PTEN (phosphatase and tensin homologue deleted on chromosome TEN) is the major negative regulator of phosphatidylinositol 3-kinase signaling and has cell-specific functions including tumor suppression. Nuclear localization of PTEN is vital for tumor suppression; however, outside of cancer, the molecular and physiological events driving PTEN nuclear entry are unknown. In this paper, we demonstrate that cytoplasmic Pten was translocated into the nuclei of neurons after cerebral ischemia in mice. Critically, this transport event was dependent on a surge in the Nedd4 family–interacting protein 1 (Ndfip1), as neurons in Ndfip1-deficient mice failed to import Pten. Ndfip1 binds to Pten, resulting in enhanced ubiquitination by Nedd4 E3 ubiquitin ligases. In vitro, Ndfip1 overexpression increased the rate of Pten nuclear import detected by photobleaching experiments, whereas Ndfip1−/− fibroblasts showed negligible transport rates. In vivo, Ndfip1 mutant mice suffered larger infarct sizes associated with suppressed phosphorylated Akt activation. Our findings provide the first physiological example of when and why transient shuttling of nuclear Pten occurs and how this process is critical for neuron survival.

Introduction

PTEN (phosphatase and tensin homologue deleted on chromosome TEN) is the major negative regulator of signaling by phosphatidylinositol 3-kinase (PI 3-K), thereby playing a central role in controlling many important cellular activities regulated by this pathway, including cell division, cell growth, cell survival, and DNA damage (Chalhoub and Baker, 2009). PTEN exerts its negative effect through its phosphatase activity on the plasma membrane lipid phosphatidylinositol 3,4,5-triphosphate (PIP3), reducing levels of phosphorylated Akt (pAkt; Maehama and Dixon, 1998; Stambolic et al., 1998). Thus, loss of PTEN, as demonstrated by genetic inactivation in human cancer or mouse knockout (KO) models, causes constitutive activation of Akt in cells, resulting in dysregulated cell proliferation, growth, and survival, which are hallmarks of tumorigenesis (Hobert and Eng, 2009; Nardella et al., 2010). PTEN can be found in both the cytoplasm and nucleus of many cell and tissue types, and its aberrant localization has been implicated in disease. The nucleocytoplasmic distribution of PTEN has been proposed to affect its tumor-suppressive function both within and outside the PI 3-K pathway (Planchon et al., 2008). However, it has remained unclear what physiological stimulus can drive PTEN into the nucleus and under what in vivo circumstances this can occur.

In the brain, PTEN is required for multiple aspects of neuronal function and development, including maintenance of neuron structure, size, synaptic plasticity, and survival (Endersby
In the current study, we demonstrate that cerebral ischemia is the stimulus for trafficking of Pten to the nucleus, leading to neuron survival. This nuclear trafficking of Pten is downstream of Nedd4 family–interacting protein 1 (Ndfip1), an adaptor for Nedd4-mediated ubiquitination (Shearwin-Whyatt et al., 2006). Interestingly, Ndfip1 up-regulation and neuronal survival were not associated with Pten degradation. Instead, Ndfip1 directly increases the rate of Pten translocation to cell nuclei, and, without Ndfip1 in vivo, Pten fails to accumulate in neuronal nuclei, resulting in larger infarct sizes in ischemia. Therefore, Pten ubiquitination and nuclear import, previously shown to be antioncogenic in the colon (Trotman et al., 2007; Wang et al., 2007), serve the unexpected function of protecting neurons from death after ischemia in the brain.

**Results and discussion**

**Nuclear trafficking of Pten in neurons is stimulated by cerebral ischemia and requires Ndfip1**

Under normal homeostasis, Pten is found predominantly in the cytoplasm of neurons in the cerebral cortex (Fig. 1, A–C). After ischemia, we observed a change in the cellular location of Pten in neurons of the periinfarct region, from the cytoplasm to the nucleus (Fig. 1, D–F; Pten antibody validation is shown in Fig. S1). This relocalization of Pten is strongly correlated with neurons up-regulating Ndfip1, an adaptor protein for the Nedd4 family of ubiquitin ligases (Fig. 1, A–F). Previously, we reported that Ndfip1 is rapidly up-regulated in surviving neurons after brain trauma (Sang et al., 2006); here, we also show that Ndfip1 is up-regulated in surviving neurons after brain ischemia (Fig. S2). The tight correlation of Pten nuclear localization with Ndfip1 up-regulation in the same neurons suggested that Ndfip1 could play a role in nuclear trafficking of Pten during ischemic stress. To determine whether Pten nuclear accumulation was Ndfip1 dependent, we conditionally deleted Ndfip1 in neurons using the Emx1-Cre transgene (Ndfip1\textsuperscript{loxP/loxP};Emx1-Cre\textsuperscript{+/+};Fig. S2; Iwasato et al., 2000). Mutant mice were viable and exhibited normal body weights and brain sizes despite Emx1-specific loss of Ndfip1 protein in neurons of the cortex (unpublished data). Similar to the wild-type (WT) animals, mutant neurons in a comparable cortical region of the nonischemic hemisphere showed a predominantly cytoplasmic distribution pattern for Pten (Fig. 1, G–I and inset). In contrast to WT littermates in D–F, Pten in the ischemic cortex of Ndfip1 conditional KO mice remains in the cytoplasm of neurons (arrows). The inset in L shows the absence of Ndfip1 and nuclear exclusion of Pten after ischemia. Bar, 30 µm.

and Baker, 2008). Thus, conditional deletion of Pten in the brain increases astrocyte proliferation and neuron hypertrophy that is associated with increased dendrites and synapses and aberrant cerebellar development (Backman et al., 2001; Kwon et al., 2001, 2006). Although these phenotypes may be attributable to increased Akt signaling, the lack of tumor formation in these brains emphasizes multifaceted roles for Pten in neurons (Endersby and Baker, 2008; Chalhoub and Baker, 2009). So although Pten status might not dictate proliferation in neurons, it still appears to be important for apoptosis during cerebral ischemia (Ning et al., 2004; Lee et al., 2009). What is entirely unclear is the molecular mechanism underscoring Pten function in neuronal ischemia, despite studies advocating Pten inhibition as a possible therapeutic route (Chang et al., 2007; Li et al., 2009).
Ndfip1 binds to and enhances ubiquitination of Pten

Next, we looked for a direct interaction between PTEN and Ndfip1. Ndfip1 acts by recruiting target proteins that lack PY motifs and facilitates their ubiquitination by Nedd4 E3 ligases (Shearwin-Whyatt et al., 2006). One member of the Nedd4 family, Nedd4-1, has been shown to be important for PTEN ubiquitination (Trotman et al., 2007; Wang et al., 2007; Drinjakovic et al., 2010), but there is controversy regarding this interaction (Fouladkou et al., 2008; Wang et al., 2008). As PTEN lacks the classical PY motifs required for Nedd4 binding, we investigated whether Ndfip1 may mediate the interaction between PTEN and the ubiquitin ligases. To determine whether Ndfip1 binds to PTEN, coimmunoprecipitation experiments were performed in human embryonic kidney (HEK-293T) cells (Fig. 2, A and B) and brain tissue (Fig. 2 C). In cells, Ndfip1 and PTEN can mutually coimmunoprecipitate each other (Fig. 2, A and B), and, in brain lysates, Ndfip1 can pull down both Pten and Nedd4-2, indicating endogenous interaction (Fig. 2 C).

The aforementioned results suggest that in the brain, Nedd4-2 may be the E3 ligase required for Pten ubiquitination. To further investigate this, we tested the role of Ndfip1 in mediating PTEN ubiquitination by either Nedd4-1 or Nedd4-2 (Fig. 2 D). Transfected PTEN alone (Fig. 2 D, lane 1) in HEK-293T cells showed a lack of ubiquitination. Overexpression of Ndfip1 with Nedd4-1 (lane 4) or Nedd4-2 (lane 6) produced the characteristic monoubiquitinated ladders together with higher molecular mass–polyubiquitinated smear. (E) Phosphorylation status of PTEN changes Ndfip1 interaction. PTEN WT and the PTEN 3A phosphorylation-defective mutant interact strongly with Ndfip1, but PTEN 4D phosphomimic mutant interacts weakly with Ndfip1. (F) Ndfip1-mediated ubiquitination of PTEN is dependent on the phosphorylation status of PTEN. In the absence of Ndfip1, little PTEN ubiquitination occurs. Coexpression with Ndfip1 results in strong ubiquitination of the PTEN 3A phosphorylation-defective mutant, but both PTEN WT and the PTEN 4D phosphomimetic mutant are weakly ubiquitinated.
In vitro nuclear import of PTEN is accelerated by Ndfip1

NEDD4-1 has been shown to control PTEN monoubiquitination and nuclear import (Trotman et al., 2007). To test whether Ndfip1 is a mediator of this process, we measured nuclear PTEN transport as a function of Ndfip1 status. To this end, we used nuclear FRAP of cherryFP-PTEN (cherry-PTEN) in the PTEN-deficient PC3 prostate cancer cell line, as previously described (Trotman et al., 2007). We first tested whether overexpression of GFP-Ndfip1 could affect cherry-PTEN nuclear transport. As shown in Fig. 3 A, recovery of nuclear PTEN fluorescence typically proceeds >800 s after nuclear bleaching. In contrast, cooverexpression of GFP-Ndfip1 strongly accelerated this process such that a plateau was reached within <500 s, with an apparent import rate greater than fourfold higher than what was observed in GFP-expressing cells (Fig. 3 E). Moreover, cells coexpressing both plasmids were readily able to reestablish the prebleach nuclear cytoplasmic signal ratios, indicating that upon Ndfip1 expression, a high fraction of PTEN in the cell is nuclear transport competent (Fig. 3 B). Next, we made use of the genetic system to test whether mouse Ndfip1 is an essential component of the PTEN import machinery and transfected Ndfip1+/− and Ndfip1−/− primary mouse embryonic fibroblasts (MEFs) with GFP-PTEN for subsequent FRAP analysis. As shown in Fig. 3 C and D, Ndfip1+/− MEFs showed PTEN import curves and apparent import rates comparable with those observed in the PC3 cells. Yet, the Ndfip1−/− cells showed only negligible signal recovery. As a result (Fig. 3 E), the KO MEFs displayed only 0.1% of the normal PTEN transport rate (2.4 × 10−6/s vs. 2.4 × 10−5/s for KO and WT cells, respectively; Fig. 3, D and E). Collectively, our in vitro analysis shows that Ndfip1 is essential for PTEN import, which can be enhanced by Ndfip1 overexpression, in full agreement with our in vivo findings using Ndfip1 KO mice.

Mice lacking Ndfip1 in the brain suffer larger infarct sizes after cerebral ischemia

The aforementioned results indicate a causal relationship between Ndfip1 up-regulation and Pten nuclear localization in surviving neurons after ischemia. If Ndfip1 induction protects neurons from ischemic death (Sang et al., 2006), elimination of Ndfip1 in neurons would be expected to increase cell death after cerebral ischemia. To test this, we quantitated the effect of neuron-specific ablation of Ndfip1 (Ndfip1loxPloxP;Nestin-Cre−). These mutant mice were compared with littermate controls (Ndfip1loxPloxP;Nestin-Cre−) 24 h after ischemia, using...
2,3,5-triphenyltetrazolium chloride (TTC) staining of coronal slices to map the extent of infarction between the two groups (Fig. 4 A). Results show increased infarct areas in all brain regions examined of Ndfip1loxP/loxP;Nestin-Cre+ mice compared with Ndfip1loxP/loxP;Nestin-Cre− controls (Fig. 4 B), suggesting an increased infarct area (unstained white tissue) in Ndfip1KO mice compared with littermate controls (significant genotype effect; two-way analysis of variance [ANOVA]). (C) PTEN levels in neuronal cell lines (SH-SY5Y) are unaffected by tamoxifen-induced overexpression of Ndfip1. (D) Western blot densitometry reveals deletion of Ndfip1 (Ndfip1loxP/loxP;Nestin-Cre−) does not alter PTEN levels in the mouse cortex compared with littermate controls (Ndfip1loxP/loxP;Nestin-Cre+; P = 0.7392; two-tailed unpaired Student’s t test; n = 5 per genotype). (E and F) Immunostaining for pAkt in WT mice (Ndfip1loxP/loxP;Nestin-Cre−) 24 h after HI (nonischemic and ischemic cortex) shows increased numbers of pAkt-positive neurons in the periinfarct region. (G and H) In Ndfip1 KO mice (Ndfip1loxP/loxP;Nestin-Cre−), fewer pAkt-positive neurons are observed in the periinfarct region after HI. (E–H) Insets show magnifications of the boxed areas. Bar, 30 µm. (I) Quantification of pAkt levels in the ischemic cortex 24 h after HI shows that fewer neurons activate Akt in the periinfarct region in Ndfip1 KO mice. ***, P < 0.005 compared with WT (two-tailed unpaired Student’s t test; n = 5 per genotype). Values are mean ± SEM throughout.
though similar ubiquitination pathways can mediate both processes. However, we are unable to resolve how Pten nuclear entry can elicit neuron survival responses at such rapid intervals, given that the known functions of PTEN in tumor suppression are quite delayed. It may be that cerebral ischemia/hypoxia represents a unique physiological context that accelerates PTEN-mediated signaling for cell survival (Ning et al., 2004). Nonetheless, our evidence demonstrating that Pten is not degraded in the cytoplasm but is imported into the nucleus for neuron survival presents a conceptual challenge to the existing dogma of promoting Pten inhibition for the treatment of stroke (Ning et al., 2004; Hong et al., 2006; Liu et al., 2010).

Our findings also provide the first in vivo example of an acute stimulus that can regulate nuclear trafficking of Pten. This has powerful implications for PTEN-mediated mechanisms that are known to operate widely in the organism under normal and disease settings, including development and cancer. Because lack of PTEN import has been demonstrated to be tumor promoting (Trotman et al., 2007), it will be of interest to determine whether Ndfip1 alteration is implicated in neoplasia. However, it remains to be seen how ischemic stimuli and tumorigenic stimuli can both be interpreted by the cell to initiate nuclear trafficking of Pten and how, in postmitotic neurons, this leads to survival; but in dividing cancer cells, this results in tumor suppressive function. Given that PTEN nuclear accumulation can occur under a variety of conditions, including cell cycle states and hormonal changes (Mutter et al., 2000; Ginn-Pease and Eng, 2003), the downstream consequence of nuclear PTEN is likely to be cell and context specific. In the current instance, we demonstrate that in neurons, ischemic damage is a trigger for subcellular distribution of Pten with survival benefit. However, it will be of interest to determine whether PTEN nuclear import in all other scenarios is also regulated by the Ndfip1/Nedd4 system.

Materials and methods

Animals

All procedures were approved by the Florey Neuroscience Institutes Animal Ethics Committee. C57BL/6 mice were obtained from the Australian Research Council. Emx1-Cre (C57BL/6) mice were provided by T. Iwasato (RIKEN Brain Science Institute, Saitama, Japan; Iwasato et al., 2000), and Nestin-Cre mice (B6.Cg-Tg(Nes-cre)1Kln/J) were obtained from The Jackson Laboratory.

Generation of Ndfip1 conditional KO mice

The targeting construct was produced using homologous recombineering of bacterial artificial chromosome clone RP23-65L6 in Escherichia coli according to the method of Liu et al. (2003). Using this approach, a 10-kb fragment of RP23-65L6, which included exons 1 of Ndfip1, was recombinated into the targeting plasmid pD253. A loxP site was inserted 962 bp upstream of exon 1 and the Neo selection cassette flanked by loxP sites, homologously recombined embryonic stem cell clones were selected for production of germine chimeras. Conditional KO Ndfip1 (Ndfip1loxPloxP/loxP Emx1-Cre) or Ndfip1loxPloxP/loxP Nestin-Cre mice were generated by crossing Ndfip1loxPloxP (C57BL/6) homozygous mice with those carrying the Emx1-Cre transgene (Iwasato et al., 2000) or the Nestin-Cre transgene, respectively. Mice were genotyped by PCR of tail biopsies with the Ndfip1 floxed allele primers across the 5‘ loxP site (forward 5’TCACTCAGATGGATGGTGC3` and reverse 5’TATCACCACCCCTGGGC3’) and the Cre transgene primers (forward 5’TTTCCCTGCAATGGCGTAATCCAA3’ and reverse 5’TGCCCTGTTCATCCAGGCAAGGCA3’).

Unilateral cerebral hypoxia-ischemia (HI)

HI was induced in 4-week-old male mice using a protocol adapted from Vannucci et al. (2001), with modifications as described. Mice were anesthetized by inhalation of isoflurane (4% induction and 2% maintenance) in a mixture of 0.05–0.1% O2 and balanced room air. A neck incision was performed, and the right common carotid artery (CCA) was exposed and dissected free of surrounding tissue and nerve before permanent double ligation with 3-0 surgical silk. The first ligation was made in 4 mm proximal to the bifurcation of the CCA into the internal and external carotid arteries with the second ligation made ~3 mm proximal to the bifurcation. After ligation, the CCA was inspected to verify that blood flow had ceased and the wound was sutured, and the animal was allowed to recover for 3 h with free access to food and water. To induce HI, conscious mice were subsequently exposed to 40 min of systemic hypoxia in a humidified incubator containing 8% O2/92% N2 (model 3130; Thermo Fisher Scientific). The incubator was equipped with an internal O2 sensor, and O2 concentration was continuously monitored throughout the hypoxic period. The incubator temperature was maintained between 35.5 and 35.7°C to preserve core body temperature during hypoxia (Vannucci et al., 2001). Mice were removed from the incubator after hypoxia and allowed to recover in a normoxic environment before returning to their cages with free access to food and water.

Immunohistochemistry

For Pten and pAkt immunostaining, mice were killed under deep anesthesia after HI by transcardial perfusion of PBS, pH 7.4, followed by 4% PFA in 0.1 M phosphate buffer. Brains for Pten staining were postfixed for 1 h in 4% PFA in 0.1 M phosphate buffer and then cryoprotected for 24 h in 20% sucrose in 0.1 M phosphate buffer at 4°C before coronal sectioning. Brains for pAkt staining were postfixed for 24 h in 4% PFA in 0.1 M phosphate buffer at 4°C before being paraffin embedded. For Ndfip1/TUNEL staining, mice were anesthetized with isoflurane and killed by decapitation after HI before brains were fresh frozen in Tissue-Tek (Sakura). 14 µm of perfusion-fixed sections was permeabilized with 0.3% Triton X-100 in 0.1 M phosphate buffer and blocked with 10% FBS in 0.1% Triton X-100 with 0.1 M phosphate buffer. 8 µm of paraffin-embedded coronal sections was deparaffinized and microwaved in 10 mM citrate buffer, pH 6.0, to unmask epitopes and treated with 0.03% hydrogen peroxide for 5 min to block endogenous peroxidase. Tissue sections were then washed in PBS (pH 7.4) and 0.1% Triton X-100 and blocked with 10% normal horse serum in PBS. 14 µm of fresh frozen coronal sections was fixed with 4% PFA in 0.1 M phosphate buffer, blocked, and permeabilized in 10% normal goat serum with 0.3% Triton X-100 in 0.1 M phosphate buffer. Sections were incubated with primary antibodies overnight followed by appropriate secondary antibodies for 1 h at room temperature. Cell death was detected using terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) as per the manufacturer’s instructions (Roche). Sections were counterstained with DAPI (1:10,000; Dako) before mounting under glass coverslips with antifade mounting reagent. For detection of pAkt, the VECTASTAIN ABC kit was used according to manufacturer’s instructions (Vector Laboratories). Fluorescent images of Pten/Ndfip1 staining were obtained using a room temperature imaging microscope with 40x objective (NA 1.3) on a laser-scanning confocal microscope (FluoView FV1000; Olympus) using FV1000-ASW software (Olympus). For Ndfip1/TUNEL staining, fluorescent images were captured at room temperature with a 10x objective (NA 0.03) on a fluorescent microscope (BX51; Olympus) equipped with a SPOT 2.3.1 camera (Diagnostic Instruments) and using Image-Pro Plus software (Media Cybernetics). Brightfield images of pAkt staining were obtained at room temperature using 10x (NA 0.30) and 40x (NA 1.00–0.50) objectives on a fluorescent microscope (DMLB2; Leica) equipped with a charge-coupled device camera (Optronix ORCA-R2; Hamamatsu Photonics) and using Picture Frame software (MBF Bioscience). All images were taken at 22°C. The primary antibodies used were purified rat monoclonal anti-Ndfip1 (clone 1G5; 1:1,000 or 1:200 for fresh frozen sections), rabbit monoclonal anti-PTEN (clone Y184; 1:500; Abcam), mouse monoclonal anti-Emx1 (clone 6H2.1; 1:500; Cascade BioScience), and rabbit monoclonal anti-p-Akt Ser 473 (1:50; Cell Signaling Technology). The secondary antibodies used were Alexa Fluor 594-conjugated goat anti-rat IgG (1:500; Invitrogen) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; Invitrogen).

Evaluation of infarct area in TTC-stained sections

Mice were anesthetized by isoflurane inhalation after HI and decapitated, and brains were removed and sectioned in a mouse coronal brain matrix at pre-defined 1-mm intervals. Sections were incubated in 2% TTC (Merck & Co., Inc.) for 30 min, rinsed in PBS, and then fixed in 4% PFA in 0.1 M phosphate buffer for 24 h before imaging sections on a flatbed color scanner. Infarct area
was measured by planimetry of scanned sections using ImageJ software (National Institutes of Health), and the infarct area was calculated and corrected for edema according to the method of Swanson et al. (1990).

**Cell culture, transfection, and plasmid constructs**

HEK-293T cells were cultured in DME (Invitrogen), 10% FCS, 4 mM l-glutamine, and 50 µg/ml PenStrep. Human SH-SY5Y neuroblastoma cells were cultured in RPMI medium (Invitrogen), 15% FCS, 2 mM l-glutamine, and 100 µg/ml PenStrep. HEK-293 cells were transiently transfected with appropriate constructs using the Effectene Transfection Reagent kit according to the manufacturer’s instructions (QiAGEN). PC3 cells or primary MEFs were cultured in DME (Mediatech, Inc.), 10% FCS, 50 µ/ml penicillin, and 100 µg/ml PenStrep and were transfected with appropriate constructs using Lipofectamine 2000 (for PC3; Invitrogen) or Effectene (for MEFs). Lentiviral infection was used to make stable SH-SY5Y cell lines of inducible Ndfip1-Flag. In brief, lentiviral particles were made through transfection of HEK-293T cells with packaging constructs plasmid cytoplasmogalovirus B8R-2 and vesicular stomatitis virus glycoprotein and the Ndfip1-Flag lentiviral plasmid using Effectene. Supernatants were harvested, and SH-SY5Y cells were infected with virus supernatant for 24 h. Successful infection was selected for with 2 mg/ml puromycin (Sigma-Aldrich) and 100 mg/ml hygromycin B (Sigma-Aldrich) to form a stable cell line for inducible Ndfip1-Flag (Howitt et al., 2009). To create N terminus Strep-Flag-tagged NDFIP1, human NDFIP1 cDNA flanked with Nhe1 and Xho1 restriction sites was generated by PCR with the primers forward 5'-GTCCTCGAGATAAATAAAGAGAACTCTG- GCTCATGGATCTGCT-3' and reverse 5'-GTCTCGAGAATATAAAGAGAACTCTG- GCTCATGGATCTGCT-3'. The purified NDFIP1 PCR fragment was digested with Nhe1 and Xho1 restriction enzymes and cloned into N-SF-TAPpcDNA3 (provided by C.J. Gloeckner, Institute of Human Genetics, Munich-Neuherberg, Germany), generating the Strep-Flag-NDFIP1 (SF-TAP-NDFIP1) pcDNA3 construct. Other constructs used were GFP-Pten in pcDNA3 (plasmid 13039; obtained from the Addgene plasmid repository), His-Ndfip1 in pcDNA3, Ndfip1-Flag in pcDNA3, PTEN WT in pcDNA3, PTEN 3A in pcDNA3, His-Ubiquitin in pcDNA5, and Nedd4-1 and Nedd4-2 in pcDNA3. Cells were lysed 20 h after transfection for immunoprecipitation assays as described in the next section. PTEN WT, PTEN 3A, and PTEN 4D were obtained from H. Zhu (Royal Melbourne Hospital, The University of Melbourne, Parkville, Victoria, Australia).

**Western blotting**

Lysates or immunoprecipitates were resolved on 10% SDS-PAGE gels followed by transfer onto Hybond-C nitrocellulose membrane (GE Healthcare). Membranes were blocked for 1 h at room temperature in 5% nonfat milk in TBS and 0.05% Tween 20. Blots were incubated overnight with primary antibodies at 4°C followed by appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Proteins were detected using ECL reagent according to the manufacturer’s instructions (GE Healthcare) and visualized by exposure to x-ray film. The primary antibodies used were rat monoclonal anti-Ndfip1 (clone 1GS; 1:2,000), rabbit monoclonal anti-PTEN (clone Y184; 1:2,000; Abcam), mouse monoclonal anti-PTEN (clone 6H2.1; 1:500; Cascade BioScience), mouse monoclonal anti–StrepMAB-Classical (1:1,000; IBA GmbH), purified rabbit polyclonal anti–Nedd4-2 (1:5,000), mouse monoclonal anti–StrepMAB (1:1,000; BD), mouse monoclonal anti–β-actin (clone AC40; 1:5,000; Sigma-Aldrich), and mouse monoclonal anti–Flag M2 (1:1,000; Sigma-Aldrich). The HRP-conjugated secondary antibodies used were goat polyclonal anti-rabbit (1:5,000 or 1:10,000; Millipore), goat polyclonal anti–rabbit (1:10,000; Millipore), and goat polyclonal anti–mouse (1:15,000; Millipore).

**Photobleaching nuclear transport assays**

PC3 or primary MEF cells were plated, transfected, and observed in glass-bottom dishes (MatTek Corporation) at 24 h after transfection (MEF cells) or between 12 and 14 h after transfection (PC3 cells; Trotman et al., 2007). Note that cotransfection of PC3 cells with cherry-PTEN and GFP-Ndfip1 leads to cell death after 14 h. Cotransfection was avoided using GFP-Ndfip1 fluorescence. FRAP assays were performed on a spinning-disk confocal microscope (UltraView VoX; PerkinElmer) using the 488- and 561-nm laser lines (set between 3 and 13% power). All experiments were performed with cells growing on a heated stage at 37°C in phenol red-free DME (Invitrogen), and imaging was performed with a 60x objective (NA 1.49 oil immersion). The FRAP protocol involved 12 s of imaging at 3-s intervals of prebleach followed by bleaching and postbleach recording at 2-s intervals for the first 40 s and 10-s intervals for the remainder of the experiment, typically using between 50 and 250 ms of exposure per channel. Images were recorded at 512 x 672 pixels and a 12-bit depth. Bleaching was performed at 100% laser intensity on the entire nuclear signal, using between 8 and 20 cycles (total of >200 ms; step size 2). Mean nuclear fluorescence intensities were measured using either the FRAP Analysis or the region of interest functions of Velocity software (v. 5.3.2; PerkinElmer) for mean nuclear fluorescence calculation with background subtraction. Note that unspecific bleaching of fluorescence signal over the imaging period was not detectable. Nuclear flux rates were calculated using nonlinear curve fitting of Prism software (v. 5; GraphPad Software) to a pseudo-first order association kinetics curve using the model equation Y = Y0 + (Yinf- Y0) e(-k*t) to determine the apparent transport rate constant K for each series. Analyses were performed on at least 10 cells or on 4 cells for GFP control overexpression with cherry-PTEN and 5 cells for Ndfip1 coexpression, whereas experiments were reproduced in independent biological replicates.

**Statistical analysis**

All data are presented as mean ± SEM unless otherwise indicated. Where appropriate, data were tested for normality before statistical analysis using the D’Agostino–Pearson test. All data were analyzed using Prism software, and P < 0.05 was considered statistically significant.

**Online supplemental material**

Fig. S1 demonstrates the specificity of the antibody Y184 for PTEN and compares it with the antibody 6H2.1 for immunostaining and Western blotting. Fig. S2 demonstrates that Ndfip1 is up-regulated in surviving neurons (TUNEL negative) of WT mice after cerebral ischemia, and the generation of Ndfip1 conditional KO mice along with a Western blot confirming the loss of Ndfip1 protein in Ndfip1 Emx-Cre mice is also shown. Fig. S3 shows the nuclear and cytoplasmic fractionation of mouse primary neurons after treatment with hypoxia to determine Pten localization. The ubiquitination of PTEN after the knockdown of endogenous Nedd4-1 and Nedd4-2, with and without overexpression of Ndfip1, is also demonstrated. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201105009/DC1.

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Figure S1. **Abcam and Cascade BioScience antibodies are specific for Pten in mouse brain tissue and fibroblasts.** (A and B) In the nonischemic mouse cortex, PTEN 6H2.1 antibody shows predominantly cytoplasmic Pten staining (arrows), similar to staining observed using PTEN Y184 antibody in Fig. 1 (A and C). (C and D) In ischemic neurons, PTEN 6H2.1 antibody shows predominantly nuclear staining (arrows), similar to staining observed using PTEN Y184 antibody in Fig. 1 (D and F). (E) In Western blots, both Y184 and 6H2.1 recognize a Pten-specific band (~52 kD) in mouse cortex. (F and G) Mouse primary neurons (E15 and 5 d in vitro) from *Pten*^lox/lox^;Nestin-Cre mice were used to test the specificity of PTEN Y184 antibody. In *Pten^+/^* neurons, Pten staining was observed in the cytoplasm and nucleus of the cell, but no staining was observed in *Pten^−/−^* neurons (arrows). Western blotting of primary cultures recognized a Pten-specific band (~52 kD) in *Pten^+/^* neurons but not in *Pten^−/−^* neurons. (H) Similar results were observed after staining with Y184 antibody in WT (*Pten^+/^*) MEFs and Pten-deficient MEFs (arrows), the generation of which has been described previously (images are in extended focus; Chen et al., 2005). Bars, 10 µm.
Figure S2. Ndfip1 in hypoxia and gene deletion in mice. (A–C) In nonischemic cortex (contralateral hemisphere), Ndfip1 shows low-level expression in neurons 24 h after right carotid artery occlusion (RCCAo), hypoxia, or HI (RCCAo + hypoxia). (D–F) In the ischemic cortex (ipsilateral hemisphere), neither carotid artery occlusion nor hypoxia alone results in Ndfip1 up-regulation or apoptosis, as detected by TUNEL. Instead, a combination of RCCAo + hypoxia is required before Ndfip1 up-regulation, and apoptosis is observed in neurons (inset in F). Bar, 50 µm. (G) A schematic diagram of the Ndfip1 WT locus, targeting construct, floxed allele, and excised allele. The targeting construct was generated by flanking exon 1 with loxP sites and insertion of a neomycin phosphotransferase selection cassette (Pgk-Neo) flanked by FRT sites into intron 1. Emx1-Cre–mediated excision of the floxed allele results in conditional deletion of exon 1 (that codes for the start codon) and the Pgk-Neo cassette. Black boxes indicate exon number. (H) Western analysis of adult cortex reveals Emx1-specific loss (pyramidal neurons only) of Ndfip1 protein in conditional KO mice (Ndfip1loxP/loxP; Emx1-Cre+) compared with control littermates (Ndfip1loxP/loxP; Emx1-Cre−; n = 4 animals per genotype). β-actin is shown for a loading control.
Figure S3. **Pten accumulates in the nucleus of neurons after hypoxia in vitro, and RNAi knockdown of either Nedd4-1 or Nedd4-2 reduces ubiquitination of PTEN.** (A) Cytoplasmic (Cyt) and nuclear (Nuc) fractionation of Ndfip1+/+ neurons under normoxic conditions shows Pten to be predominantly in the cytoplasmic fraction (using HSP70 as a cytoplasmic marker). Under hypoxic conditions (<1% O2), an increase in the level of Pten in the nuclear fraction is observed. In Ndfip1−/− neurons under normoxic conditions, Pten is found in the cytoplasmic fraction. These neurons do not elevate Pten in the nuclear fraction under hypoxic conditions. (B) PTEN ubiquitination (Ub) assay shows increased ubiquitination of PTEN with coexpression of Ndfip1 (compare lanes 1 and 2). When Nedd4-1 was reduced in the presence of Ndfip1 (compare lanes 2 and 4), a reduction in the ubiquitin profile of PTEN was observed. A similar effect was also observed with Nedd4-2 knockdown (compare lanes 2 and 6). IB, immunblotted; IP, immunoprecipitated.

Reference