The ubiquitin ligase Nedd4-1 is dispensable for the regulation of PTEN stability and localization

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Communicated by Aaron J. Ciechanover, Technion-Israel Institute of Technology, Bat Galim, Haifa, Israel, April 4, 2008 (received for review December 5, 2007)

PTEN is a tumor suppressor frequently mutated in cancer. Recent reports implicated Nedd4-1 as the E3 ubiquitin ligase for PTEN that regulates its stability and nuclear localization. We tested the physiological role of Nedd4-1 as a PTEN regulator by using cells and tissues derived from two independently generated strains of mice with their Nedd4-1 gene disrupted. PTEN stability and ubiquitination were indistinguishable between the wild-type and Nedd4-1-deficient cells, and an interaction between the two proteins could not be detected. Moreover, PTEN subcellular distribution, showing prominent cytoplasmic and nuclear staining, was independent of Nedd4-1 presence. Finally, activation of PKB/Akt, a major downstream target of cytoplasmic PTEN activity, and the ability of PTEN to transactivate the Rad51 promoter, a measure of its nuclear function, were unaffected by the loss of Nedd4-1. Taken together, our results fail to support a role for Nedd4-1 as the E3 ligase for PTEN that regulates its stability and nuclear localization. Instead, PTEN seems to be regulated by PTEN-independent mechanisms.

Results

Analysis of PTEN Levels and Ubiquitination in Nedd4-1-Deficient Cells.

To examine the physiological function of Nedd4-1, its gene in the mouse was disrupted by using two independent approaches. First, by using gene-trap methodology, a β-gal cDNA was inserted into the mouse Nedd4-1 genomic locus between exons 6 and 7 (ES trap clone BX786; BayGenomics) (F.F. and D.R., unpublished observations; H.K., A.N., and N.B., unpublished observations). Surprisingly, by using a comprehensive analysis of PTEN levels, subcellular distribution, and cytoplasmic and nuclear function in tissues and cells from Nedd4-1 knockout mice, we demonstrate here that Nedd4-1 is dispensable for regulation of PTEN stability, subcellular localization, and activity.


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0803233105/DCSupplemental.

PNAS | June 24, 2008 | vol. 105 | no. 25 | 8585–8590

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the expression of its close relative Nedd4-2 remained unaffected (Fig. 1).

By using in vitro ubiquitination assays and monitoring PTEN ubiquitination upon overexpression or knockdown of Nedd4-1 in cultured cells, a direct role of Nedd4-1, but not Nedd4-2, in regulation of PTEN ubiquitination and stability was recently proposed (21). To explore this relationship in our system, we examined PTEN levels in Nedd4-1−/− MEFs. Judged by immunoblotting, PTEN protein levels were independent of the presence of Nedd4-1 (Fig. 2A Top and Bottom). Moreover, PTEN protein levels in the hearts of the Nedd4-1−/− embryos (a tissue that expresses high levels of Nedd4-1; Fig. 2A Middle) were indistinguishable from those in Nedd4-1 WT or heterozygote hearts (Fig. 2A Top). To determine overall levels of PTEN ubiquitination, Nedd4-1−/−/ trap and control WT MEFs were pretreated with a proteasome inhibitor (MG132) for 3 h and lysed. Lysates were then boiled in SDS (to denature proteins obtained from cell lysates of the indicated MEFs were probed with anti-PTEN antibodies. (Upper) and anti-Nedd4-1 antibodies (Lower), respectively. Actin levels were determined with anti-actin antibodies. (C) Immunoblotting of lysates from Nedd4-1+/+ and Nedd4-1−/−/exon9,10 MEFs. Ten or 40 µg of proteins obtained from cell lysates of the indicated MEFs were probed for Nedd4-1 by immunoblotting with anti-Nedd4-1 antibodies, as in B. Actin levels were determined with anti-actin antibodies.

Fig. 1. Loss of Nedd4-1 expression in MEFs derived from Nedd4-1 knockout mice. (A) Genotyping of MEFs generated from Nedd4-1−/−, Nedd4-1−/−/ trap, and Nedd4-1−/−/ trap embryos. PCR analysis of genomic DNA from the indicated cells is shown. A 447-bp fragment corresponds to the WT allele and a 680-bp fragment to the trapped allele. (B) Genotyping of MEFs generated from Nedd4-1+/+ and Nedd4-1+/−/ traps. Cell lysates were probed for Nedd4-1 and Nedd4-2 by using anti-Nedd4-1 antibodies (Upper) and anti-Nedd4-2 antibodies (Lower), respectively. Actin levels were determined with anti-actin antibodies. (C) Immunoblotting of lysates from Nedd4-1+/+ and Nedd4-1−/−/exon9,10 MEFs. Ten or 40 µg of proteins obtained from cell lysates of the indicated MEFs were probed for Nedd4-1 by immunoblotting with anti-Nedd4-1 antibodies, as in B. Actin levels were determined with anti-actin antibodies.
was immunoprecipitated from Nedd4-1 endogenous Nedd4-1; such reduction, however, did not alter PTEN abundance (Fig. 2). Further, we determined PTEN levels in Nedd4-1 RNA interference. As seen in Fig. 2 which the endogenous Nedd4-1 level was acutely reduced with development), we tested PTEN stability in HEK293T cells in which the endogenous Nedd4-1 level was acutely reduced with RNA interference. As seen in Fig. 2B, microRNA-adapted short hairpin RNA (shRNA/mirs) directed toward three different regions of human Nedd4-1 led to a ≈70% reduction in levels of endogenous Nedd4-1; such reduction, however, did not alter PTEN abundance (Fig. 2B). To investigate this observation further, we determined PTEN levels in Nedd4-1+/+ MEFs with their Nedd4-1 knocked down and in Nedd4-1+/− exon9,10 MEFs reconstituted to express Nedd4-1 by transfection. As seen in Fig. 2C, knockdown of endogenous Nedd4-1 by shRNA (≈50%) in WT MEFs did not lead to increased levels of PTEN. Moreover, GFP-Nedd4-1 transfection into Nedd4-1+/− exon9,10 MEFs did not alter PTEN levels (Fig. 2D). Thus, chronic loss of Nedd4-1 by gene knockout or acute loss by gene knockdown did not affect PTEN stability.

Fig. 3. PTEN ubiquitination is comparable in Nedd4-1+/+ and Nedd4-1+/− MEFs and lack of association between PTEN and Nedd4-1. (A) PTEN ubiquitination: Nedd4-1+/+ and Nedd4-1+/− MEFs were pretreated with a proteasome inhibitor (20 μM MG132). They were then lysed, and the lysate was boiled in SDS to remove PTEN-associated proteins. After dilution of the SDS, PTEN was immunoprecipitated from the lysates, and immunoprecipitates were immunoblotted with anti-ubiquitin antibody to detect ubiquitinated PTEN (PTEN-Ub; Upper) or anti-PTEN antibody (Lower). Control (Ctr): Immunoprecipitate with beads alone (without anti-PTEN antibodies). The left two lanes represent total ubiquitination of the MEF lysates used for the experiment. (B) PTEN stability is unchanged in the absence of Nedd4-1. Nedd4-1+/+ MEFs (Top Left) or Nedd4-1+/− MEFs (Bottom Left) were exposed to cycloheximide (CHX) and the amount of PTEN remaining was analyzed over time (0, 3, 6, 9 h) by immunoblotting. Identical results were obtained in the Nedd4-1exons9,10 MEFs (data not shown). Quantification of the pulse–chase data from both the Nedd4-1+/− MEFs and Nedd4-1+/− exons9,10 MEFs (and their wild-type controls) is shown in the Right (mean ± SD, n = 4). (C and D) PTEN does not bind Nedd4-1. (C) PTEN was immunoprecipitated from Nedd4-1+/+ and Nedd4-1+/− MEFs, and the precipitates were immunoblotted for Nedd4-1 (Upper) or PTEN (Lower) with their respective antibodies. Control (Ctr): beads alone (without anti-PTEN antibodies). (D) Upper) Immobilized GST-PTEN, GST alone (negative control), or GST-LAPTMS(C terminus) (GST-LAPTMS(Cter; positive control) were incubated with lysates from Nedd4-1+/+ and Nedd4-1+/− MEFs, and the precipitate was immunoblotted for Nedd4-1. (Lower) GST fusion proteins (arrowheads) used for the pulldowns, analyzed by Ponceau S staining. Binding experiments were repeated two to four times with identical results.
Lack of Physical Interaction Between PTEN and Nedd4-1. By using pulldowns of purified GST-PTEN and Nedd4-1 proteins, as well as coimmunoprecipitation from HEK293 cells transfected with PTEN and Nedd4-1, Wang et al. (21) reported a direct interaction between PTEN and Nedd4-1. We thus tested whether endogenous PTEN and Nedd4-1 can coimmunoprecipitate from WT MEFs. As shown in Fig. 3C, no association between endogenous Nedd4-1 and PTEN in WT MEFs could be detected. To test further for Nedd4-1–PTEN interactions, we performed a pulldown experiment whereby lysates from Nedd4-1+/− or Nedd4-1−/− trap MEFs were incubated with immobilized GST-PTEN, GST alone (negative control), or GST-LAPTM5 (C terminus), which readily interacts with Nedd4-1 and was used as a positive control (32). As shown in Fig. 3D, unlike the GST-LAPTM5 (C terminus), GST-PTEN failed to precipitate Nedd4-1 from cell lysates. Thus, by using two robust methods of detecting Nedd4-1–protein–protein interactions, we were unable to detect its association with PTEN.

Normal PI3K Signaling Throughput in Nedd4-1-Deficient Cells. Cytoplasmic activity of PTEN has been shown to antagonize the PI3K pathway throughput via direct dephosphorylation of PI(3,4,5)P3, a second messenger product of PI3K activity (4, 6). We reasoned that if Nedd4-1 promoted ubiquitination and degradation of PTEN (21), then the absence of Nedd4-1 might lead to impairment of the PI3K signaling pathway. To test this possibility, we investigated the activation-specific phosphorylation of PKB/Akt, a major downstream target of PI3K activity, in our Nedd4-1-deficient cells. As shown in Fig. 4, there were no differences in PKB/Akt phosphorylation under either serum starvation or serum restimulation conditions between the Nedd4-1+/−, Nedd4-1−/−, or Nedd4-1+/+ MEFs, suggesting no change in PTEN cytoplasmic function upon Nedd4-1 loss.

Characterization of Nuclear Localization and Function of PTEN in Nedd4-1-Deficient Cells. It was recently shown that in both HeLa cells and Trp53−/− MEFs, PTEN nuclear localization depended on its Nedd4-1-mediated monoubiquitination (22). These findings infer that in cells lacking Nedd4-1, nuclear translocation of PTEN should be impaired. Contrary to this prediction, however, we found that endogenous PTEN was localized in the nucleus and in the cytoplasm of both Nedd4-1−/− and Nedd4-1+/− MEFs (Fig. 5). Quantitation of PTEN subcellular localization revealed that 100% of the Nedd4-1-deficient cells exhibited both cytosolic and nuclear localization. Namely, 213 of 213 Nedd4-1+/− trap MEFs and 290 of 290 Nedd4-1−/− exon9,10 MEFs used as controls, and 155 of 155 Nedd4-1−/− trap MEFs and 297 of 297 Nedd4-1−/− exon9,10 MEFs exhibited indistinguishable, coordinate PTEN cytoplasmic and nuclear localization (Fig. 5).

One of the proposed nuclear functions of PTEN is its influence on the activity of the promoter for Rad51, a protein essential for the repair of double-strand DNA breaks and maintenance of genomic integrity (20, 33–35). Consistent with previous studies in PC-3 cells, we observed transactivation of the Rad51 promoter by PTEN upon coexpression of E2F1 (Fig. 6). We then tested the activity of the Rad51 promoter in response to PTEN in Nedd4-1-deficient cells. Significantly, the presence of Nedd4-1 had no effect on the ability of PTEN to activate the Rad51 promoter (Fig. 6). Accordingly, cellular levels of transfected PTEN were indistinguishable between WT and Nedd4-1-deficient cells (data not shown). Thus, Nedd4-1 disruption failed to prevent PTEN nuclear localization or affect its nuclear function.

Discussion
In contrast to a comprehensive understanding of PTEN genetic alterations in cancer and a generally accepted role as a major negative regulator of PI3K signaling, relatively little is known about modes of PTEN regulation. PTEN C-terminal phosphorylation has been implicated in control of PTEN stability (36–40), whereas phosphorylation-dependent polyubiquitination was proposed as a potential molecular mechanism leading to PTEN degradation (41). Recent work has postulated that PTEN is subject to both poly- and monoubiquitination mediated by the ubiquitin ligase Nedd4-1, leading to PTEN degradation and nuclear localization, respectively (21, 22). Importantly, a potential phosphatase-independent nuclear function of PTEN in tumor suppression and the monoubiquitination-dependent molecular mechanism of PTEN nuclear translocation have been described, revealing a nuclear molecular cascade involving PTEN (20).

By using cells deficient for Nedd4-1, we demonstrate here that Nedd4-1 is dispensable for the regulation of PTEN stability, localization, or activity. There are several possible reasons for the apparent discrepancy between our results and published findings (21, 22). Our work used two independently generated Nedd4-1 knockout mouse models and MEFs derived from them, whereas the previous reports relied entirely on overexpression and knockdown sequences leading to PTEN modulation. In an attempt to assess the effects of transient, acute knockdown of Nedd4-1 on PTEN, we performed such an analysis in HEK293T cells and WT MEFs (Fig. 2). Regardless of the experimental system, we failed to detect the effect of rapid reduction of Nedd4-1 levels on PTEN. The fact that the high levels of expression of particular siRNAs may lead to substantial off-target effects raises the possibility that such events may have contributed to the discrepancy of the data reported by Wang et al. and Trotman et al. (21, 22) with our results.

Our genetic approach to disrupt the Nedd4-1 gene in all mouse tissues and the analysis of cells and tissues derived from these mice offers a more physiological system for the analysis of Nedd4-1 ubiquitin ligase targets. This is exemplified by the fact that even

![Fig. 4.](image-url) PKB/Akt activation does not depend on Nedd4-1. Subconfluent Nedd4-1−/−, Nedd4-1−/− trap, and Nedd4-1−/− trap MEFs (A) or Nedd4-1+/+ and Nedd4-1−/− exon9,10 MEFs (B) were starved overnight followed by either no treatment, 10-min stimulation with 10% FBS, or 50 μM LY294002 treatment for 1 h followed by FBS stimulation. Equal amounts of protein lysates were immunoblotted with anti-phospho-PKB (Ser-473), anti-PKB, anti-PTEN, or anti-GAPDH antibodies. Results are representative of three independent experiments.
though there was a statistically significant inverse correlation between Nedd4-1 mRNA and PTEN protein levels in human bladder cancer samples (21), complete loss of Nedd4-1 expression in cells and tissues did not affect PTEN levels (Fig. 2). Thus, our results are not consistent with the existence of a linear relationship between Nedd4-1 and regulation of PTEN. We did notice, however, that primary Nedd4-1-deficient MEFs grow more slowly than their wild-type counterparts (data not shown), consistent with a potential role of Nedd4-1 in control of cell proliferation and transformation, likely via a yet unidentified substrate. These observations may explain the ability of overexpressed Nedd4-1 to enhance Ras-induced transformation of p53-deficient fibroblasts (21) and the apparent negative effect of Nedd4-1 knockdown on growth of certain prostate cancer cell lines as xenografts (21).

Our biochemical analysis did not support a role for Nedd4-1 in PTEN ubiquitination or binding. Although endogenous Nedd4-1 from WT MEFs readily interacted with the GST fusion protein containing the C terminus of its known substrate LAPTMS (32), GST-PTEN could not precipitate Nedd4-1 from the same cells (Fig. 3). Unlike known Nedd4-1 substrates, including LAPTMS, which typically contain PY motifs (27–30, 32), PTEN does not possess this motif, possibly explaining the observed lack of its interaction with Nedd4-1 (Fig. 3 C and D) and unaffected PTEN ubiquitination in Nedd4-1-deficient cells (Fig. 3A).

The proposition that PTEN is subject to monoubiquitination-dependent nuclear translocation, leading to profound effects on its tumor suppressor function, deserves further attention because it may have significant clinical implications (21, 22); namely, PTEN nuclear localization has been associated with various stages of tumorigenesis. For instance, in the thyroid, normal follicular cells display preferential nuclear staining, which gradually weakens as the normal cells progress toward follicular adenoma and eventually carcinoma (12). These progressive changes in PTEN nuclear localization seem to precede the eventual loss of PTEN expression associated with more advanced disease, likely because of genetic and epigenetic means. The observed lack of coordinate increase in cytoplasmic staining accompanying decreased nuclear PTEN localization during genesis of thyroid tumors argues against a single activity regulating PTEN nuclear localization and cytoplasmic turnover. Contrary to this view, however, in both normal pancreatic islet cells and melanocytes, PTEN is preferentially found in the nucleus, whereas in tumors originating from these cells, PTEN nuclear localization diminishes and is associated with increased cytoplasmic accumulation (10, 14). Thus, it is conceivable that PTEN mono- and polyubiquitination may be subject to regulation by multiple ubiquitin ligases, possibly acting in cell- and tissuespecific manners. In that context, the discovery of potential phosphatase-independent nuclear roles of PTEN in tumor suppression (20) and the monoubiquitination-dependent molecular mechanism of PTEN nuclear translocation (22) offers a significant insight into this largely underappreciated aspect of PTEN function. As our knowledge in this area continues to expand, the identification of the physiological ubiquitin ligase(s) for PTEN remains a major challenge on the road to a comprehensive understanding of cellular roles of this tumor suppressor.

Experimental Procedures

Materials. The sources of all reagents can be found in supporting information (SI) Experimental Procedures.

Methods. The detailed experimental procedures can be found in SI Experimental Procedures.

Generation of Nedd4-1 Knockout Mice and MEFs. For generation of Nedd4-1−/− mice, the Nedd4-1-trapped ES clone (XB786) was obtained from BayGenomics; the Nedd4-1−/− exon9,10 conventional knockout mice were generated by homologous recombination in ES cells (H.K., A.N., and N.B., unpublished observations). Nedd4-1−/− trap MEFs from E14.5 were generated as described in ref. 31. Nedd4-1−/− exon9,10 MEFs were prepared from E13.5 embryos and immortalized after infection with retroviruses expressing the SV40 Large T-antigen (44).

12. 11-fold specificity of the lysate with lysis buffer (to dilute the SDS), PTEN was precipitated from the boiled lysate with 10 μg of anti-PTEN antibody and 25 μl of protein G-Sepharose beads (GE Healthcare) followed by immunoblotting with anti-ubiquitin and anti-PTEN antibodies.

13. Determination of PKB/Akt Activation. PKB/Akt activation was determined as described in ref. 6, and in the SI.

14. Analysis of Rad51 Promoter Activity. The activity of the pG3-hRad51-luc promoter in PC-3 cells and Nedd4-1+/− or Nedd4-1−/− MEFS was assessed as described in ref. 20.

ACKNOWLEDGMENTS. We thank C. Jiang and A. Griffin for technical support and Dr. Yuxin Yin (Columbia University, New York) for the gift of pG3-hRad51-luc and pCMV-E2F1 plasmids. This work was supported by grants from the Canadian Institute of Health Research (177834-3186S) and the National Cancer Institute of Canada, with support from the Canadian Cancer Society (to D.R.) and the Canadian Breast Cancer Foundation (to V.S.). D.R. and V.S. hold Canada Research Chairs.
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SI Experimental Procedures

Reagents. All materials were obtained from Sigma unless otherwise stated. Monoclonal anti-PTEN antibody (clone 6H2.1), anti-ubiquitin antibody, and anti-β-actin antibody (clone AC-74) were obtained from Cascade Biotechnology, Covance, and Sigma, respectively. Nedd4-1 antibodies were either purchased from BD Biosciences (monoclonal, clone 15, which recognizes Nedd-4-1), or described previously (polyclonal) (1). Polyclonal Nedd4-2 antibody was described in ref. 2. Polyclonal anti-phospho-PKB (Ser-473) and anti-total PKB antibodies were obtained from Cell Signaling (antibodies 9271 and 9272, respectively). Monoclonal anti-GAPDH antibody was obtained from Abcam.

Generation of Nedd4-1 Knockout Mice and Mouse Embryonic Fibroblasts (MEFs). All described experiments conform to the institutional regulatory standards.

Nedd4-1−/−trap. The Nedd4-1 trapped ES clone (XB786) was obtained from BayGenomics. The formation of the fusion transcript of Nedd4-1 (first 6 exons) and the β-gal present in the trapping vector was confirmed by PCR. The trapping vector is inserted in the C2 coding region. ≈7,000 bp 3′ of exon 6. Genotyping was performed by PCR of genomic DNA, with one set of primers flanking the insertion region and a second set directed toward the inserted β-gal gene (Fig. L1). PCR amplification of the wild-type and knockout regions yields 447- and 680-bp fragments, respectively. MEFs from E14.5 were generated as described in ref. 3.

Nedd4-1−/−exons9,10. Nedd4-1 conventional knockout mice were generated by homologous recombination in ES cells (H.K., A.N., and N.B., unpublished observations). The targeting vector, a derivative of pTK-neo vector (Novagen), contained the Nedd4-1 genomic region spanning exons 9 and 11 in which the sequence corresponding to exons 9 and 10 of the Nedd4-1 gene (Ensemble NC_000075.4) was replaced by the Neo cassette. After electroporation and selection, ES cell clones positive for the homologous recombination as judged by Southern blotting (not shown) were injected into mouse blastocysts to obtain chimaeric mice and germ-line transmission. MEFs of the indicated genotypes were prepared from E13.5 embryos and immortalized after infection with retroviruses expressing the SV40 Large T-antigen (4). All MEFs were maintained in DMEM supplemented with 10% FBS, glutamine (1:100, Invitrogen 25030-149), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cloning and Expression of GST Fusion Proteins. Mouse PTEN cDNA was cloned in-frame with the GST coding sequence of pGEX4T3 (Amersham Biosciences) to generate pGEX-PTEN, GST, GST-PTEN, and GST-LAPTM5(C terminus) (5) purified by standard procedures (6).

Immunofluorescence Confocal Microscopy. MEFs were fixed with 4% paraformaldehyde in PBS, followed by permeabilization with 0.1% Triton X-100. Fixed and permeabilized cells were incubated with blocking buffer (1.5% BSA and 1.5% goat serum in PBS) to block nonspecific antibody binding. To visualize PTEN, cells were stained with anti-PTEN antibody (1:50) followed by Cy3-conjugated donkey anti-mouse IgG secondary antibody (1:1,000; Jackson Immunoresearch). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) before viewing. Images of stained cells were acquired on a Quorum spinning disk confocal microscope Zeiss Axiovert 200 equipped with a Hamamatsu C9100-13 CCD camera and Tokagawa spinning disk confocal scan head. Each image acquired corresponds to a focal volume of ≈0.7 μm.

Pulldown and Coimmunoprecipitation (Co-IP) Assays. Nedd4-1+/+ and Nedd4-1−/−trap MEFs were lysed with 1 ml of lysis buffer [50 mM Hepes (pH 7.5); 150 mM NaCl; 1% Triton X-100; 10% (vol/vol) glycerol; 1.5 mM MgCl2; 1 mM EDTA; 10 μg/ml leupeptin, aprotinin, and pepstatin each; plus 1 mM PMSF; 50 mM NaF; 1 mM NaVO4; 50 μM LnLL(N-acetyl-Leu-Leu-norleucinal); and 0.4 mM chloroquine] and cleared by centrifugation at 10,600 × g for 15 min. The cleared supernatants were used for pulldown and co-IP experiments. For pulldown experiments, 1 mg of total cell lysate protein was incubated for 2 h at 4°C with 25 μg of GST, GST-PTEN (described above), or GST-LAPTM5(C terminus) (5) immobilized on glutathione-Sepharose beads (GE Healthcare). Beads were washed twice with 1 ml of lysis buffer and twice with 1 ml of HNTG [20 mM Hepes (pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, and 0.1% Triton X-100]. Bound proteins were eluted from the beads with SDS/PAGE sample buffer, resolved by SDS/PAGE, and transferred to nitrocellulose membrane. Bound Nedd4-1 was identified by immunoblotting with anti-Nedd4-1 antibody (1:1,000, clone 15; BD Biosciences) followed by horseradish peroxidase-linked anti-mouse IgG secondary antibody and ECL detection (GE Healthcare). For co-IP experiments, 1 mg of total cell lysate protein was incubated with 10 μg of anti-PTEN antibody and 25 μl of protein G-Sepharose beads (GE Healthcare) for 2 h, and beads were washed three times with HNTG. Bound proteins were eluted from beads with SDS/PAGE sample buffer, resolved by SDS/PAGE, transferred to nitrocellulose membrane, and bound Nedd4-1 was detected with anti-Nedd-1 antibody, as above.

Knockdown Experiments. For knockdown of endogenous Nedd4-1, three different shRNAmirs directed toward the human Nedd4-1 RNA were purchased from OpenBiosystems: V2LHS.254872 [shRNA (construct 1)], V2LHS.72555 [shRNA (construct 2)], and V2LHS.72555 [shRNA (construct 3)] (all in pGIPZ) as well as nonsilencing control, and plasmids transfected into HEK293T cells by using the calcium phosphate method. Uptake of the plasmid was verified by fluorescence microscopy (GFP expression), and knockdown of Nedd4-1 and levels of PTEN and actin were tested 72 h after transfection. Levels of PTEN and the remaining Nedd4-1 after Nedd4-1 knockdown were analyzed by densitometry (using Adobe Photoshop). For knockdown of Nedd4-1 in WT MEFs (Nedd4-1−/−exons9,10), shRNAmir directed against mouse Nedd4-1 (V2LMM.17409; OpenBiosystems) was used, and the experiment was performed as described for HEK293T cells. For reexpression of Nedd4-1 in Nedd4-1−/−exons9,10 MEFs, GFP-tagged Nedd4-1 (described in ref. 5) was transfected into these MEFs, and analysis of levels of Nedd4-1, PTEN, and actin was determined 24 h after transfection.

Ubiquitination Assays. Nedd4-1+/+ and Nedd4-1−/−trap MEFs were pretreated with 20 μM MG132 for 3 h. They were then lysed in lysis buffer supplemented with the proteasome inhibitor LLnL as well as protease inhibitors, as above. Cell lysate (1 mg of protein) was then boiled for 5 min in 1% SDS to ensure dissociation of any PTEN-associated proteins. After 11-fold dilution of the lysate with lysis buffer (to dilute the SDS), PTEN was precipitated from the boiled lysate with 10 μg of anti-PTEN.
antibody and 25 μl of protein G-Sepharose beads (GE Healthcare). Beads were then washed twice with lysis buffer and twice with HNTG, and proteins were separated on SDS/PAGE and immunoblotted with anti-ubiquitin antibody to detect ubiquitated PTEN, or anti-PTEN antibody.

**Pulse-Chase Analysis of PTEN.** MEFs grown to 80% confluence were treated with 50 μg/ml cycloheximide for 0, 3, 6, and 9 h, and then lysed with lysis buffer supplemented with proteasome and lysosomal inhibitors as above. After clarification of the lysate by centrifugation, 30 μg of each sample was analyzed by immunoblotting for PTEN and β-actin.

**Determination of PKB/Akt Activation.** To examine the effect of Nedd4-1 deficiency on PI3K signaling, Nedd4-1−/−trap, Nedd4-1−/−exons9,10, and corresponding control WT MEFs were plated at 70% confluence. After 18-h starvation in medium containing 0.2% FBS (lot 1275281; Invitrogen), cells were either left untreated, stimulated with 10% FBS for 10 min, or pretreated with 50 μM PI3K inhibitor LY294002 for 1 h before serum stimulation. Cells were lysed in CHAPS buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM MgCl2, 1 mM EGTA, 0.5% CHAPS, 10% (vol/vol) glycerol, 50 mM NaF, 100 μM NaVO4, and protease inhibitor mixture (Calbiochem), and the protein concentration was determined. Twenty micrograms of total protein from each sample was separated by SDS/PAGE, transferred to PVDF membrane, and analyzed by immunoblotting with anti-phospho-PKB (Ser-473), anti-PKB, anti-PTEN, and anti-GAPDH antibodies.

**Analysis of Rad51 Promoter Activity.** PC-3 cells and Nedd4-1+/+ or Nedd4-1−/−exons9,10 MEFs were transiently transfected by Lipofectamine 2000 (Invitrogen) with the pGL3-hRad51-luc plasmid alone or in combination with pCMV-E2F1 (7) or pCMV-E2F1 and pcDNA3.1-HA-PTEN (8) in the presence of trace amounts of pCMV-β-gal. Luciferase assays were performed by using the luciferase assay system (Promega), and activities were normalized to β-galactosidase activity.