EMBO reports

scientific report

# Role of Varp, a Rab21 exchange factor and TI-VAMP/VAMP7 partner, in neurite growth

*Andrea Burgo[1,2*], Emmanuel Sotirakis[1,2*], Marie-Christine Simmler[1,2], Agathe Verraes[1,2], Christophe Chamot[2,3], Jeremy C. Simpson[4], Letizia Lanzetti[5], Véronique Proux-Gillardeaux[1,2] & Thierry Galli[1,2+]*

[1]Membrane Traffic in Neuronal & Epithelial Morphogenesis, INSERM U950, [2]Program in Development & Neurobiology, Institut Jacques Monod, CNRS UMR7592, University Denis Diderot/Paris 7, and [3]RIO Imaging Platform, Paris, France, [4]School of Biology & Environmental Science and Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Dublin, Ireland, and [5]Department of Oncological Sciences, Institute for Cancer Research & Treatment, University of Torino, Turin, Italy

**The vesicular soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) tetanus neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP/VAMP7) was previously shown to mediate an exocytic pathway involved in neurite growth, but its regulation is still largely unknown. Here we show that TI-VAMP interacts with the Vps9 domain and ankyrin-repeat-containing protein (Varp), a guanine nucleotide exchange factor (GEF) of the small GTPase Rab21, through a specific domain herein called the interacting domain (ID). Varp, TI-VAMP and Rab21 co-localize in the perinuclear region of differentiating hippocampal neurons and transiently in transport vesicles in the shaft of neurites. Silencing the expression of Varp by RNA interference or expressing ID or a form of Varp deprived of its Vps9 domain impairs neurite growth. Furthermore, the mutant form of Rab21, defective in GTP hydrolysis, enhances neurite growth. We conclude that Varp is a positive regulator of neurite growth through both its GEF activity and its interaction with TI-VAMP.**

Keywords: neurite growth; Rab; SNARE; Varp; TI-VAMP/VAMP7

*EMBO reports* (2009) **10**, 1117–1124. doi:10.1038/embor.2009.186

## INTRODUCTION

The vesicular exocytic traffic is an essential mechanism to sustain membrane expansion in several processes in eukaryotic cells, including neurite growth (Futerman & Banker, 1996; Pfenninger, 2009). It is widely accepted that the general mechanisms underlying membrane traffic can be divided into four essential steps—that include vesicle budding, transport, tethering and fusion (Bonifacino & Glick, 2004)—and are regulated by Rab GTPases (Zerial & McBride, 2001; Grosshans *et al*, 2006) and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Jahn & Scheller, 2006). Although much is known about the functions of Rabs and SNAREs in these processes, so far only a few members of these two families have been shown to have documented roles in neurite growth (Ng & Tang, 2008). The tetanus neurotoxin insensitive vesicle-associated membrane protein (TI-VAMP/VAMP7) is a vesicular SNARE (v-SNARE) that mediates an exocytic pathway that is crucial for neurite growth in PC12 cells and in neurons in primary culture (Martinez-Arca *et al*, 2000, 2001; Alberts *et al*, 2003). In yeast two-hybrid screens, we identified the Vps9 domain and ankyrin-repeat-containing protein (Varp) as a new partner for TI-VAMP. Varp was recently shown to be a guanine nucleotide exchange factor (GEF) for Rab21 (Zhang *et al*, 2006), a small GTPase that regulates phagocytosis (Khurana *et al*, 2005), cell adhesion, cell migration and cytokinesis by controlling the endosomal trafficking of β1-integrins (Pellinen *et al*, 2006, 2008). These findings prompted us to study the biological meaning of the interaction between Varp and TI-VAMP and to characterize the function of Varp and Rab21 in neurite growth. Our results show that, similarly to TI-VAMP, Varp and Rab21 are positive regulators of neurite growth.

[1]Membrane Traffic in Neuronal & Epithelial Morphogenesis, INSERM U950, [2]Program in Development & Neurobiology, Institut Jacques Monod, CNRS UMR7592, University Denis Diderot/Paris 7, and [3]RIO Imaging Platform, Paris, F-75013 France
[4]School of Biology & Environmental Science and Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Dublin 4, Ireland
[5]University of Torino, Department of Oncological Sciences, Institute for Cancer Research & Treatment, Turin, Italy
*These authors contributed equally to this work
+Corresponding author. Tel: +33 157 278 039; Fax: +33 157 278 036;
E-mail: thierry.galli@inserm.fr

**Received 29 April 2009; revised 13 July 2009; accepted 14 July 2009; published online 11 September 2009**

## RESULTS

### The GEF Rab21 Varp is a new TI-VAMP-interacting protein

To identify the partners of TI-VAMP, we carried out a yeast two-hybrid screen using the full cytoplasmic domain of TI-VAMP as bait in a human placental library. Among already characterized partners of TI-VAMP, such as syntaxin 3, SNAP23, AP-3δ and HRB (Martinez-Arca *et al*, 2003; Scheuber *et al*, 2006; Chaineau *et al*, 2008), this screen identified Varp as a new TI-VAMP-interacting protein. The screen was repeated using a human fetal brain library and produced similar results. The region

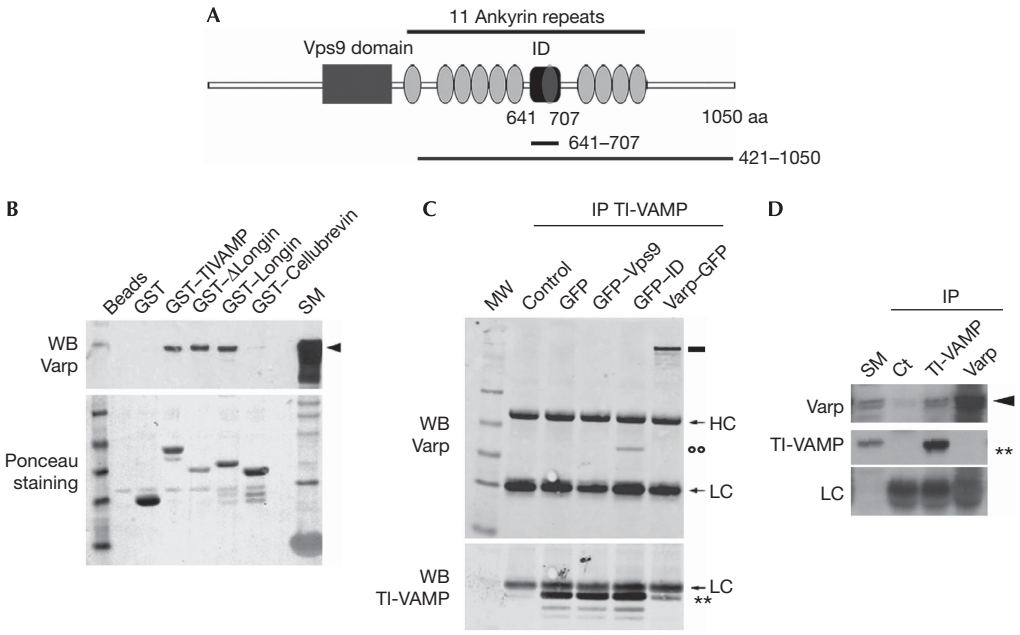


**Fig 1** | The GEF Rab21 Varp is a new TI-VAMP-interacting protein. (**A**) Schematic structure of Varp. The longer black line (421–1050) highlights the total coverage of all prey clones identified in the yeast two-hybrid screens and the shorter black line (641–707) highlights the minimal domain required for Varp interaction with TI-VAMP (ID, black box). (**B**) *In vitro* translated full-length Varp (arrowhead) interacts with the GST-tagged full cytoplasmic domain of TI-VAMP (GST–TIVAMP), GST-tagged amino-terminal domain (GST–Longin) and the protein with this domain deleted (GST–ΔLongin) but not to GST alone or GST-tagged cytoplasmic domain of cellubrevin. (**C**) TI-VAMP (double asterisk) precipitates GFP–ID (double circle) and GFP-tagged Varp (dash) from HeLa cell extracts. (**D**) Varp (arrowhead) precipitates with TI-VAMP (double asterisk) in differentiated PC12 cells. TI-VAMP is not immunoprecipitated by the Varp antibody, due to the fact that Varp antibody was raised against ID. Ct, immunoprecipitation with Pan-mouse IgGs; Control, untransfected HeLa cells; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HC, IgG heavy chain; ID, interaction domain; IP, immunoprecipitation; LC, IgG light chain; SM, 10% of starting material; TI-VAMP, tetanus neurotoxin-insensitive vesicle-associated membrane protein; WB, western blot.

from amino acids 641 to 707 was present in all clones corresponding to Varp isolated in both yeast two-hybrid screens, defining the probable minimal domain responsible for the interaction with TI-VAMP (Fig 1A), herein called the interacting domain (ID). To gain insight into the TI-VAMP–Varp interaction, we generated a Varp antibody directed against ID (supplementary Fig S1A online) and confirmed the interaction using *in vitro* biochemical assays (Fig 1B). In glutathione-*S*-transferase (GST) pull-down experiments, Varp interacted with the full-length TI-VAMP, its amino-terminal domain (Longin), and the protein with this domain deleted (ΔLongin) corresponding to the SNARE domain, suggesting that Varp has two binding sites in TI-VAMP. Furthermore, TI-VAMP co-precipitated green fluorescent protein (GFP)-tagged ID and Varp from HeLa cells (Fig 1C) and endogenous Varp from PC12 cells (Fig 1D), further confirming the interaction *in vivo*. The N-terminal region of Varp includes a Vps9 domain that has recently been shown to have Rab21 GEF activity (Zhang *et al*, 2006). Using a wild-type (wt) myc-tagged Rab21 form or locked either in the GDP-bound (T33N) or GTP-bound (Q78L) state, we showed that endogenous Varp precipitated with Rab21, preferentially with Rab21-T33N (supplementary Fig S1B online) as expected for a GEF (Boguski & McCormick, 1993; Delprato & Lambright, 2007). It has been shown that Rab21-T33N concentrates in the *trans*-Golgi network (TGN; Simpson *et al*, 2004; and data not shown), thus interaction between Rab21 and Varp is likely to occur in the TGN. We studied the localization of untagged and GFP-tagged Varp in cells moderately overexpressing the proteins to bypass the weak staining produced by immunohistochemistry using Varp antibody. As shown in Fig 2A, Varp strongly localized with TI-VAMP mainly in the perinuclear region of HeLa cells. Both proteins co-localized in vesicles, often in close proximity to microtubules (Fig 2A, Merge), suggesting a potential role of Varp in the transport of TI-VAMP. Using live-cell imaging, we observed that Varp–GFP and red fluorescent protein (RFP)–TI-VAMP were partially transported in the same vesicles in mouse hippocampal neurons in culture both under control conditions and in Brefeldin A washout experiments (Jareb & Banker, 1997), suggesting that this interaction might be related to transport from the cell body into growing neurites (Fig 2B; supplementary Movies S1 and S2 online). To gain insight into the potential TI-VAMP–Varp–Rab21 connection, we analysed their distribution in PC12 cells and in mouse hippocampal neurons in culture. Rab21, Varp and TI-VAMP largely co-localized in the perinuclear region and partially along the neurites, both in mouse hippocampal neurons (Fig 2C) and in differentiated PC12 cells (supplementary Fig S2 online). The peripheral region of the growth cone was largely devoid of Varp and Rab21, whereas TI-VAMP was present and dynamic in filopodia (Fig 2D,E; supplementary Movie S3 online). This suggests a potential role of the Varp and Rab21 complex

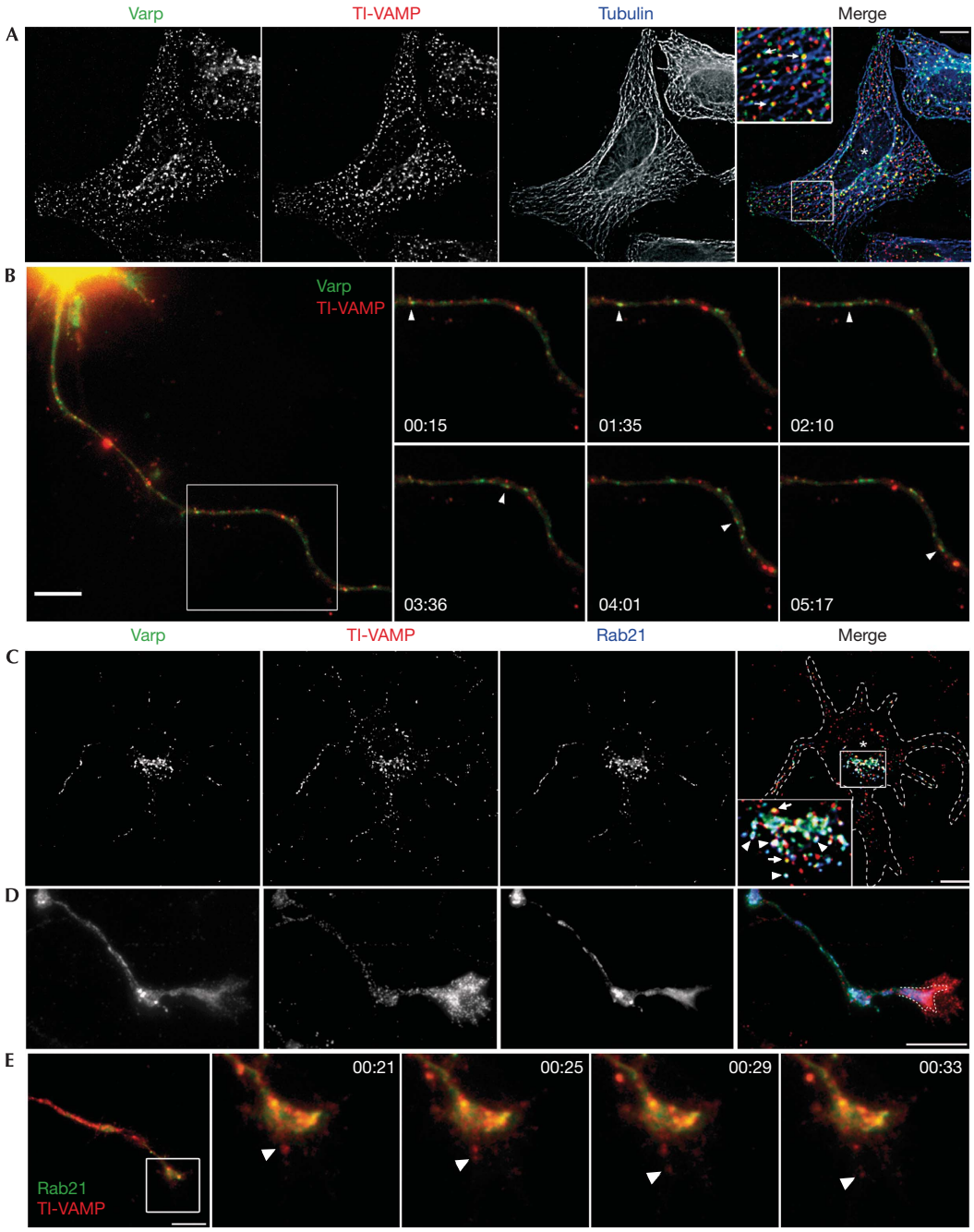


**Fig 2** | Varp, TI-VAMP and Rab21 co-localization. (**A**) Varp and TI-VAMP partially localize in HeLa cells co-transfected with RFP–TI-VAMP and Varp–GFP. Images were deconvoluted. Arrows indicate co-localization of Varp and TI-VAMP. Asterisk indicates nucleus. (**B**) Vesicular co-transport of Varp–GFP and RFP–TI-VAMP (arrowheads) in anterograde and retrograde (data not shown) directions in mouse hippocampal neurons (images from supplementary Movies S1 and S2 online). (**C**) DIV1 mouse hippocampal neurons were transfected with GFP–Rab21-wt and Varp. Images were deconvoluted. Overlay and inset show co-localization of Varp and TI-VAMP (arrows) or Rab21, Varp and TI-VAMP (arrowheads). The edges of the cellular body and neurites are emphasized by a white dashed line. Asterisk indicates nucleus. (**D**) Detail of distal axon and growth cone of transfected mouse hippocampal neurons. The probable transition zone of growth cone is emphasized by a white dashed line. (**E**) RFP–TI-VAMP vesicles were tracked in an axon expressing GFP–Rab21. Vesicular movements of TI-VAMP vesicle are shown in the series of frames corresponding to the boxed region. TI-VAMP-positive vesicles (arrowheads) moved into the peripheral region of the axon, whereas Rab21 was retained in the central region (images from supplementary Movie S3 online). Scale bars, 10 μm. Time in minutes. DIV, days *in vitro*; GFP, green fluorescent protein; RFP, red fluorescent protein; TI-VAMP, tetanus neurotoxin-insensitive vesicle-associated membrane protein; Varp, Vps9 domain and ankyrin-repeat-containing protein; wt, wild type.

in regulating TI-VAMP trafficking in the microtubule-rich region of the growing neurite but not in the actin-rich region of the growth cone. In differentiated PC12 cells, Varp and TI-VAMP co-localized with Rab21 to a greater extent than with Rab5 and Rab11 (supplementary Fig S2 online), reinforcing the connection established here.

### Varp regulates neurite growth

As TI-VAMP is involved in neurite growth (Martinez-Arca *et al*, 2000, 2001; Alberts *et al*, 2003), we examined the function of Varp by silencing its expression in PC12 cells and mouse hippocampal neurons in culture. PC12 cells have the advantage of being easily transfected and able to develop long neurites in response to staurosporine, as previously described by Martinez-Arca *et al* (2000). By using this model, we showed that neurite length is reduced by approximately 20% in Varp and TI-VAMP silenced cells. This is in sharp contrast with control (scramble and luciferase) or unrelated small interfering RNA (siRNA; Syb2; Fig 3A,B). Furthermore, in developing mouse hippocampal neurons, silencing of Varp by two different siRNA duplexes reduced axonal growth significantly (Fig 3C–F), further establishing the role of Varp in neurite and axon growths. As GFP–ID interacts with TI-VAMP *in vivo* (Fig 1C), it was reasonable to think that the expression of ID might inhibit axonal growth by preventing the formation of the endogenous TI-VAMP–Varp complex. We thus expressed Varp–GFP and GFP–ID, GFP alone being our control, in developing mouse hippocampal neurons. We found that GFP–ID inhibited axonal growth (Fig 3G–I). We also tested the effect of a mutant of Varp lacking the Vps9 domain (ΔVps9-Varp–GFP) because this domain has been shown to be responsible for the GEF activity of Varp (Zhang *et al*, 2006). This truncated protein, which includes ID, still interacted with TI-VAMP (supplementary Fig S3C online) and had a dominant-negative effect on neurite growth (supplementary Fig S3 online). We conclude that the interaction of Varp with TI-VAMP and its GEF activity have an important function in neurite growth.

### Varp is required for TI-VAMP–Rab21 co-localization

The inactivated form of Rab21, Rab21-T33N, has previously been shown to concentrate in TGN (Simpson *et al*, 2004) and TI-VAMP has been shown to regulate the exocytosis of post-Golgi vesicles (Alberts *et al*, 2006; Scheuber *et al*, 2006; Sander *et al*, 2008). TI-VAMP and Rab21 co-localized to a significant extent in the perinuclear region of HeLa (Fig 4A), PC12 cells (supplementary Fig S2 online) and hippocampal neurons (Fig 2C), suggesting a conserved mechanism of sorting. As TGN is an important site for protein sorting in the exocytic pathway, we analysed the staining of TI-VAMP, Rab21 and the marker TGN46. We saw partial and significant co-localization in HeLa cells (Fig 4A; supplementary Fig S4 online), suggesting that TGN is a site at which Varp might regulate a functional link between TI-VAMP and Rab21. To further test this hypothesis, we silenced Varp and measured the co-localization between TI-VAMP and Rab21. Varp was efficiently depleted by a selected mix of siRNA duplexes (Varp_h2), but not by control siRNAs (Luc, Sc; Fig 4B). The efficiency of gene silencing was also assessed by real-time RT–PCR, confirming a reduction of 75.1 ± 2.7% in Varp messenger RNA expression in HeLa cells (supplementary Table S1 online). GFP–Rab21-wt was distributed in a punctuate pattern and significantly localized with TI-VAMP mainly in the perinuclear region (overlapping with the TGN marked with TGN46, see Fig 4A; supplementary Fig S4 online) in control siRNA (scramble, Fig 4A, left panels), whereas this co-localization was reduced in Varp siRNA-treated cells (Fig 4A, right panels). A statistical analysis confirmed the reduction of co-localization between TI-VAMP and Rab21 in Varp siRNA-treated cells (Fig 4C,D). Therefore, despite the lack of evidence for a tripartite complex (data not shown), the Rab21 GEF Varp might establish a functional link between TI-VAMP and Rab21, probably occurring at TGN sites.

### Rab21 regulates neurite growth

As TI-VAMP and Varp are both involved in neurite growth, and the Vps9 domain of Varp is required for this function, we then investigated the role of Rab21, studying the effect of the expression of constitutively active and inactive mutants of Rab21 on neurite growth in PC12 cells. As shown in Fig 5A,B, Rab21-Q78 L significantly stimulated the growth of long neurites. By contrast, Rab21-T33N had no effect on the growth of neurites longer than 40 μm (Fig 5B) but induced abundant small protrusions (Fig 5A, middle panel), as previously seen in *Dictyostelium* (Khurana *et al*, 2005). Indeed, a quantification of the number of neurites (> 5 μm) in cells expressing Rab21 mutants showed a significant increase in the amount of short protrusions in Rab21-T33N compared with Rab21-wt or Rab21-Q78L (Fig 5C, see percentage of neurites between 5 and 10 μm, first points of percentile graph). Altogether these results suggested that the activation of Rab21, which is downstream from Varp, mediates the extension of long neurites. We did not see a significant effect on the average neurite length in Rab21-silenced PC12 cells and mouse hippocampal neurons (data not shown). A likely explanation might be that other isoforms of Rab21—or other closely related Rab proteins—are able to compensate for the lack of Rab21, as in the cases of Rab3 (Schluter *et al*, 2004) and Rab32/38 (Wasmeier *et al*, 2006).

## DISCUSSION

Here we show that Varp is a positive regulator of neurite growth and particularly of axonal growth in hippocampal neurons. Varp is able to interact with TI-VAMP, and expressing ID strongly impairs neurite growth. Furthermore, Varp activates Rab21 through its Vps9 domain, and a form of Varp lacking this domain inhibits neurite growth. Silencing the expression of Varp also inhibits neurite growth. Altogether these data suggest that Varp is required for neurite growth and that both Vps9 domain and ID are important for its function. Our demonstration of a positive function of Varp and Rab21 in neuritogenesis, distinct from the negative roles of Rab5 and Rab11, is an important step towards a precise characterization of the function of these highly homologous Rab proteins. Indeed, it has been recently proposed that nerve-growth-factor-mediated neuritogenesis requires the down-regulation of Rab5 by RabGAP5, a Rab5 GTPase-activating protein that interacts with the nerve growth factor receptor TrkA (Liu *et al*, 2007). Structural studies further support the idea that Rab5 and Rab21 are likely to have different effectors (Delprato & Lambright, 2007). In addition, protrudin is required for neurite growth and operates through interaction with the GDP-bound form of Rab11 (Shirane & Nakayama, 2006). The characterization of the upstream signalling pathways that control Varp and the downstream effectors of Rab21 is now crucial to understand how

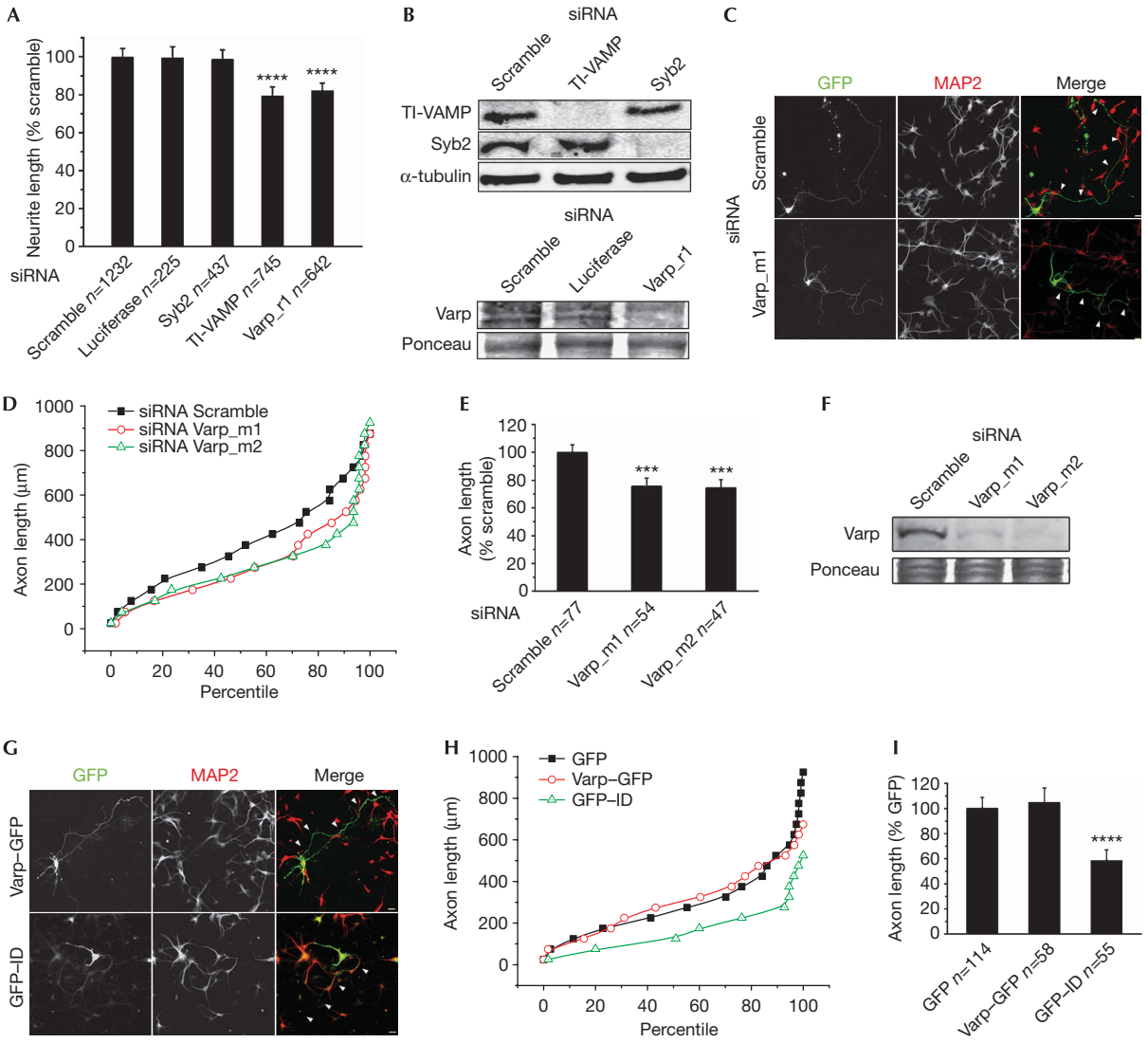


**Fig 3** | Varp regulates neurite growth in PC12 cells and mouse hippocampal neurons. (**A**) Rat PC12 cells were treated with siRNAs against scramble, luciferase, Syb2, TI-VAMP or Varp (Varp_r1) for 72 h, differentiated with 100 nM staurosporine for 2–12 h and then immunostained alternatively for TI-VAMP, Syb2 or Varp and tubulin (data not shown). Neurite growth was impaired after the depletion of TI-VAMP and Varp but not of Syb2. (**B**) The degree of silencing of TI-VAMP, Syb2 and Varp expression in PC12 cells was assessed by western blot analysis. (**C**) DIV1 mouse hippocampal neurons were co-transfected either with two Varp siRNA oligonucleotides (Varp_m1 and Varp_m2) or scramble siRNA and EGFP as a reporter gene and fixed after a further 72 h. The structure of the longest process, that is, the axon (MAP2 negative) is indicated by arrowheads in the merge. (**D**,**E**) Quantification of the effect of Varp silencing on axonal length in GFP-positive cells represented as percentile (**D**) and average (**E**). (**F**) Efficiency of mouse Varp siRNA oligonucleotides assessed by western blotting on mouse L-929 cells. (**G**) DIV1 mouse hippocampal neurons were transfected with Varp–GFP, GFP–ID or GFP, and stained for GFP (green) and MAP2 (red) after a further 48 h. (**H**,**I**) Quantification of the effect of Varp and ID overexpression on axonal length represented as percentile (**H**) and average (**I**). Percentile representations in (**D**,**H**): values on the *x*-axis indicate the percentage of axons shorter than the length indicated on the *y*-axis. The shift towards the bottom indicates decreased axonal growth. See also supplementary information for the mean of axon length. Significance determined by two-tailed unpaired *t*-test ****$P<0.0001$, ***$P<0.005$. Data are shown as mean ± s.e.m. Scale bars, 20 μm. DIV, days *in vitro*; EGFP; enhanced green fluorescent protein; GFP; green fluorescent protein; ID, interaction domain; MAP2, microtubule-associated protein 2; *n*, number of neurites or axons; siRNA, small interfering RNA; Syb2, synaptobrevin 2; TI-VAMP, tetanus neurotoxin-insensitive vesicle-associated membrane protein; Varp, Vps9 domain and ankyrin-repeat-containing protein.

vesicular trafficking might be regulated by neurotrophins and guidance cues, and how it might fine-tune neurite growth.

In conclusion, we have previously shown that the v-SNARE TI-VAMP mediates a membrane trafficking pathway involved in neurite growth (Martinez-Arca *et al*, 2000, 2001; Alberts *et al*, 2003; Tsaneva-Atanasova *et al*, 2009), and in this study we have shown that the Rab21 GEF Varp, one of its partners, positively regulates neurite growth. The precise molecular role of Varp is

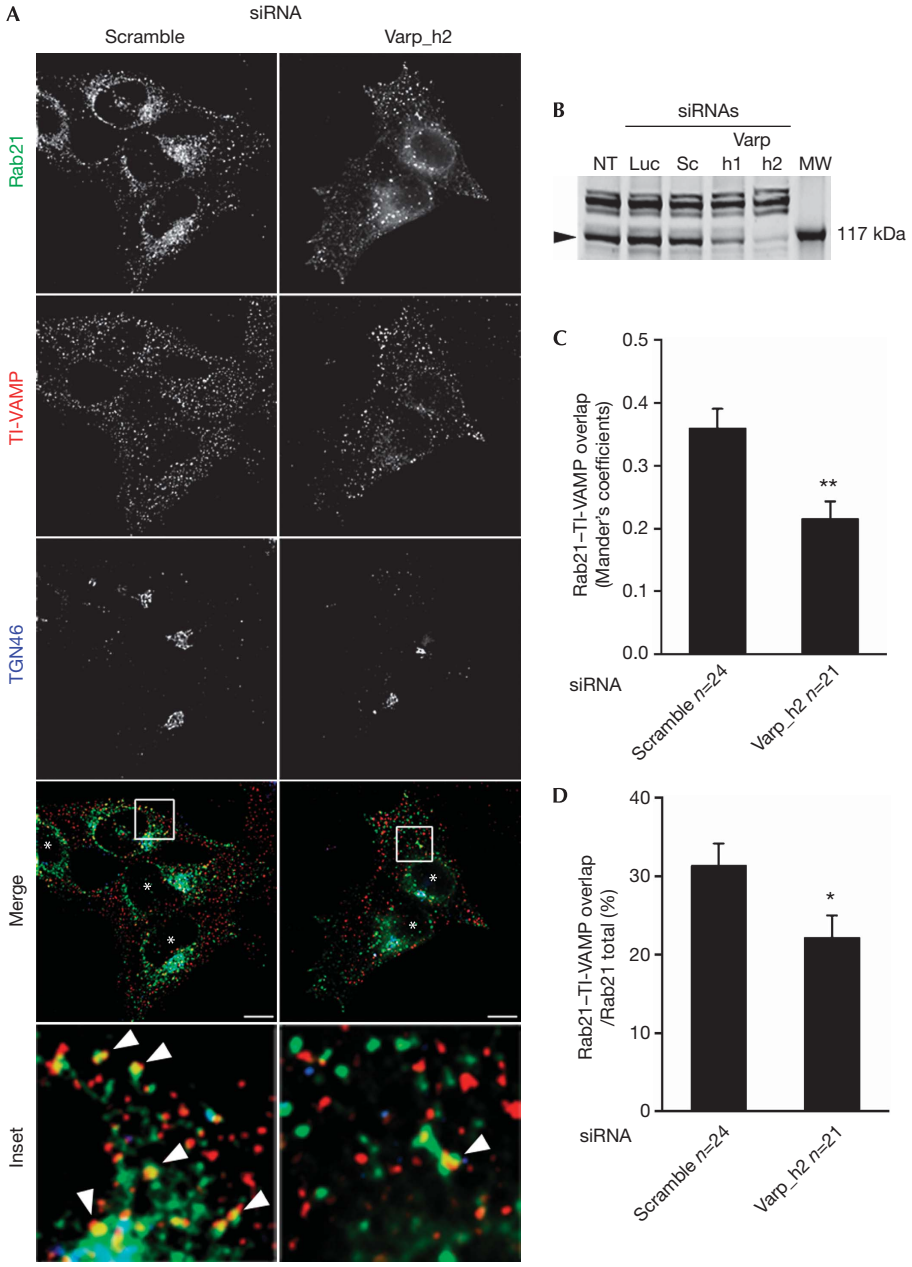


**Fig 4** | Varp regulates co-localization of TI-VAMP and Rab21. (**A**) HeLa cells were treated with scramble or Varp (Varp_h2) oligonucleotides for 72 h and then transfected with GFP–Rab21-wt. After 24 h, cells were fixed and labelled for GFP, TI-VAMP and TGN46. Images were processed for deconvolution. Asterisks indicate nuclei. Arrowheads in the inset indicate co-localization of TI-VAMP and Rab21. Scale bar, 10 μm. (**B**) Western blot showing the efficiency of Varp silencing in HeLa cells transfected with siRNA oligonucleotides against luciferase (Luc), Scramble (Sc) or Varp (Varp_h1 or _h2) compared with not transfected cells. Arrowhead, Varp. (**C**,**D**) Co-localization of Rab21 and TI-VAMP in control cells (Scramble) and in Varp-silenced cells represented as Mander's coefficients (**C**) and as percentage of the number of pixels that overlap between TI-VAMP and Rab21–GFP staining on the total number of pixels for Rab21–GFP staining (**D**). Significance determined by two-tailed unpaired *t*-test $*P<0.05$, $**P<0.01$. Data are shown as mean ± s.e.m. GFP, green fluorescent protein; *n*, number of cells; NT, not transfected cells; TGN, *trans*-Golgi network; siRNA, small interfering RNA; TI-VAMP, TI-VAMP, tetanus neurotoxin-insensitive vesicle-associated membrane protein; Varp, Vps9 domain and ankyrin-repeat-containing protein.

likely to be more complicated than activating Rab21 and regulating TI-VAMP function. Indeed, Varp contains several ankyrin repeats (Mosavi *et al*, 2004). Thus, Varp is likely to interact with yet more proteins through these domains. Furthermore, Varp has also been shown to be an effector of Rab32/38, this interaction being mediated by the first ankyrin repeat (Wang *et al*, 2008; Tamura *et al*, 2009). This is reminiscent of the function of Sec2 in yeast, a GEF of Sec4; the targeting of Sec4 to secretory vesicles

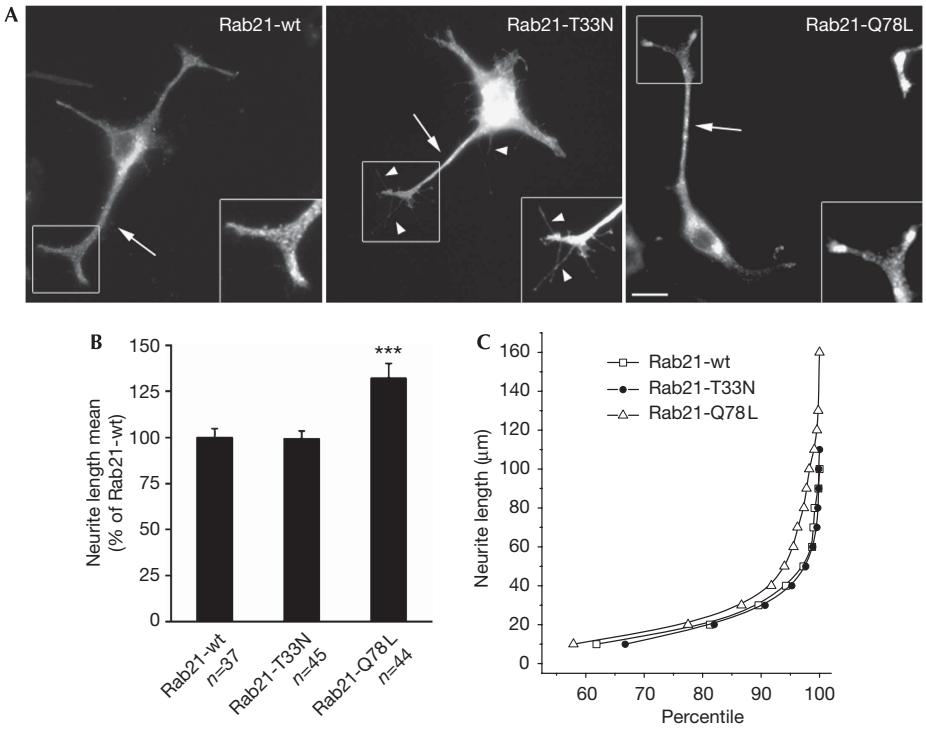


**Fig 5** | Rab21 regulates neurite growth. (**A**) Effects of Rab21 mutants in differentiating PC12 cells transfected with either GFP–Rab21-wt or -T33N or -Q78L. Arrows indicate neurites and arrowheads indicate small protrusions. Scale bar, 20 μm. (**B**) Quantification of the neurites longer than 40 μm in differentiated PC12 cells expressing Rab21 mutants. Significance determined by two-tailed unpaired *t*-test ***$P<0.005$. Data are shown as mean ± s.e.m. (**C**) Quantification of the length of all neurites (>5 μm) represented as percentile. Significance determined by Kolmogorov test. Rab21 Q78L vs Rab21 wt $P<0.05$. Rab21 T33N vs Rab21 wt $P<0.01$. GFP, green fluorescent protein; *n*, number of neurites; wt, wild type.

is regulated by Ypt32, another Rab (Ortiz *et al*, 2002). In this context, Varp might seem to be an important molecular adaptor, functionally connecting and/or activating several proteins involved in different important processes that require vesicular trafficking, including neurite growth. Thus, we anticipate that Varp is likely to regulate other functions mediated by TI-VAMP-dependent membrane trafficking routes (Proux-Gillardeaux *et al*, 2005). Future studies will therefore be required to characterize the molecular functions of Varp, particularly how the activation by Rab32/38, the activation of Rab21 and binding to TI-VAMP are regulated in time and space.

## METHODS

**Cell culture and complementary DNA transfection.** HeLa and L-929 cells were grown in Dulbecco's modified Eagle medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum. PC12 cells were grown in RPMI (Roswell Park Memorial Institute) medium (Invitrogen) with 10% horse serum and 5% fetal calf serum. Hippocampal neurons were prepared from newborn P0 mice and grown on poly-Lysine-coated coverslips in Neurobasal medium supplemented with 2% B27 and 2 mM glutamine. HeLa, PC12 and hippocampal neurons at 1 day *in vitro* (DIV) were transfected with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

**siRNA knockdown.** Varp, Syb2 and TI-VAMP RNA interference were achieved by using specific pre-designed siRNA duplexes (see supplementary information online for details). For every experiment carried out, non-targeting siRNAs and mock transfection were used as controls. Human HeLa cells and mouse L-929 cells were transfected once with oligonucleotides by using oligofectAMINE (Invitrogen) according to the manufacturer's instructions and cultured for a further 72–96 h. PC12 cells were transfected by using AMAXA Nucleofector™ technology (Amaxa, Köln, Germany) twice on two consecutive days. At 48 h after the second transfection, cells were differentiated with staurosporine at 37 °C, fixed and processed for analysis. Mouse hippocampal neurons were co-transfected at DIV1 with oligonucleotides and enhanced GFP by using lipofectAMINE 2000, according to the manufacturer's instructions, and cultured for an additional 72 h before being processed for immunofluorescence.

**Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

T.G. and V.P.-G. are both senior authors on this paper. We thank F. Saudou, B. Goud (Institut Curie, France), François Darchen (Institut de Biologie Physico-Chimique, France) and A. Zahraoui (CEA Saclay, France) for helpful comments; L. Cabanié and F. Cordelières (Institut Curie, France) for technical assistance. This study was funded in part by grants from Institut National de la Santé et de la Recherche Médicale (INSERM; Avenir Program), the European Commission ('Signalling and Traffic' STREP 503229), the Association pour la Recherche sur le Cancer,

the Agence Nationale de la Recherche, the Association Française contre les Myopathies (AFM), the Mairie de Paris Medical Research and Health Program and the Fondation pour la Recherche Médicale (FRM, to T.G.). A.B. was supported by an INSERM and AFM post-doctoral fellowship. E.S. was supported by the Ministère de la Recherche et des Technologies and FRM.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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