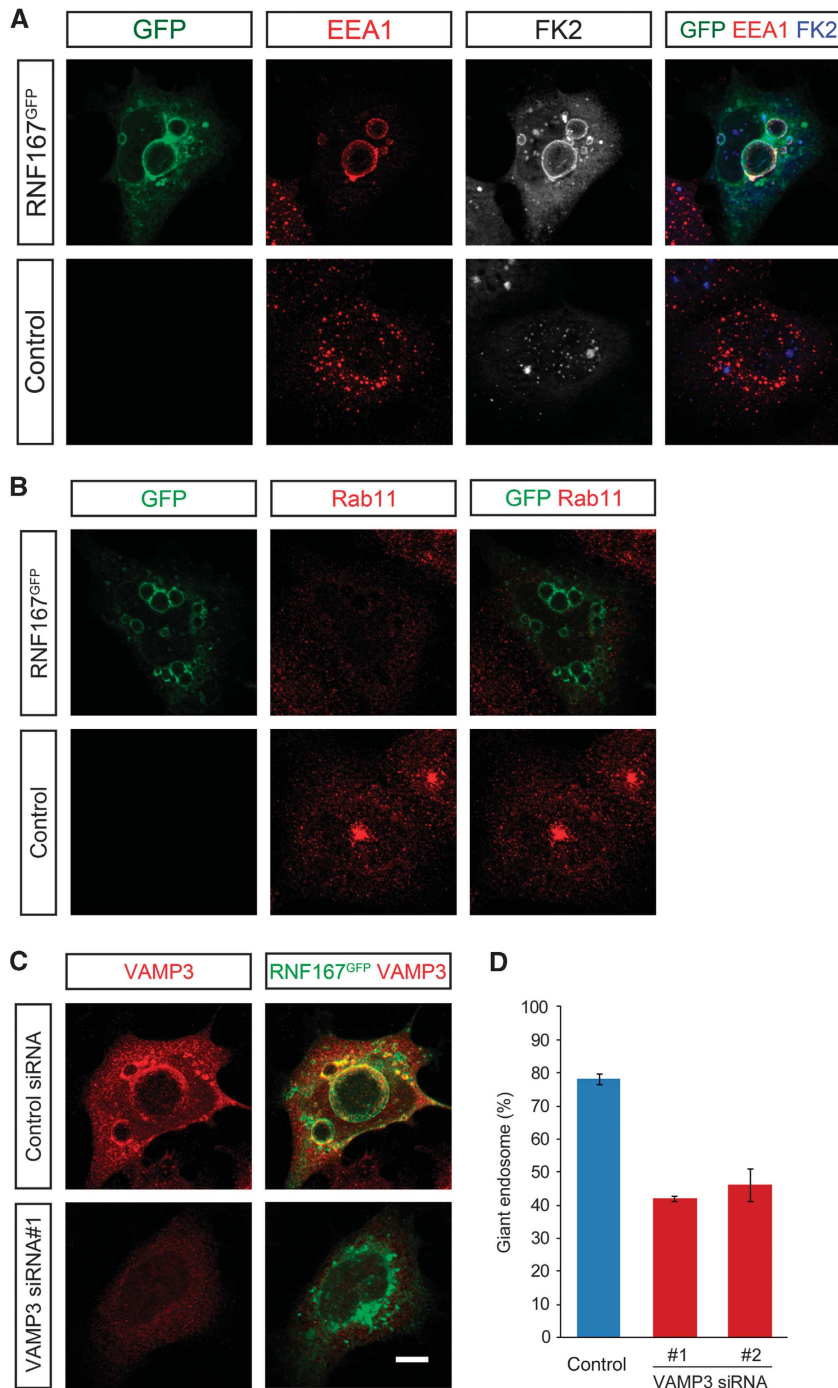
Figure 6 RNF167, a human PA-TM-RING E3 ligase, also affects endosomal trafficking. (**A**, **B**) The endosomal phenotype induced by human RNF167 resembles that of Godzilla. HEK293 cells were transfected with RNF167-C-GFP. RNF167 overexpression induces the formation of EEA1 and FK2-positive giant early endosomes (**A**), as well as loss of Rab11 recycling endosomes (**B**). (**C**, **D**) HEK293 cells expressing human RNF167-C-GFP exhibit robust accumulation of giant endosomes, which are VAMP3 positive. VAMP3 knockdown results in the rescue of the giant endosome phenotype induced by RNF167, quantified in (**D**). Quantified results are given as mean ± s.d. of four independent experiments. Bar 10 μm.

ium, and maintained at room temperature unless otherwise stated. Experimental crosses were performed at 25°C. The FRT strains used to generate *gl* deletion mutants were WHf06772 and RBe01478; FRT strains for the *godzilla*[CG10277] deletion (Δ*godzilla*[1]; Supplementary Figure S5) were XPd01485 and WHf07224, which were obtained from the Harvard Exelixis collection (Thibault *et al*, 2004). Deficiency lines Df(3R)Tpl10 (BL1990), Df(3R)Tpl9 (BL1986) and Df(3R)WIN11 (BL2393) were obtained from the Bloomington stock Center. The P-element line used to generate *godzilla*[CG10277] deletion

mutant (Δ*godzilla*[2]; Supplementary Figure S5) was EP705, from the Bloomington stock Center. The deletion was confirmed by southern blotting and DNA sequencing. As *godzilla*[CG10277] mutant larva cannot survive on agar-based fly food, mutant larvae were maintained with yeast paste on apple juice plate. UAS::mCherry::FYVE[Hrs] (Velichkova *et al*, 2010) and UAS-GFP[caax] (Finley *et al*, 1998) were used in subcellular localization studies. For mutant clone analysis, Δ*godzilla*[2] was recombined with P{neoFRT}82B ry[506] (BL2035). The resulting Δ*godzilla*[2], P{neoFRT}82B ry[506] was crossed
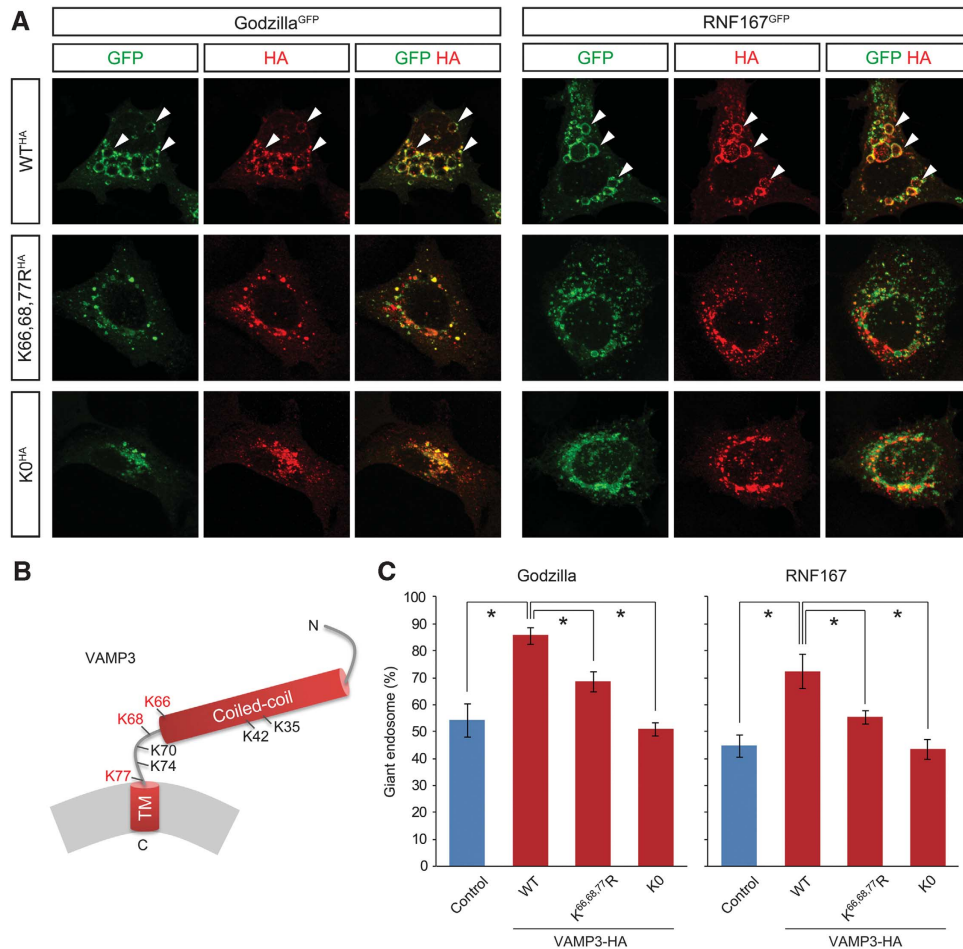
Figure 7 Mutation of Godzilla target ubiquitylation in VAMP3 disrupts VAMP3 protein function. (**A**) The giant endosome phenotype induced by Godzilla or RNF167 is supported by wild-type VAMP3, but not by Lys-substituted VAMP3 mutants. HA-tagged VAMP3 mutants were co-transfected with Gozilla-C-GFP (left) or RNF167-C-GFP (right) after knockdown of endogenous VAMP3. The giant endosome phenotype is supported by wild-type VAMP3 (WT^{HA}), but only partially by K66, 68, 77R^{HA} and not by K0^{HA}. (**B**) Schematic structure of VAMP3. hVAMP3 contains seven Lys residues in total; two are in the coiled-coil region, while other five are located in the linker region. The Lys residues identified as ubiquitylated are indicated in *red*. (**C**) Statistical analysis of (**A**). Quantified results are given as mean ± s.d. for three independent experiments. Asterisk $P < 0.05$.

to P{hsFLP}12; Sco/CyO (BL1929). Progeny was crossed to P{neoFRT}82B P{Ubi-GFP(S65T)nls}3R P{A92}RpS3[Plac92]/ TM6B, Tb[1] (BL5627), and larvae were heat-shocked for 30 min at 37°C, prior to analysis.

Genomic rescue and transgenic constructs

The genomic rescue construct harbouring *godzilla*^{CG10277} was constructed by φ31 integrase-based methodology. Briefly, a *Drosophila* genomic DNA fosmid clone (FlyFos028285), corresponding to the region containing *CG42564*, *CG42537*, *CG1024*, *TfIIFalpha*, *Godzilla* and *CG1021* was obtained from FlyFos project (http://transgeneome.mpi-cbg.de/index.php?id = 42). To remove *CG1021*, the fosmid was digested with *Aat*II and religated. The resulting clone was subjected to transgenesis to P[acman] strain 9750 (65B2). Since complementation analyses suggest that Δ*godzilla*^{2} harbours an additional lethal mutation on the third chromosome, Δ*Godzilla*^{1}/ Δ*Godzilla*^{2} are employed as *Godzilla* mutant in this study. pUAST-Goliath-GFP and pUAST-Godzilla-GFP constructs were generated from Goliath WT-C-GFP and Godzilla WT-C-GFP by digestion with *Bgl*II and *Bam*HI. The obtained fragments were ligated into *Bgl*II site in pUAST. The direction of the insert was confirmed by restriction enzyme digestion and the constructed vectors were subjected to embryo injection (BestGene, CA).

Immunostaining and antibodies

The following antibodies were used: goat anti-mouse Cy3 (1:1000, Jackson), goat anti-mouse Cy2 (1:1000, Amersham), goat anti-mouse Cy5 (1:200, Jackson), goat anti-rabbit Cy2 (1:1000, Amersham), and goat anti-rabbit Cy3 (1:1000, Amersham), donkey anti-rabbit Cy5 (1:200, Jackson), donkey anti-guinea pig Cy3 (1:500, Jackson), mouse anti-HA (Covance, 1:1000), rabbit anti-HA (SantaCruz, 1:100), mouse anti-GFP (Clontech, 1:5000), rabbit anti-Myc (Abcam, 1:2000), rabbit anti-EEA1 (1:100, SantaCruz), mouse anti-mannose 6 phosphate receptor (1:500, Abcam), rabbit anti-LC3B (1:400, Cell Signaling), mouse anti-conjugated ubiquitin FK2 (1:200, Enzo Life Science), rabbit anti-*Drosophila* Rab5 antibody (2 µg/ml, Abcam), guinea-pig anti-Godzilla (1:1,500; this work); rabbit anti-Rab11 (1:8000), anti-pJNK (1:250, Promega). Anti-VAMP3 antibodies were obtained from Synaptic Systems (Synaptic Systems, Germany), rabbit anti-Rab11 (1:100) from Cell Signaling and anti-pan-ERK (1:5000) was obtained from BD Biosciences. MitoTracker and LysoTracker were from Invitrogen. Disc staining was carried out according to Grabbe *et al* (2004). For immunostaining of HEK cells, cells were grown on collagen-coated coverslips in 24-well plates, fixed with 4% paraformaldehyde/ DMEM and blocked with 50 mM NH$_4$Cl/PBS. After permeabilization with 0.3% Triton X-100 and 5% goat serum containing PBS, cells were incubated with primary antibody
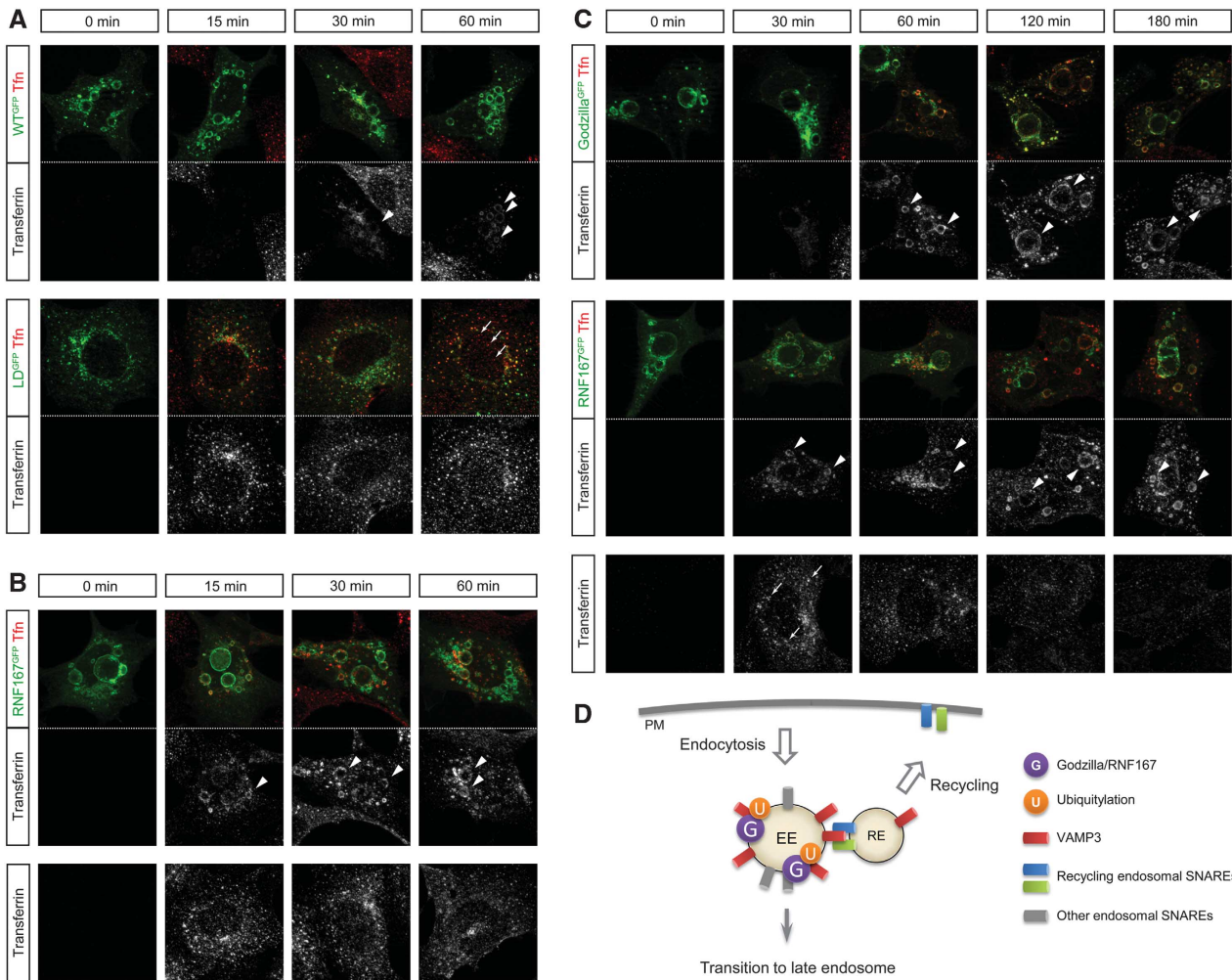
Figure 8 Transferrin recycling is disrupted in Godzilla- or RNF167-transfected cells. (**A**) Transferrin is accumulated at enlarged endosomes in Godzilla-transfected, but not in ligase-dead mutant transfected cells. Cells were exposed to Alexa Fluor 555-labelled transferrin in wild-type (top panels) or ligase-dead Godzilla (bottom panels) transfected HEK293 cells for the indicated period. In ligase-dead Godzilla transfected cells, transit of transferrin to endosomes is observed at 15 and 30 min, with appearance in recycling vesicles (segregated signal from ligase-dead Godzilla at 60 min, *arrows*). In contrast, Alexa Fluor 555-labelled transferrin is accumulated at enlarged endosomes in wild-type Godzilla-transfected cells (*arrowheads*). (**B**) Transferrin is accumulated at enlarged endosomes in RNF167-transfected, but not in mock-transfected cells. Cells were exposed to Alexa Fluor 555-labelled transferrin in RNF67 (top panels) or control (bottom panels) transfected HEK293 cells for the indicated time. Alexa Fluor 555-labelled transferrin is accumulated in enlarged endosomes in RNF167-transfected cells. (**C**) Transferrin recycling is disrupted in Godzilla- or RNF167-transfected cells. Cells were exposed to Alexa Fluor 555-labelled transferrin in Godzilla (top panels) or RNF167 (middle panels) transfected HEK293 cells and chased for the indicated period. In mock-transfected cells (bottom panels), transit of transferrin to endosomes is strongly observed at 30 min (*arrows*), and has almost disappeared at 120 and 180 min. In contrast, Alexa Fluor 555-labelled transferrin is accumulated at enlarged endosomes in Godzilla- or RNF167-transfected cells (*arrowheads*). (**A–C**) The results shown here are typical images from four independent experiments. (**D**) Schematic model visualizing the role of the Goliath family E3 ligases in regulation of endosomal trafficking. In normal conditions, a significant portion of endocytosed proteins on early endosomes are recycled *via* recycling endosomes, while the remaining proceed to lysosomal degradation *via* transition to the late endosome. In the budding process from the early endosome to recycling endosome, VAMP3/Syb on the early endosome forms a SNARE complex with recycling endosome SNAREs, such as Syntaxin1 and SNAP-25. Ubiquitylation of VAMP3/Syb by Godzilla/RNF167 plays a role in the regulation of recycling endosome traficking.

overnight. For visualization, cells were further incubated with fluorescence-labelled secondary antibody followed by analysis by ApoTome fluorescence microscopy (Zeiss, Germany) and Leica TCS SPE confocal microscope (Leica, Germany).

Antibody generation

Antigen was produced as bacterial recombinant protein in BL21 Star (DE3). Briefly, cDNA corresponding to C-terminal part of the Godzilla protein (residues 291–536) was synthesized (GenScript, NJ) and ligated into *Nco*I-*Bam*HI site of pETHis1a vector. As the original sequence contains *Nco*I site in this region, the corresponding nucleotide was replaced to synonymous nucleotide. Purified proteins by using Ni-NTA agarose (Qiagen, Germany)

were immunized in guinea pig (Eurogentec, Belgium). Antibody reactivity was verified by western blotting and immunofluorescence (1:1500).

Proteomics

UbiSCAN analysis (Cell Signaling), involving K-GG peptide immunoprecipitation and LCMS/MS, was performed on HEK293 cells expressing either wild-type Godzilla or ligase-dead Godzilla as control. Briefly, Godzilla WT-C-GFP or Godzilla LD-C-GFP was transfected with lipofectamine according to manufacturer's protocol. After 24 h, cells were harvested with urea lysis buffer. Resulting cell extracts (~40 mg protein) were used in the subsequent UbiSCAN analysis (Xu *et al*, 2010).

Bioinformatics sequence analysis

Protein sequence analysis was performed using SMART program from the EMBL, Heidelberg (http://smart.embl-heidelberg.de). For the generation of phylogenetic tree, the multiple nucleotide sequences of ORF were aligned by using ClustalW and then the tree was generated by PhyML.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

Conflict of interest

The authors declare that they have no conflict of interest.

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