PTEN couples Sema3A signalling to growth cone collapse

Neil H. Chadborn[1,*], Aminul I. Ahmed[1,*], Mark R. Holt[2], Rabinder Prinjha[3], Graham A. Dunn[2], Gareth E. Jones[2] and Britta J. Eickholt[1,‡]

[1]MRC Centre for Developmental Neurobiology and [2]Randall Division of Cell and Molecular Biophysics, King's College London, Guy's Campus, London, SE1 1UL, UK
[3]GlaxoSmithKline, Neurodegeneration Research, Neurology and GI CEDD, Third Avenue, Harlow, Essex, CM19 5AW, UK
*These authors contributed equally to this work
‡Author for correspondence (e-mail: Britta.J.Eickholt@kcl.ac.uk)

Accepted 18 November 2005
Journal of Cell Science 119, 951-957 Published by The Company of Biologists 2006
doi:10.1242/jcs.02801

Summary

Distinct changes in glycogen synthase kinase-3 (GSK-3) signalling can regulate neuronal morphogenesis including the determination and maintenance of axonal identity, and are required for neurotrophin-mediated axon elongation. In addition, we have previously shown a dependency on GSK-3 activation in the semaphorin 3A (Sema3A)-mediated growth-cone-collapse response of sensory neurons. Regulation of GSK-3 activity involves the intermediate signalling lipid phosphatidylinositol 3,4,5-trisphosphate, which can be modulated by phosphatidylinositol 3-kinase (PI3K) and the tumour suppressor PTEN. We report here the involvement of PTEN in the Sema3A-mediated growth cone collapse. Sema3A suppresses PI3K signalling concomitant with the activation of GSK-3, which depends on the phosphatase activity of PTEN. PTEN is highly enriched in the axonal compartment and the central domain of sensory growth cones during axonal extension, where it colocalises with microtubules. Following exposure to Sema3A, PTEN accumulates rapidly at the growth cone membrane suggesting a mechanism by which PTEN couples Sema3A signalling to growth cone collapse. These findings demonstrate a dependency on PTEN to regulate GSK-3 signalling in response to Sema3A and highlight the importance of subcellular distributions of PTEN to control growth cone behaviour.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/5/951/DC1

Key words: PI3K, GSK-3, PTEN, Axon guidance, Semaphorin 3A, Cytoskeleton, Signal transduction

Introduction

During the development of the nervous system a complex network of events determines the shape and functional connectivity of neurons, resulting in the known complexity of neuronal circuits. Phosphatidylinositol 3-kinases (PI3K) have emerged as key enzymes in contributing to the appropriate integration of neurons into functional circuits. They mediate intracellular signalling, which influences neuronal migration and neurite extension, as well as mechanisms that control the function of guidance molecules (Atwal et al., 2003; Beffert et al., 2002; Bock et al., 2003; Campbell and Holt, 2001; Zhou et al., 2004). Activation of PI3K results in the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3). Through protein effectors containing pleckstrin-homology (PH) domains, PIP3 is known to modulate the activities of a number of signalling molecules, such as guanine nucleotide exchange factors (GEFs), phosphoinositide-dependent kinases (PDKs) and Akt (Downward, 2004). Locally, these proteins may cause structural rearrangements of the cytoskeleton, for example modulating actin polymerisation and causing localised zones of membrane protrusions. Levels of PIP3 are additionally controlled through de-phosphorylation by a PI3-phosphatase activity, which is mediated by PTEN (phosphatase and tensin homologue deleted on chromosome ten). PTEN is a tumour suppressor that has been shown to antagonise motility, inhibit cell cycle progression, and induce apoptosis in different cell types (Leslie and Downes, 2004), mainly through inhibition of PI3K signalling, but also through mechanisms independent of its lipid phosphatase activity (Raftopoulou et al., 2004). Patients carrying mutations of PTEN show a predisposition to develop tumours that can coincide with defects in neural development such as macrocephaly, mental retardation, cerebellar hypertrophy, ataxia and seizures (Li et al., 1997), and conditional-knockout mice have indicated that PTEN may control neuronal migration and/or neurite extension (Marino et al., 2002).

We have investigated the function of PTEN in sensory growth cones during the growth cone collapse response mediated by semaphorin 3A (Sema3A). Our findings provide evidence that neuronal PTEN associates with microtubules and functions as an essential mediator of PI3K and glycogen synthase kinase-3 (GSK-3) signalling in response to Sema3A.

Results

We have previously shown that GSK-3 activation is required for the Sema3A-induced growth cone collapse in DRG neurons (Eickholt et al., 2002). This led to the characterisation of an inactive pool of GSK-3 at the leading edge of growth cones. Here, we investigate the mechanisms that regulate GSK-3 during growth cone advance, and evaluate their requirements for growth cone collapse. GSK-3 is a downstream signalling mediator of the PI3K/Akt pathway in many cell types (Chung

Journal of Cell Science

et al., 2001) and consistent with a role for Akt in the negative regulation of GSK-3 in neurons, it is well established that inhibition of PI3K leads to the activation of GSK-3 (Cross et al., 1995; Eickholt et al., 2002). We have used time-lapse analysis to explore the dynamics of the growth cone collapse through acute PI3K inhibition and reveal two features. First, highly motile growth cones that migrated at an average speed of around 1 μm/minute collapsed within minutes after application of 10 μM LY294002, but fully recovered their outspread morphology by 30 minutes while still in the presence of LY294002 (Fig. 1A,B, supplementary material Movie 1). Second, after the collapse response, a retraction in the axon was evident within the first 10 minutes of LY294002 application, followed by the rapid recovery of the growth cone and continuation of forward migration (Fig. 1A,B, supplementary material Movie 1). In control experiments, applications of DMSO (Fig. 1B), or direct inhibition of casein kinase-2, an additional target of LY294002, using DRB did not mimic the collapse response (data not shown). Next, we set out to test whether GSK-3 is a cellular substrate of PI3K inhibition. Prior to application of 10 μM LY294002, cultures were treated with the GSK-3 inhibitors LiCl (20 mM), SB216763 (10 μM) or SB415286 (40 μM). The LY294002-mediated growth cone collapse was greatly reduced in the presence of the GSK-3 inhibitors (Fig. 1C).

An increase in PIP3 antagonises Sema3A-induced growth cone collapse

If inhibition of a basal activity of PI3K is sufficient to cause a collapse of growth cones, it would be predicted that increasing the production of PIP3 results in growth cones becoming more resistant to extracellular ligands known to induce a growth cone collapse, such as Sema3A (Eickholt et al., 1997). Levels of PIP3 are modulated by the activities of PI3K and PTEN, the PI3-specific phosphatase, and we took two different approaches to increase endogenous PIP3 in dorsal root ganglion (DRG) neurons. Firstly, stimulation of PI3K was achieved using a small phosphopeptide derived from the PDGF receptor (740Y-P). 740Y-P has previously been shown to mimic receptor-dependent activation of PI3K in vitro and in vivo (Bron et al., 2003; Rordorf-Nikolic et al., 1995). When synthesised in tandem with the known internalisation motif ‘antennapedia’, the peptide can enter freely into cells, and has been demonstrated to stimulate neuronal survival in cerebellar neurons in a PI3K-dependent manner (Williams and Doherty, 1999). During growth cone collapse, application of 740Y-P resulted in a decreased sensitivity to Sema3A (Fig. 2A). Normal Sema3A-induced collapse was restored when 740Y-P was added together with LY294002, highlighting the specific action of the 740Y-P peptide on PI3K (Fig. 2A). Moreover, a peptide corresponding to the 1309Y-P Shc SH2 binding site on the ErbB3 receptor (Dunican et al., 2001) did not interfere with the Sema3A-induced growth cone collapse (Fig. 2A). In the second approach we altered PIP3 levels by expression of a dominant-negative PTEN. The tumour suppressor PTEN is known to antagonise the PI3K signalling pathway by reducing PIP3 levels (Ono et al., 2001). A naturally occurring mutation is the single residue substitution within the catalytic domain (PTEN C124S) (Marsh et al., 1997), which inactivates enzymatic function and has been shown to inhibit endogenous PTEN in a dominant-negative manner (Ono et al., 2001). In

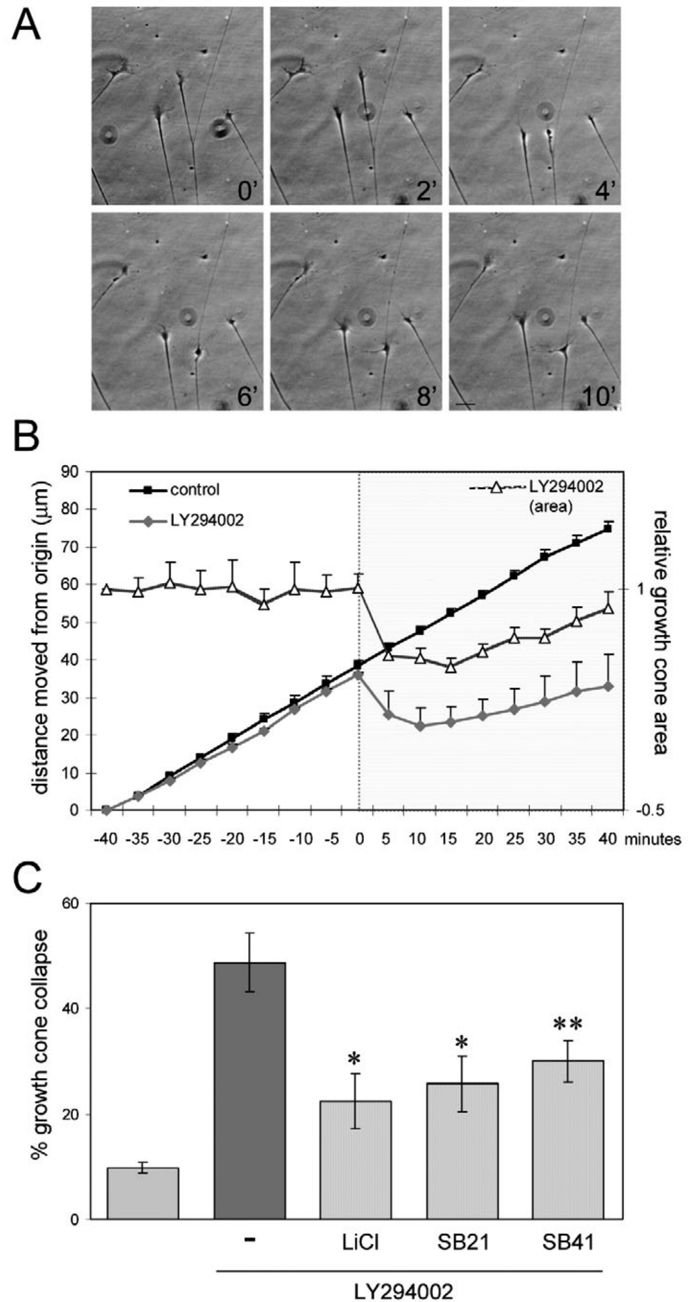

Fig. 1. Inhibition of PI3K is sufficient to induce a growth cone collapse. (A) Single frames show a DRG growth cone before (0’), and following incubation with 10 μM LY294002 every 2 minutes (see Movie 1 in supplementary material). (B) Data charting the displacement (in μm + s.e.m.) of at least 15 growth cones in each of three independent experiments. LY294002 (10 μM) or control (DMSO) was applied to DRG cultures after 40 minutes (dotted line). Treatment with the inhibitor induces a retraction within 5 minutes and neurons start extending at half the original rate within 15 minutes. This response coincides with a drop in growth cone area, followed by the recovery in area during recovery of forward movement. (C) In the presence of the GSK-3 inhibitors, LiCl (20 mM), SB216763 (10 μM) or SB415286 (40 μM), the LY294002-induced growth cones collapse (10 μM, 10 minutes) is reduced. Each data point is the mean ± s.e.m. of six experiments, with $n \geqslant 60$ growth cones in each experiment. *$P \leqslant 0.006$; **$P \leqslant 0.01$. Scale bar, 10 μm.

DRG neurons, overexpression of GFP-PTEN C124S significantly attenuated the Sema3A response (Fig. 2B) whereas overexpression of wild-type GFP-PTEN showed a similar response to expression of control GFP.

PTEN is required for Sema3A mediated Akt/GSK-3 signalling

The results from the previous experiments suggest that PI3K activity is a determining event during growth cone expansion and growth cone collapse, and thus Sema3A-mediated growth cone collapse may directly involve the inhibition of this pathway. To monitor changes in activity we challenged cultures of N1E-115 neuroblastoma cells, which have previously been shown to exhibit morphological changes reminiscent of a collapse response when stimulated with Sema3A (Brown et al., 2004; van Horck et al., 2002). In control experiments, NIE-115 cells overexpressing GFP, Sema3A application at 1 μg/ml for 5, 10, 20 and 30 minutes induced marked decreases in phosphorylation levels of Akt in a time-dependent manner (P-Ser473, Fig. 3A). We also probed for P-GSK-3β (P-Ser9) and found similar decreases to those we reported previously (Eickholt et al., 2002). These data might indicate a direct interference of PI3K signalling as has been suggested for Sema3F-induced growth cone collapse (Atwal et al., 2003). Alternatively, decreases in P-Akt and P-GSK-3 in response to Sema3A may require the lipid phosphatase activity of PTEN. To test this possibility we overexpressed GFP-PTEN and the phosphatase-deficient GFP-PTEN C124S. Overexpression of GFP-PTEN did not alter the ability of Sema3A to decrease phosphorylation of Akt and GSK-3β. By contrast, overexpression of GFP-PTEN C124S rendered these cells insensitive to changes in phosphorylation (Fig. 3C,F), although LY294002 treatment still decreased Akt and GSK-3β phosphorylation (Fig. 3E,F). We also decreased PTEN protein levels in NIE-115 cells using specific siRNA, which, as expected, resulted in the desensitisation of the cells to exhibit decreases in Akt and GSK-3β phosphorylation in response to Sema3A (Fig. 3D).

In addition to growth cones and N1E-115 neuroblastoma cells, several other cell types are responsive to Sema3A (Eickholt et al., 1999; Eickholt et al., 2002), and we have previously used MDA-MB-231 cells expressing the 'neuronal' Sema3A receptor complex to substantiate the biochemical parameters of Sema3A-induced activation of GSK-3 (Eickholt et al., 2002). Similarly to the result in N1E-115 cells, Sema3A application decreased the levels of P-Akt and P-GSK-3 substantially (supplementary material Fig. S1). We also tested MDA-MB 468 cells, which are known to be deficient in PTEN (Howells et al., 2002) (supplementary material Fig. S2). Application of Sema3A to these cells had no effect on either Akt or GSK-3 phosphorylation. MDA-MB 468 cells were transfected with GFP-PTEN and showed a decrease in both P-Akt and P-GSK-3 levels following stimulation with Sema3A. This is in contrast to expression of the phosphatase-deficient GFP-PTEN C124S, which did not rescue the response to Sema3A (supplementary material Fig. S1).

These results, in combination with our observation that the Sema3A collapse is significantly reduced in the presence of inactive PTEN, suggests that Sema3A-mediated attenuation in PI3K/GSK-3 signalling may also depend on the lipid phosphatase in neurons.

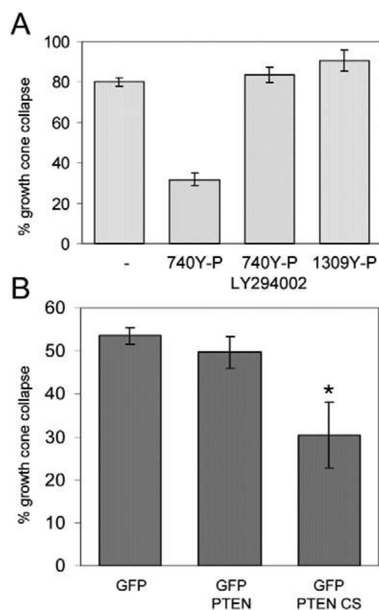

Fig. 2. Increases in PIP3 by activation of PI3K or inactivation of PTEN antagonises Sema3A-induced growth cone collapse. (A) Chick DRG explants were cultured overnight in the presence of NGF. Prior to application of Sema3A-Fc at 1 μg/ml, the cultures were incubated with the PI3K-activating 740Y-P peptide at 40 μg/ml for 30 minutes, with the 740Y-P peptide and LY294002 (10 μM), or with the Y-P-peptide 1309 (at 40 μg/ml). (B) GFP, GFP-PTEN or phosphatase-deficient GFP-PTEN C124S were nucleofected into chick DRG neurons, cultured for 24 hours and stimulated with Sema3A as before. Each data point is the mean ± s.e.m. of at least three independent experiments; **P*<0.05.

PTEN colocalises with microtubules in DRG neurons

PTEN is an evolutionary conserved phosphatase implicated in several aspects of nervous system development and/or function (Lachyankar et al., 2000; Li et al., 2003). In the CNS, PTEN protein is expressed during late embryonic and adult stages and is enriched in different neuronal populations (Lachyankar et al., 2000). In order to evaluate the distribution of PTEN in DRG neurons we sectioned embryonic rat tissue and probed for PTEN using a specific anti-PTEN antibody. Analysis of the subcellular distributions by confocal microscopy revealed the presence of PTEN in the cytoplasm of DRG neurons and, at low levels, in the nucleus (Fig. 4A). Noticeably, PTEN levels were elevated in the peripheral projections of the DRG neurons relative to the signal detected within the cell body demonstrating an axonal enrichment of PTEN protein. To elucidate the subcellular distribution in growth cones we also labelled dissociated DRG neurons cultured from E18 rat embryos with the PTEN antibody by confocal microscopy. We detected little PTEN in the peripheral (P)-domain and/or PTEN that associated with the plasma membrane (Fig. 4B); instead, PTEN protein was highly enriched in the central (C)-domain of the growth cone where it accumulated with βIII-positive microtubules (Fig. 4B). A similar co-distribution with βIII-immunolabelling was detected in NIE-115 neuroblastoma cells (Fig. 5A). Specificity of the PTEN antibody was validated by the absence of immunolabelling following siRNA depletion of PTEN in NIE-115 (Fig. 5B,C). We also analysed the

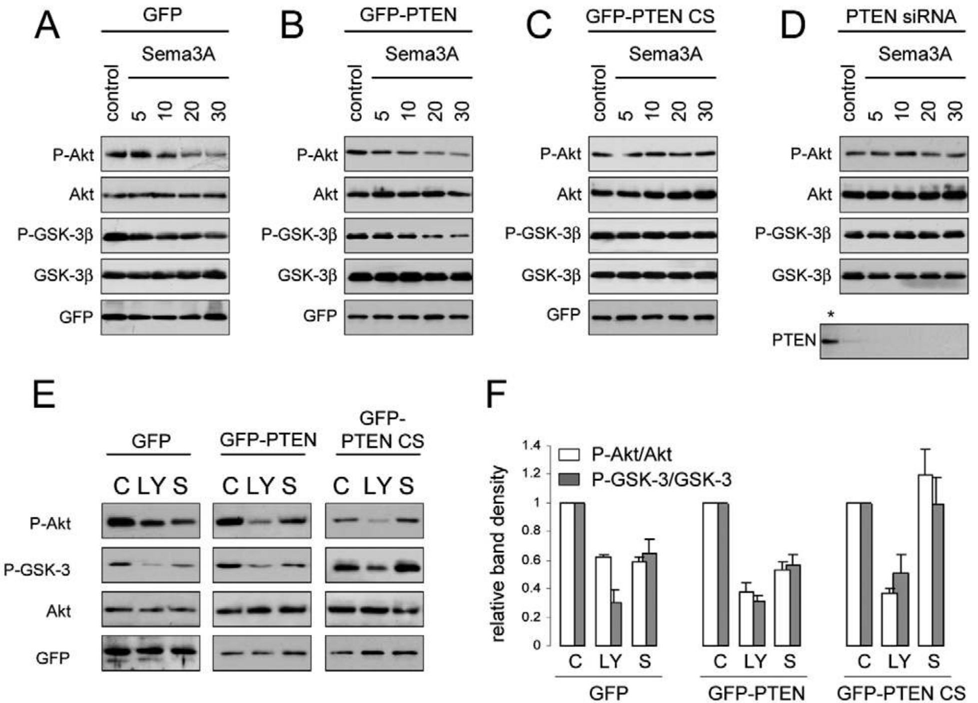

Fig. 3. PTEN is required for Sema3A-mediated Akt/GSK-3 signalling in N1E-115 cells. N1E-115 cells were transfected with GFP, GFP-PTEN or GFP-PTEN C124S (GFP-PTEN CS) and stimulated with Sema3A (1 μg/ml) for 5, 10, 20 or 30 minutes (A,B,C). Western blots using P(Ser473)-Akt and P(Ser9)GSK-3β antibodies reveal a decrease in phosphorylation following Sema3A treatment in GFP- and GFP-PTEN-expressing cells, but not in cells expressing GFP-PTEN C124S. (D) N1E-115 cells were transfected with a nonspecific siRNA (*) or PTEN siRNA, and stimulated with Sema3A as previously. Knockdown of PTEN rendered NIE-115 cells insensitive to changes in GSK-3 and Akt phosphorylation in response to Sema3A. (E) In addition, the Sema3A response at 10 minutes (S) was compared with LY294002 treatments (LY, at 10 μM for 10 minutes). (F) Normalised relative band density (P-Akt/Akt, P-GSK-3/GSK-3) of each transfection experiment (mean + s.e.m. of three experiments).

distribution of PTEN by expression of a GFP-tagged PTEN in NIE-115 cells and found an enrichment with microtubules (Fig. 5D).

PTEN accumulates at the membrane during Sema3A-induced growth cone collapse

If PTEN mediates the Sema3A-induced growth cone collapse and functions to antagonise PI3K signalling, then an increase or a more stable recruitment to the growth cone membrane would be predicted. To test this hypothesis we expressed GFP-PTEN as previously, and monitored putative changes in subcellular distribution during the Sema3A growth cone collapse (Fig. 6). We used a Nipkow spinning-disc confocal microscope, which has the advantage over other systems of causing minimal phototoxicity while providing confocal vertical resolution (0.8 μm). Similar to the distribution of endogenous PTEN, GFP-PTEN is enriched in the axonal compartment and the C-domain of the growth cone, with relatively low levels seen in the P-domain (Fig. 6A, supplementary material Movie 2). In order to preserve the spatial resolution needed to analyse signalling events, we induced a partial-collapse response, by culturing neurons on glass coverslips, which provide a more adhesive substratum. Following treatment with Sema3A under these conditions we find all the features of a full growth-cone collapse; however, the small decreases in spread morphology (rather than full collapse) allow analysis by time-lapse microscopy (supplementary material Movie 2). Application of Sema3A

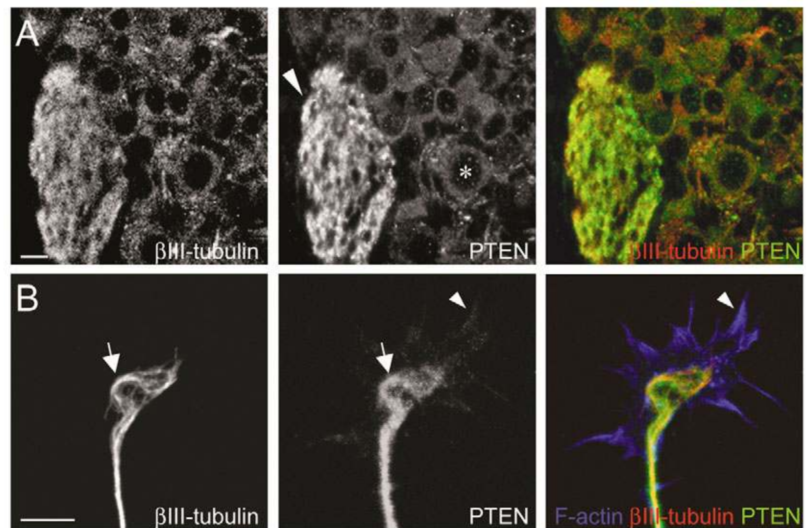

Fig. 4. PTEN is enriched in the axon of DRG neurons where it accumulates with microtubules. (A) A single confocal section taken through an E18 trunk of a rat embryo labelled with anti-βIII-tubulin (left) and anti-PTEN (middle) antibodies. PTEN is present in the nucleus of DRG neurons (*) and the surrounding cytosol and in the axons of the peripheral projection of the DRGs (arrowhead). (B) Rat E18 DRG neurons were triple-labelled with anti-βIII-tubulin (left), anti-PTEN (middle) antibodies and phalloidin (blue channel, right). In the growth cone, PTEN colocalises with the microtubule in the C-domain (arrow), whereas little signal is detected in the P-domain or the growth cone membrane (arrowhead). Bars, 10 μm.

Journal of Cell Science

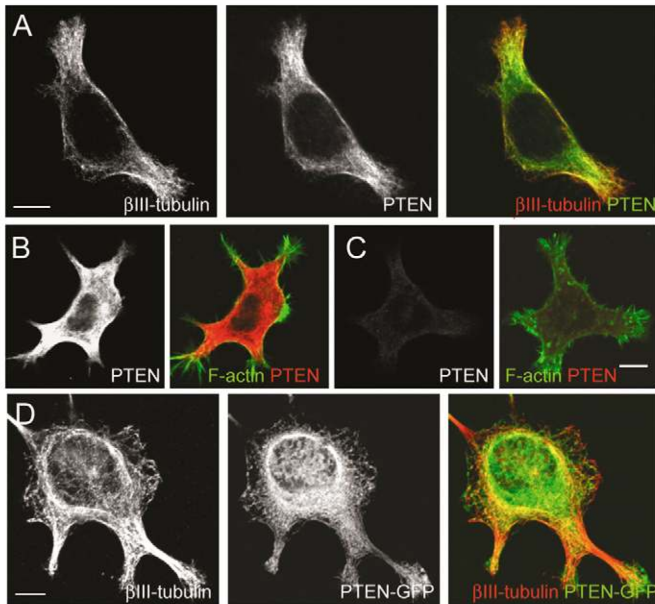

Fig. 5. PTEN and GFP-PTEN are enriched at microtubules in N1E-115 cells. (A) NIE-115 cells were labelled with anti-βIII-tubulin (left) and anti-PTEN (middle) antibodies. Similarly to primary DRG neurons, there is an accumulation of endogenous PTEN with microtubules in N1E-115 cells. (B,C) NIE-115 cells were transfected with a control siRNA (B) or PTEN specific siRNA (C), and labelled with the anti-PTEN antibody (left) and Phalloidin to visualise F-actin. Confocal micrographs, taken with identical scanning parameters, demonstrate the loss of PTEN signal in the PTEN siRNA-treated cells. (D) Similarly to the distribution of endogenous PTEN, GFP-PTEN accumulates with microtubules in N1E-115 cells. Bars, 10 μm.

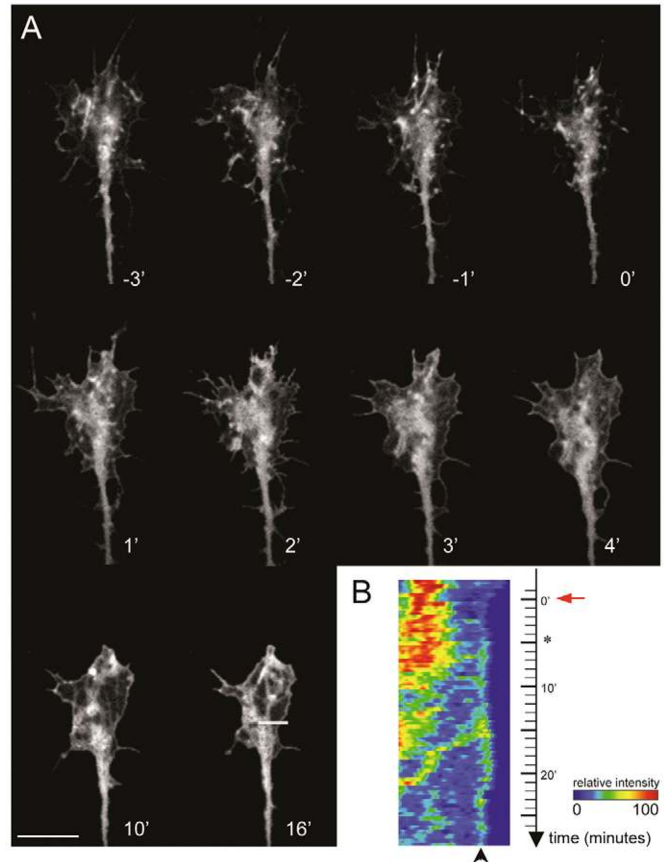

Fig. 6. PTEN accumulates at the growth cone membrane during Sema3A mediated growth cone collapse. (A) DRG neurons from E18 rat embryos were nucleofected with GFP-PTEN and cultured overnight in vitro. Growth cones were imaged by confocal time-lapse microscopy at 1 frame every 30 seconds. The presented selection of images shows a growth cone 3 minutes before (–3', –2', –1') and during the response to Sema3A, which was applied at 0'. Following Sema3A application, fluorescent signals of GFP-PTEN increase uniformly at the membrane. See also Movie 2 in supplementary material. (B) A 3-pixel section in each frame was taken at the level indicated at 16' in A and pasted side by side. To facilitate the visualisation of movement and changes in relative fluorescence intensity at the membrane over time, we applied pseudo colour (see arrowhead). Following Sema3A addition at 0' (arrow) relative levels of PTEN increased at the membrane after 4 minutes (asterisk). Bar, 10 μm.

induces a rapid increase of GFP-PTEN signals within the P-domain and accumulations at the membrane that were clearly visible after approximately 4 minutes (Fig. 6). We created a kymograph along a 3-pixel-wide line region in each frame of a time-lapse series that were pasted side by side. To facilitate the visualisation of movement and changes in intensity level over time we applied pseudo colour. Before addition of Sema3A, a high accumulation is seen in the C-domain, whereas the P-domain shows levels only just above background (Fig. 6B, blue, location of the membrane is indicated by the arrowhead). Four minutes after addition of Sema3A (asterisk), the membrane signal increases (Fig. 6B, blue to green). These data indicate that PTEN localises to the membrane of the growth cone upon Sema3A addition and highlights a novel mechanism by which PI3K signalling is attenuated during Sema3A-induced growth cone collapse.

Discussion

Cellular polarisation and migratory responses require signal integration between the plasma membrane, actin and microtubules. Recent experiments indicate a key role for the lipid products of PI3K in this process because perturbation of PI3K function by pharmacological inhibitors impairs cell polarity, chemotaxis and/or motility in several cell types including human neutrophils and *Dictyostelium* (Funamoto et al., 2002; Grabham et al., 2003; Iijima and Devreotes, 2002; Parent, 2004; Sawyer et al., 2003; Wang et al., 2002). Similarly, our results in DRG growth cones show that following application of PI3K inhibitor, a transitory collapse-response is induced with a decrease in forward movement following the morphological recovery of the growth cone. Although our experiments did not examine effects in response to chemotactic gradients, extending neurites exhibit an inherently polarised morphology, by the presence of the 'trailing' axon that separates the highly motile growth cone from the stationary neuronal cell body. Examination of the localisation of PTEN revealed that the vast majority of PTEN accumulates with the microtubules in the C-domain. This sequestration results in the relative inaccessibility of the phosphatase to its substrate required for catalysis and may explain why in our experiments overexpression of GFP-PTEN did not lead to any obvious defect in growth cone migration behaviour. Similarly,

overexpression of GFP-PTEN in NIE-115 cells did not decrease basal levels of Akt or GSK-3 phosphorylation in comparison with control GFP-expressing cells.

The importance of spatial restriction of PTEN during cell polarisation and chemotaxis has previously been demonstrated in *Dictyostelium* (Funamoto et al., 2002; Iijima and Devreotes, 2002). Here, PTEN localises to the sides and the rear of chemotacting cells, whereas PI3K accumulates toward the stimulus resulting in a reciprocal localisation. This has been speculated to reinforce PIP3 enrichment, with the highest concentration in the membrane domain towards the front of the cell. In neuronal growth cones, sequestration to microtubules may fulfil a similar function to create a reciprocal relationship between PI3K activity and PTEN. It is interesting to speculate whether in the growth cone PTEN may be delivered by microtubule localisation to the membrane domain to increase membrane dynamics necessary for growth cone advance. However, low concentrations of both microtubule-stabilising and -destabilising drugs inhibit free dynamic microtubule ends in the peripheral domain of the growth cone, without altering neuronal extension or the growth cone morphology (Buck and Zheng, 2002), which would argue against such a model.

Upon stimulation with Sema3A we observed a marked decrease in Akt phosphorylation, an indication of decreased PI3K activity. This repression requires the lipid phosphatase PTEN, based on two lines of evidence: firstly, overexpression of a phosphatase-deficient PTEN desensitises NIE-115 cells to antagonise PI3K signalling in response to Sema3A. Secondly, PTEN-deficient MDA-MB-468 breast carcinoma cells respond to Sema3A only when PTEN was expressed. Interestingly, upon acute exposure to Sema3A in DRG growth cones, PTEN accumulates within minutes at the plasma membrane highlighting a mechanism by which PTEN couples Sema3A signaling to characteristic morphological changes in neurons. Despite the suggested importance in the Sema3A-mediated growth cone collapse, the question remains how PTEN accumulation to the membrane is regulated. Upon Sema3A stimulation PTEN may be released from dynamic microtubules exploring the periphery of growth cones, which allows the membrane targeting and antagonistic effect on PI3K signalling. In this context it is interesting to note that inhibition of microtubule dynamics was indeed found to antagonise repulsive turning at substrate border (Challacombe et al., 1996; Tanaka and Kirschner, 1995; Williamson et al., 1996) and in gradient of inhibitory guidance molecules (Buck and Zheng, 2002).

A connection between neuronal polarity and neuronal elongation to regulate the assembly of microtubules by NGF has recently been shown (Zhou et al., 2004), which linked membrane-localised PI3K activation downstream through a localised inactivation of GSK-3 to the microtubule plus-end-binding protein APC. Our results may be consistent with the putative integration of signals from the back (microtubules) to the front (membrane) that may function to reinforce or re-sensitise PI3K-dependent signalling during growth-factor-promoted elongation and to mediate rapid changes in migration behaviour in response to guidance signals. Since other signals have recently been shown to mediate neuronal migration and axonal remodelling by using PI3K and/or GSK-3 signalling (Beffert et al., 2002; Bock et al., 2003; Del Rio et al., 2004; Gonzalez-Billault et al., 2004; Jiang et al., 2005; Zhou et al., 2004), it is interesting to speculate whether PTEN could be a common neuronal mediator in this pathway.

Materials and Methods

Neuronal cultures

DRGs were dissected from E7 chick embryos (Bovine Goldline, Henry Stewart, Norfolk, UK) or E18 rat embryos in L15 media (Gibco). Extra-mesenchymal tissue was removed using a sharpened 0.2 mm tungsten wire and DRGs were plated on either glass coverslips previously coated with poly-L-lysine followed by laminin (both 20 μg/ml; Sigma) or plated on Labtech permanox chamberslides (Nunc) coated with laminin. They were then incubated at 37°C/5% CO_2 for 20-24 hours in DMEM/10%FCS/Penicillin/Streptomycin supplemented with 20 ng/ml NGF (Promega). Cultures were fixed with pre-warmed 4% paraformaldehyde containing 10% sucrose. Pharmacological inhibitors were used as described in the text; LY294002 was purchased from Calbiochem, the GSK-3 inhibitors SB216763, SB415286 were kindly provided by GlaxoSmithKline and LiCl was from Sigma. The Sema3A-Fc chimera was purified as previously described (Eickholt et al., 1997).

Nucleofection

We used an electroporation-based gene transfer method that is low in toxicity but highly efficient, which relies on the direct introduction of the DNA into the nucleus ('nucleofection'). DRGs from E18 rat embryos were dissected as described previously. DRGs were washed in Hank's medium (without Ca^{2+}/Mg^{2+}), and then incubated in trypsin (1 mg/ml, Worthington) for 10 minutes at 37°C. Neurons were dissociated using a fire-polished Pasteur pipette and then centrifuged at 1000 rpm in a bench microfuge. The medium was removed and replaced with 100 μl nucleofector solution (Amaxa Biosystems). 1×10^6 cells were mixed with 5 μg DNA and nucleofected (program G-13, Nucleofector, Amaxa Biosystems). For live-cell imaging, nucleofected cells were cultured on poly-L-lysine (50 μg/ml)/laminin (20 μg/ml)-coated glass-bottom dishes (MatTek Corporation) for 15 hours.

Video imaging and analysis

DRG explants or nucleofected neurons were cultured for 15 hours on laminin-coated glass-bottom dishes. 20 mM HEPES was added, and cultures were allowed to adapt for at least 2 hours. A layer of mineral oil (Sigma) was added to the dish to prevent evaporation and to stabilise the pH by reducing gaseous exchange. The dishes were transferred to a heated stage mounted on an inverted microscope. Time-lapse phase-contrast images (×10 objective, Axiovert, Zeiss) or GFP fluorescence images (×63 objective, Leica DM IRB with CARV spinning disc confocal) of the growth cones were taken using a Hamamatsu Orca ER Camera. Acquisition software (AQM v6, Kinetic Imaging) collated all images, and the movement of individual growth cones was recorded. Analysis of growth cone behaviour was achieved using an in-house MS-DOS tracking program and subsequent data processing using MS Excel.

Immunocytochemistry

Fixed neuronal cultures (4% paraformaldehyde) were washed twice with PBS, and permeabilised for 5 minutes using 1% Triton X-100/PBS. Phalloidin-Alexa Fluor 488 (in 0.5% Triton X-100/PBS; Molecular Probes) was added for 30 minutes. After three washes, the cultures were mounted with Mowiol (15% Mowiol 4-88, 30% glycerol in PBS with DABCO) onto slides. For each experiment, growth cones were counted as either collapsed (no more than one filopodia) or uncollapsed, and represented as percentages. For immunocytochemistry, fixed cultures were washed twice with PBS, incubated for 10 minutes with 0.5% Triton/PBS, and then blocked for 30 minutes in blocking buffer (2% BSA/0.5% Triton/PBS). They were then incubated with anti-βIII-tubulin (1:500, Covance), anti-PTEN (1:100, Santa Cruz Biotechnology) and phalloidin-Alexa Fluor 568 (or 488) diluted in blocking buffer at 4°C overnight. Following two further washes with PBS, Alexa-Fluor-conjugated secondary antibodies (Molecular Probes) were added for 1 hour. Cultures were then mounted in Mowiol and analysed by confocal microscopy (optical slice size 0.8 μm, Zeiss LSM 5 META confocal laser-scanning microscope). For the PTEN staining of DRGs in situ, E18 rat trunks were embedded in 20% gelatin, sectioned on a vibratome (80 μm) and imaged with a 3 μm confocal slice size.

Peptide synthesis

Peptides were synthesised as previously described (Dunican et al., 2001; Williams and Doherty, 1999) or custom made (Chiron Technologies). Amino hexanoic acid (AHX) functions as a spacer, and the biotin at the N-terminus allows for capturing of the peptides. The Antennapedia internalisation sequence (RQIKIWFQNRRMKWKK) was synthesised in tandem with sequences present in the ErbB3 (AA1309), or the PDGF receptor (AA740). The sequences were as follows: ErbB3 1309, Biot-AHX-RQIKIWFQNRRMKWKKFDNPDY(p)WHSR; PDGF 740, Biot-AHX- RQIKIWFQNRRMKWKKSCGGY(p)MDMS. These peptides have previously been shown to translocate across the membrane of intact cells and show binding to the respective target sequences (the 1309Y-P peptide targets the SH2 domain of Shc, and the 740Y-P targets the SH2 domain of p85).

Journal of Cell Science

Western blots

MDA-MB 231 cells, MDA-MB 468 cells (University of Michigan Human Breast Cell Bank, Michigan, MI) and N1E-115 neuroblastoma cells (kindly provided by Christine Hall, Institute of Neurology, UCL, UK) were grown in DMEM, 10% FCS (Gibco). N1E-115 and 231 cells were transfected with PTEN siRNA (Cell Signaling Technology) by incubating Lipofectamine 2000 (Invitrogen) for 40 hours. 3 hours prior to transfection, N1E-115 cells were transferred to DMEM only. 468 cells were transfected with GFP-PTEN and GFP-PTEN C124S, by nucleofection as above, using program T-20. All cells were treated with Sema3A-Fc (1 μg/ml) or LY294002 (10 μM) and incubated for the indicated time. After lysis [in 250 mM sucrose, 10 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 25 mM NaF, 2 mM sodium orthovanadate, 0.5% NP40, 0.1 mM DTT, 'Complete' protease inhibitor cocktail (Roche, town, Switzerland)] and centrifugation, total protein content was determined by BioRad protein assay. Equal quantities (10 μg) of total protein were loaded onto a 10% polyacrylamide gel. After blotting onto nitrocellulose membrane (Amersham Pharmacia) and blocking (5% milk, 1 hour RT), blots were incubated with anti-P(Ser473)Akt, anti-Akt (Cell Signaling), anti-PTEN (Santa Cruz Biotechnology), anti-actin (Roche), or anti-P(Ser9)GSKβ-3 (Cell Signaling), followed by HRP-conjugated secondary antibody (Vector Labs), both for 1 hour at RT. Blots were developed with ECL plus (Amersham Pharmacia).

We would like to thank Jon Gilthorpe and Nick Leslie for the provision of GFP and PTEN-GFP expression constructs, respectively. We also thank Nick Leslie for much helpful discussion, and Mathias Gautel for the use of his CARV spinning-disc confocal set-up. We would like to thank Patrick Doherty for the provision and advice on the use of peptides. This work was funded by the Wellcome Trust (B.J.E., N.H.C., G.E.J.), King's Trustees (A.A.) and the Medical Research Council (G.E.J., M.H., G.A.D.).

References

Atwal, J. K., Singh, K. K., Tessier-Lavigne, M., Miller, F. D. and Kaplan, D. R. (2003). Semaphorin 3F antagonizes neurotrophin-induced phosphatidylinositol 3-kinase and mitogen-activated protein kinase kinase signaling: a mechanism for growth cone collapse. *J. Neurosci.* **23**, 7602-7609.

Beffert, U., Morfini, G., Bock, H. H., Reyna, H., Brady, S. T. and Herz, J. (2002). Reelin-mediated signaling locally regulates protein kinase B/Akt and glycogen synthase kinase 3beta. *J. Biol. Chem.* **277**, 49958-49964.

Bock, H. H., Jossin, Y., Liu, P., Forster, E., May, P., Goffinet, A. M. and Herz, J. (2003). Phosphatidylinositol 3-kinase interacts with the adaptor protein Dab1 in response to Reelin signaling and is required for normal cortical lamination. *J. Biol. Chem.* **278**, 38772-38779.

Bron, R., Klesse, L. J., Shah, K., Parada, L. F. and Winter, J. (2003). Activation of Ras is necessary and sufficient for upregulation of vanilloid receptor type 1 in sensory neurons by neurotrophic factors. *Mol. Cell Neurosci.* **22**, 118-132.

Brown, M., Jacobs, T., Eickholt, B., Ferrari, G., Teo, M., Monfries, C., Qi, R. Z., Leung, T., Lim, L. and Hall, C. (2004). Alpha2-chimaerin, cyclin-dependent Kinase 5/p35, and its target collapsin response mediator protein-2 are essential components in semaphorin 3A-induced growth-cone collapse. *J. Neurosci.* **24**, 8994-9004.

Buck, K. B. and Zheng, J. Q. (2002). Growth cone turning induced by direct local modification of microtubule dynamics. *J. Neurosci.* **22**, 9358-9367.

Campbell, D. S. and Holt, C. E. (2001). Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. *Neuron* **32**, 1013-1026.

Challacombe, J. F., Snow, D. M. and Letourneau, P. C. (1996). Actin filament bundles are required for microtubule reorientation during growth cone turning to avoid an inhibitory guidance cue. *J. Cell Sci.* **109**, 2031-2040.

Chung, C. Y., Potikyan, G. and Firtel, R. A. (2001). Control of cell polarity and chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKa. *Mol. Cell* **7**, 937-947.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789.

Del Rio, J. A., Gonzalez-Billault, C., Urena, J. M., Jimenez, E. M., Barallobre, M. J., Pascual, M., Pujadas, L., Simo, S., La Torre, A., Wandosell, F. et al. (2004). MAP1B is required for Netrin 1 signaling in neuronal migration and axonal guidance. *Curr. Biol.* **14**, 840-850.

Downward, J. (2004). PI 3-kinase, Akt and cell survival. *Semin. Cell Dev. Biol.* **15**, 177-182.

Dunican, D. J., Williams, E. J., Howell, F. V. and Doherty, P. (2001). Selective inhibition of fibroblast growth factor (FGF)-stimulated mitogenesis by a FGF receptor-1-derived phosphopeptide. *Cell Growth Differ.* **12**, 255-264.

Eickholt, B. J., Morrow, R., Walsh, F. S. and Doherty, P. (1997). Structural features of collapsin required for biological activity and distribution of binding sites in the developing chick. *Mol. Cell Neurosci.* **9**, 358-371.

Eickholt, B. J., Mackenzie, S. L., Graham, A., Walsh, F. S. and Doherty, P. (1999). Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. *Development* **126**, 2181-2189.

Eickholt, B. J., Walsh, F. S. and Doherty, P. (2002). An inactive pool of GSK-3 at the leading edge of growth cones is implicated in Semaphorin 3A signaling. *J. Cell Biol.* **157**, 211-217.

Funamoto, S., Meili, R., Lee, S., Parry, L. and Firtel, R. A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell* **109**, 611-623.

Gonzalez-Billault, C., Del Rio, J. A., Urena, J. M., Jimenez-Mateos, E. M., Barallobre, M. J., Pascual, M., Pujadas, L., Simo, S., La Torre, A., Gavin, R. et al. (2004). A role of MAP1B in reelin-dependent neuronal migration. *Cereb. Cortex* **15**, 1134-1145.

Grabham, P. W., Reznik, B. and Goldberg, D. J. (2003). Microtubule and Rac 1-dependent F-actin in growth cones. *J. Cell Sci.* **116**, 3739-3748.

Howells, L. M., Gallacher-Horley, B., Houghton, C. E., Manson, M. M. and Hudson, E. A. (2002). Indole-3-carbinol inhibits protein kinase B/Akt and induces apoptosis in the human breast tumor cell line MDA MB468 but not in the nontumorigenic HBL100 line. *Mol. Cancer Ther.* **1**, 1161-1172.

Iijima, M. and Devreotes, P. (2002). Tumor suppressor PTEN mediates sensing of chemoattractant gradients. *Cell* **109**, 599-610.

Jiang, H., Guo, W., Liang, X. and Rao, Y. (2005). Both the establishment and the maintenance of neuronal polarity require active mechanisms: critical roles of GSK-3beta and its upstream regulators. *Cell* **120**, 123-135.

Lachyankar, M. B., Sultana, N., Schonhoff, C. M., Mitra, P., Poluha, W., Lambert, S., Quesenberry, P. J., Litofsky, N. S., Recht, L. D., Nabi, R. et al. (2000). A role for nuclear PTEN in neuronal differentiation. *J. Neurosci.* **20**, 1404-1413.

Leslie, N. R. and Downes, C. P. (2004). PTEN function: how normal cells control it and tumour cells lose it. *Biochem. J.* **382**, 1-11.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R. et al. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943-1947.

Li, L., Liu, F. and Ross, A. H. (2003). PTEN regulation of neural development and CNS stem cells. *J. Cell Biochem.* **88**, 24-28.

Marino, S., Krimpenfort, P., Leung, C., van der Korput, H. A., Trapman, J., Camenisch, I., Berns, A. and Brandner, S. (2002). PTEN is essential for cell migration but not for fate determination and tumourigenesis in the cerebellum. *Development* **129**, 3513-3522.

Marsh, D. J., Dahia, P. L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R. J. and Eng, C. (1997). Germline mutations in PTEN are present in Bannayan-Zonana syndrome. *Nat. Genet.* **16**, 333-334.

Menager, C., Arimura, N., Fukata, Y. and Kaibuchi, K. (2004). PIP3 is involved in neuronal polarization and axon formation. *J. Neurochem.* **89**, 109-118.

Ono, H., Katagiri, H., Funaki, M., Anai, M., Inukai, K., Fukushima, Y., Sakoda, H., Ogihara, T., Onishi, Y., Fujishiro, M. et al. (2001). Regulation of phosphoinositide metabolism, Akt phosphorylation, and glucose transport by PTEN (phosphatase and tensin homolog deleted on chromosome 10) in 3T3-L1 adipocytes. *Mol. Endocrinol.* **15**, 1411-1422.

Parent, C. A. (2004). Making all the right moves: chemotaxis in neutrophils and Dictyostelium. *Curr. Opin. Cell Biol.* **16**, 4-13.

Raftopoulou, M., Etienne-Manneville, S., Self, A., Nicholls, S. and Hall, A. (2004). Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. *Science* **303**, 1179-1181.

Rordorf-Nikolic, T., Van Horn, D. J., Chen, D., White, M. F. and Backer, J. M. (1995). Regulation of phosphatidylinositol 3′-kinase by tyrosyl phosphoproteins. Full activation requires occupancy of both SH2 domains in the 85-kDa regulatory subunit. *J. Biol. Chem.* **270**, 3662-3666.

Sawyer, C., Sturge, J., Bennett, D. C., O'Hare, M. J., Allen, W. E., Bain, J., Jones, G. E. and Vanhaesebroeck, B. (2003). Regulation of breast cancer cell chemotaxis by the phosphoinositide 3-kinase p110delta. *Cancer Res.* **63**, 1667-1675.

Tanaka, E. and Kirschner, M. W. (1995). The role of microtubules in growth cone turning at substrate boundaries. *J. Cell Biol.* **128**, 127-137.

van Horck, F. P., Lavazais, E., Eickholt, B. J., Moolenaar, W. H. and Divecha, N. (2002). Essential role of type I(alpha) phosphatidylinositol 4-phosphate 5-kinase in neurite remodeling. *Curr. Biol.* **12**, 241-245.

Wang, F., Herzmark, P., Weiner, O. D., Srinivasan, S., Servant, G. and Bourne, H. R. (2002). Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat. Cell Biol.* **4**, 513-518.

Williams, E. J. and Doherty, P. (1999). Evidence for and against a pivotal role of PI 3-kinase in a neuronal cell survival pathway. *Mol. Cell Neurosci.* **13**, 272-280.

Williamson, T., Gordon-Weeks, P. R., Schachner, M. and Taylor, J. (1996). Microtubule reorganization is obligatory for growth cone turning. *Proc. Natl. Acad. Sci. USA* **93**, 15221-15226.

Zhou, F. Q., Zhou, J., Dedhar, S., Wu, Y. H. and Snider, W. D. (2004). NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. *Neuron* **42**, 897-912.