

Review Article

A vicious partnership between AKT and PHLDA3 to facilitate neuroendocrine tumors

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Neuroendocrine tumors (NET) are malignancies derived from diffuse neuroendocrine systems (DNES): for example, lung, pancreas, pituitary, stomach, duodenum and the small intestine. Among these, PanNET is a rare cancer that affects 0.32 in 1 000 000 people per year, but has a very poor prognosis compared to other NET cancers, with a 5-year survival rate of 27–43%.^(1,2) The drug everolimus, which targets the mammalian target of rapamycin (mTOR), has been shown to improve the progression-free survival of patients with advanced PanNET.⁽³⁾ Everolimus was first approved for cancer therapy in 2009 in the USA and 2010 in Japan. mTOR is a critical regulator that activates cell proliferation, growth and anti-apoptosis pathways. The efficacy of everolimus against PanNET strongly indicates that PanNET cells proliferate in a manner that involves the mTOR cascade.

Pancreatic neuroendocrine tumors (PanNET) are rare cancers that generally have a poor prognosis. Accurate diagnosis and proper treatment of these tumors requires a better understanding of the molecular mechanisms underlying the development of PanNET. It has been shown that the mTOR inhibitor everolimus can improve the progression-free survival of PanNET patients, suggesting that inhibition of the PI3K-Akt-mTOR pathway may suppress the progression of PanNET. PHLDA3 is a novel tumor suppressor protein that inhibits Akt activation by competition for binding to PIP₃. Our analysis of PanNET revealed frequent loss-of-heterozygosity and DNA methylation at the *PHLDA3* locus, resulting in strong suppression of *PHLDA3* transcription. Such alterations in the *PHLDA3* gene were also frequently found in lung neuroendocrine tumors (NET), suggesting the possibility that various types of NET have in common the functional loss of the *PHLDA3* gene.

We have previously shown that Pleckstrin homology-like domain family A, member 3 (PHLDA3) is a novel p53-regulated repressor of Akt.⁽⁴⁾ In addition, we found that loss-of-heterozygosity (LOH) as well as hyper-methylation at the *PHLDA3* gene are frequently observed in PanNET specimens.⁽⁵⁾ In this paper, we will review the significance and molecular modes of action of PHLDA3 and Akt in neuroendocrine tumors.

PI3K-Akt-mTOR Cascade

Phosphatidylinositols (PI) are pivotal factors that control the PI3K (phosphoinositide 3-kinase)-Akt (also known as protein kinase B [PKB]) pathway.⁽⁶⁾ PI are phospholipids that contain an inositol ring and are a significant lipid component of the

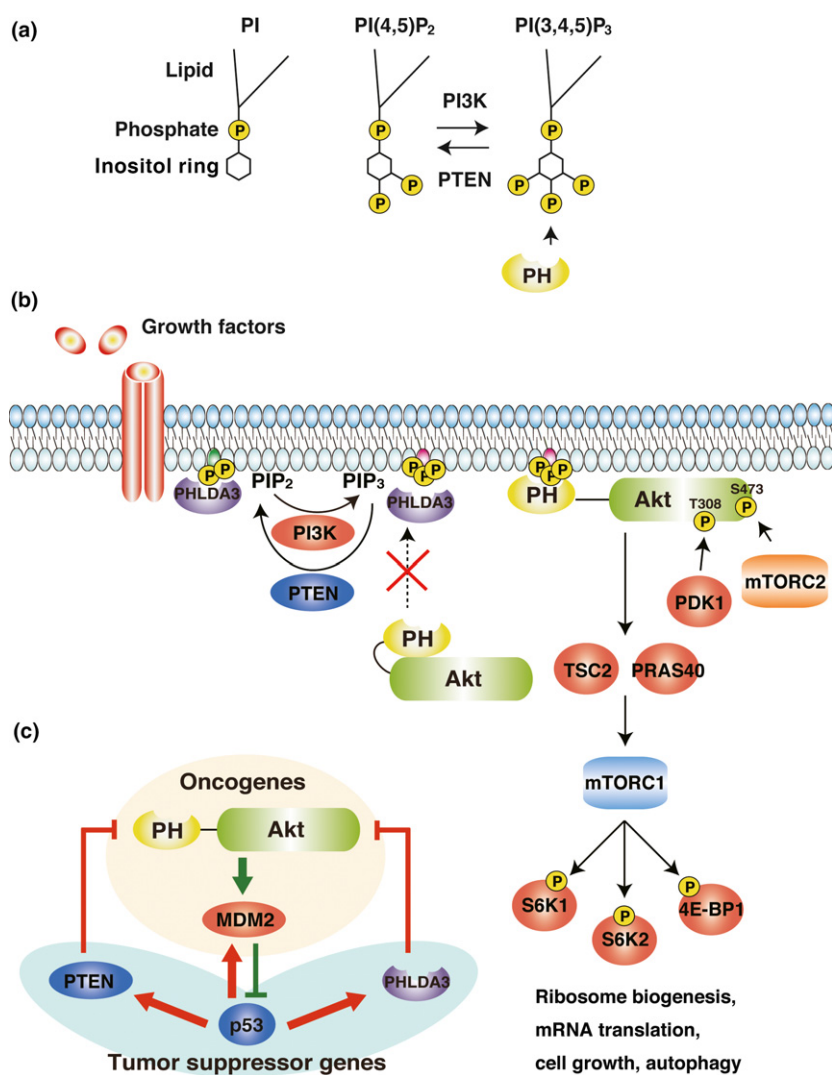


Fig. 1. PI3K-Akt-mTOR cascades and the p53-Akt network. (a) Schematic models of PI, PI(4,5)P₂ and PI(3,4,5)P₃. (b) PI3K-Akt-mTOR cascade and the model of competitive Akt suppression by PHLDA3. (c) The Akt-p53 pathway. Akt and p53 regulate each other. The molecules shown here are either oncoproteins or tumor suppressor proteins.

cellular membrane. Combinations of phosphorylated 3', 4'- or 5'-hydroxyl groups on the inositol ring define the different PIP subtypes (phosphatidylinositol phosphates). PI(3,4,5)P₃ (phosphatidylinositol-3,4,5-trisphosphate) has a particularly important role as a biologically active lipid. PI3K are lipid kinases that catalyze the conversion of PI(4,5)P₂ (phosphatidylinositol-4,5-bisphosphate) to PI(3,4,5)P₃.⁽⁷⁻⁹⁾ Conversely, PTEN (phosphatase and tensin homolog) is a lipid phosphatase that converts PI(3,4,5)P₃ back to PI(4,5)P₂.⁽¹⁰⁾ Proteins with PH, PX or ENTH domains localize to the inner membrane via binding to PIP.⁽¹¹⁾ Akt possesses one PH domain that specifically binds to PIP with high affinity (Fig. 1a).⁽¹²⁾

Akt acts as an oncogene that stimulates cell proliferation.⁽⁶⁾ In quiescent cells, in the absence of mitogen stimulation, Akt is catalytically inactive, and its activation involves multiple steps. The first step involves the activation of PI3K by one or more signaling events, such as ligand binding to RTK (receptor tyrosine kinases), activation of G-protein-coupled receptors or activation of Ras.⁽¹³⁾ In the second step, activated PI3K selectively converts PI(4,5)P₂ to PI(3,4,5)P₃.⁽¹⁴⁻¹⁶⁾ Next, Akt binds to PI(3,4,5)P₃ via its PH domain and re-localizes to the plasma membrane. There, two residues of Akt, Thr308 and Ser473, are phosphorylated by PDK1 (3-phosphoinositide-dependent kinase 1) and mTORC2 (mTOR complex 2), respectively.^(17,18) Once phosphorylated, Akt is active and, in turn,

activates multiple proteins related to cell proliferation, cell growth and suppression of apoptosis (Fig. 1b).⁽¹⁹⁾

Among the targets activated by Akt are TSC2 and PRAS40, which are responsible for the activation of mTORC1.^(20,21) mTORC1 is a rapamycin-sensitive protein complex that activates S6K1, S6K2 and 4E-BP1, which regulate ribosome biogenesis, mRNA translation, cell growth and autophagy (Fig. 1b).^(20,22-24) Akt also activates MDM2, a ubiquitin E3 ligase, that leads to degradation of p53 protein via the proteasome system.^(25,26) p53 regulates the transcription of PTEN and PHLDA3, both of which are responsible for the suppression of Akt activity.^(4,27) Thus, the Akt oncogenic pathway and the p53 tumor suppressive pathway regulate each other to fine-tune cell proliferation (Fig. 1c).

PHLDA3 Suppresses the Akt Signal Pathway

The transcription factor p53 regulates several genes related to the suppression of cancer progression.⁽²⁸⁾ Various stresses such as DNA damage, hypoxia and oncogene activation can trigger p53 activation. We became interested in *PHLDA3* as it was identified in a screen for p53 target genes.⁽²⁹⁾ Murine *PHLDA3* was first identified in 1999 (then designated *Tih1*) as the closest paralog of the imprinted gene *Ipl*.⁽³⁰⁾ *PHLDA3* is comprised of 127 amino acids, and contains one PH domain. We

found that p53 localizes to the transcription start site of *PHLDA3* and that p53 transcriptionally activates *PHLDA3*.⁽⁴⁾ We also found that overexpression of *PHLDA3* results in an increase in the apoptotic cell fraction. We further demonstrated the important relationship between the PH domain of Akt and the *PHLDA3* protein as follows.

- 1 An *in vitro* phosphatidylinositol phosphate (PIP) binding assay revealed that *PHLDA3* binds to all combinations of PIP (PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, PI(4,5)P₂, PI(3,5)P₂ and PI(3,4,5)P₃), whereas the PH domain of Akt selectively binds to PI(3,4)P₂ and PI(3,4,5)P₃ (Fig. 2a).
- 2 We found high rates of apoptosis in cells expressing either GFP-*PHLDA3* or GFP-PH-Akt (PH domain of Akt) compared to controls (Fig. 2b). The amount of phosphorylated Akt (S473) was consistently lower in both cells (Fig. 2c).
- 3 Overexpression of *PHLDA3* results in inhibition of Akt translocation to the plasma membrane (Fig. 2d).
- 4 Protein-lipid overlay assays revealed that *PHLDA3* binding to PI(3,4)P₂ and PI(3,4,5)P₃ inhibits the binding of Akt to these PIP (Fig. 2e).

PHLDA3 appears to function as if it is an isolated PH domain of Akt, and thereby acts as a dominant-negative form of Akt. Thus, we concluded that *PHLDA3* inhibits Akt activity via competitive binding to PIP (Fig. 1b).

Neuroendocrine Tumors, Endocrine Cells and Akt

In many cancer cells, factors related to the PI3K-Akt-mTOR cascade are highly activated and contribute to rampant cell proliferation.^(31,32) Accordingly, suppression of this pathway may be an effective approach for cancer therapy and molecules targeting this cascade may be candidate anticancer drugs. Indeed, several inhibitors against PI3K-Akt-mTOR pathway have been examined for PanNET therapy.⁽³³⁾ Everolimus and temsirolimus, which are derivatives of rapamycin, specifically inhibit the kinase activity of mTORC1. Everolimus (RAD001) has been tested on patients with PanNET, metastatic renal cell carcinoma and subependymal giant cell astrocytoma accompanied with tuberous sclerosis complex.^(3,34,35) In RADIANT-3, a double-blind phase 3 study, PanNET patients were randomly assigned to treatment with oral everolimus or a matching placebo.⁽³⁾ Progression-free survival was significantly prolonged in patients receiving everolimus (11.0 months) compared to patients receiving placebo (4.6 months), representing a 65% reduction in the estimated risk of progression or death.⁽³⁾ The efficacy of everolimus against PanNET strongly indicates that PanNET cell proliferation depends on the PI3K-Akt-mTOR cascade.

In addition, Jiao *et al.* performed whole exomic sequencing to analyze genetic alterations in 68 non-familial PanNET samples. They revealed that 44% of the samples had mutations in *MEN1* (encoding menin, a component of a histone methyltransferase), and 43% had mutations in either of the two subunits of the transcription/chromatin remodeling complex *DAXX* (death-domain-associated protein) and *ATRAX* (α thalassemia/mental retardation syndrome X-linked). They also revealed that 15% of the specimens harbored mutations in genes related to the mTOR pathway, such as *PTEN* or *TSC2*.⁽³⁶⁾ This report consistently indicates that aberrant activations in mTOR pathway are related to PanNET tumorigenicity.

Several studies in mouse models have further indicated the importance of the PI3K-Akt-mTOR pathway in the regulation of pancreatic islet cell proliferation. Overexpression of active

Akt1 in mouse islet β cells resulted in increased cell mass and hyperplasia,^(37,38) and a conditional *PTEN* deletion in islet β cells resulted in increased β cell proliferation, cell size and mass.^(39,40) These reports indicate the possibility that the proliferation of pancreatic endocrine cells is dependent on Akt signaling, suggesting that aberrant activation of PI3K-Akt-mTOR leads to PanNET oncogenicity.

Neuroendocrine Tumors and Akt-*PHLDA3*

PHLDA3 suppresses activity of the Akt oncoprotein, suggesting the possibility that *PHLDA3* is a tumor suppressor gene. It is known that activation of the PI3K-Akt pathway is related to oncogenicity in lung cancer.⁽⁴¹⁾ Using several kinds of lung cancer specimens, we investigated copy number alterations in the *PTPN7* gene locus, which is adjacent to *PHLDA3* (Fig. 3a, b).⁽⁴⁾ We found frequent chromosome loss at the *PTPN7* locus in 12 out of 29 large-cell neuroendocrine carcinoma (LCNEC), 8 out of 13 carcinoid samples, and 1 out of 8 small cell lung carcinomas (21 out of 50 lung NET in all). However, copy number alteration at this locus was very rare in other kinds of lung cancers (Fig. 3b).⁽⁴⁾ Consistently, *PHLDA3* mRNA abundance was significantly reduced in 10 out of 11 LCNEC (Fig. 3c). Elevated phospho-Akt staining was detected in 27 out of 32 LCNEC (Fig. 3d). These observations are consistent with our hypothesis that aberrant cell proliferation of lung neuroendocrine tumor/carcinoma depends on Akt hyper-activation resulting from *PHLDA3* inactivation. This indicates that *PHLDA3* is a tumor suppressor gene in LCNEC. We also analyzed the epistatic relationship between p53 and *PHLDA3*. We found that 63% (5/8) of the samples with WT p53 showed LOH at *PHLDA3*, whereas only 13% (3/24) of the samples with nonfunctional p53 showed LOH at *PHLDA3*. These results further support the notion that *PHLDA3* is an important downstream mediator of p53 in tumor suppression.

Loss-of-heterozygosity is frequently found at the 1q31 locus in PanNET specimens derived from insulinomas and gastrinomas, both of which are pancreatic NET.^(42,43) Because we found frequent LOH at the *PHLDA3* locus in lung NET, we speculated that the *PHLDA3* gene, located at 1q31, may also undergo LOH in PanNET. We analyzed 54 PanNET samples to determine the frequency of LOH.⁽⁵⁾ We found LOH in 36 out of 50 samples (72%) at the *PHLDA3* locus (Fig. 3e). Furthermore, we investigated both genetic and epigenetic alterations at the *PHLDA3* locus. Although no mutations were observed in the coding region of *PHLDA3*, DNA methylation was observed at the exon 1 locus (7 out of 7 samples, which had undergone LOH at the *PHLDA3* loci; Fig. 3f). We consistently observed strikingly lower abundance of *PHLDA3* mRNA in the *PHLDA3* LOH-positive cells (Fig. 3g). These results indicate that concomitant alterations in LOH and DNA methylation (two-hit inactivation) result in the suppression of *PHLDA3* expression, which, in turn, promotes the development of PanNET. Taken together, these data indicate that *PHLDA3* is a tumor suppressor gene in neuroendocrine tumors.

In addition to our study showing importance of *PHLDA3* in the suppression of NET, Brady *et al.* have also reported a tumor suppressive function for *PHLDA3*.⁽⁴⁴⁾ The authors constructed knock-in mice expressing p53 with mutations in the first, second or both of the TAD (transactivation domains). They found that MEF cells with p53 mutated in the first TAD lost the ability to arrest cell cycle at G1 and that apoptosis was significantly decreased in thymus and small intestine in response to acute DNA damage. However, HrasV12-induced

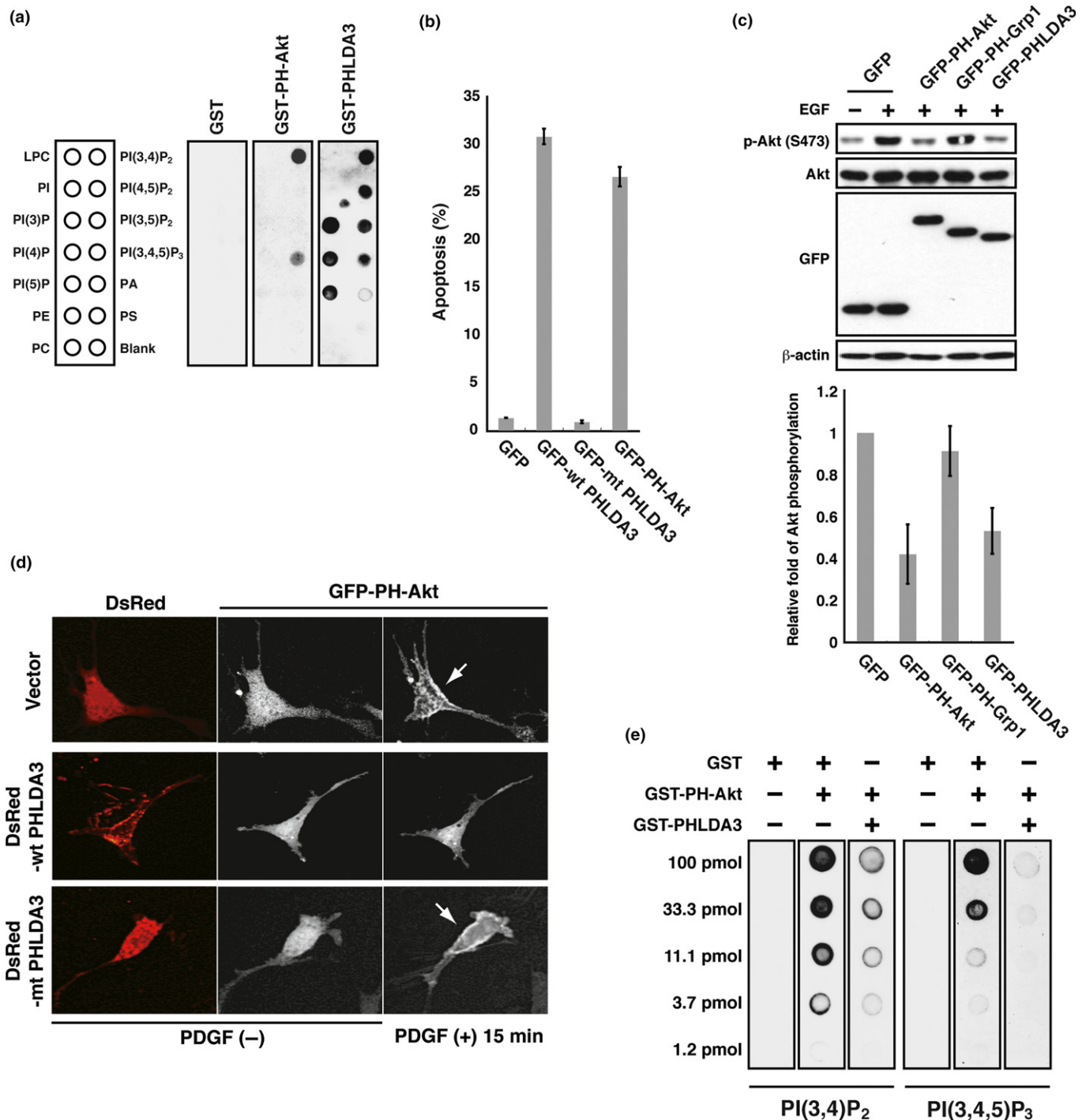


Fig. 2. PHLDA3 competes with the PH domain of Akt. (a) Binding of GST-PHLDA3, GST-PH-Akt or GST to immobilized PIP was assessed by protein-lipid overlay assay. Nitrocellulose membranes spotted with 100 pmol of different phospholipids were used. Bound proteins were detected with anti-GST antibody. Note that GST alone produced no signal under the conditions employed. LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. (b) 293T cells were transfected with GFP, GFP-WT PHLDA3, GFP-mtPHLDA3 (a PHLDA3 mutant with a small deletion within the PH domain), or GFP-PH-Akt and analyzed for GFP-positive cells 48 h post-transfection. The apoptotic rate, measured by PI-positive cells (cells stained with PI without fixation), is shown. Mean apoptotic rates ± SD from three experiments are shown. (c) PHLDA3 inhibits Akt activation. COS7 cells were transfected with the indicated fusion proteins for 24 h and subsequently stimulated with EGF for 5 min. Induction of Akt phosphorylation upon EGF treatment was detected in control cells expressing GFP. Akt activity after EGF treatment was analyzed by western blotting, and Akt activity relative to the GFP-transfected control was calculated. The mean ± SD from three experiments is shown. GFP fusion protein levels were also analyzed by western blotting. (d) Akt translocation to the plasma membrane upon PDGF treatment was analyzed by live-cell imaging. NIH 3T3 cells were transfected with GFP-PH-Akt together with DsRed, DsRed-WT PHLDA3 or DsRed-mtPHLDA3. GFP-PH-Akt subcellular localization was monitored before and after PDGF treatment (15 min). Note that Akt is localized at the plasma membrane in cells expressing DsRed or DsRed-mtPHLDA3 (shown by arrows). (e) PHLDA3 inhibits PH-Akt binding to PIP₂ and PIP₃. Binding of GST-PH-Akt to immobilized PIP was assessed by protein-lipid overlay assay. Nitrocellulose membranes spotted with serially diluted PIP₂ and PIP₃ were incubated with the indicated proteins. While GST did not interfere with Akt binding to PIP, PHLDA3 significantly interfered. Bound Akt was detected with anti-Akt PH domain antibody.

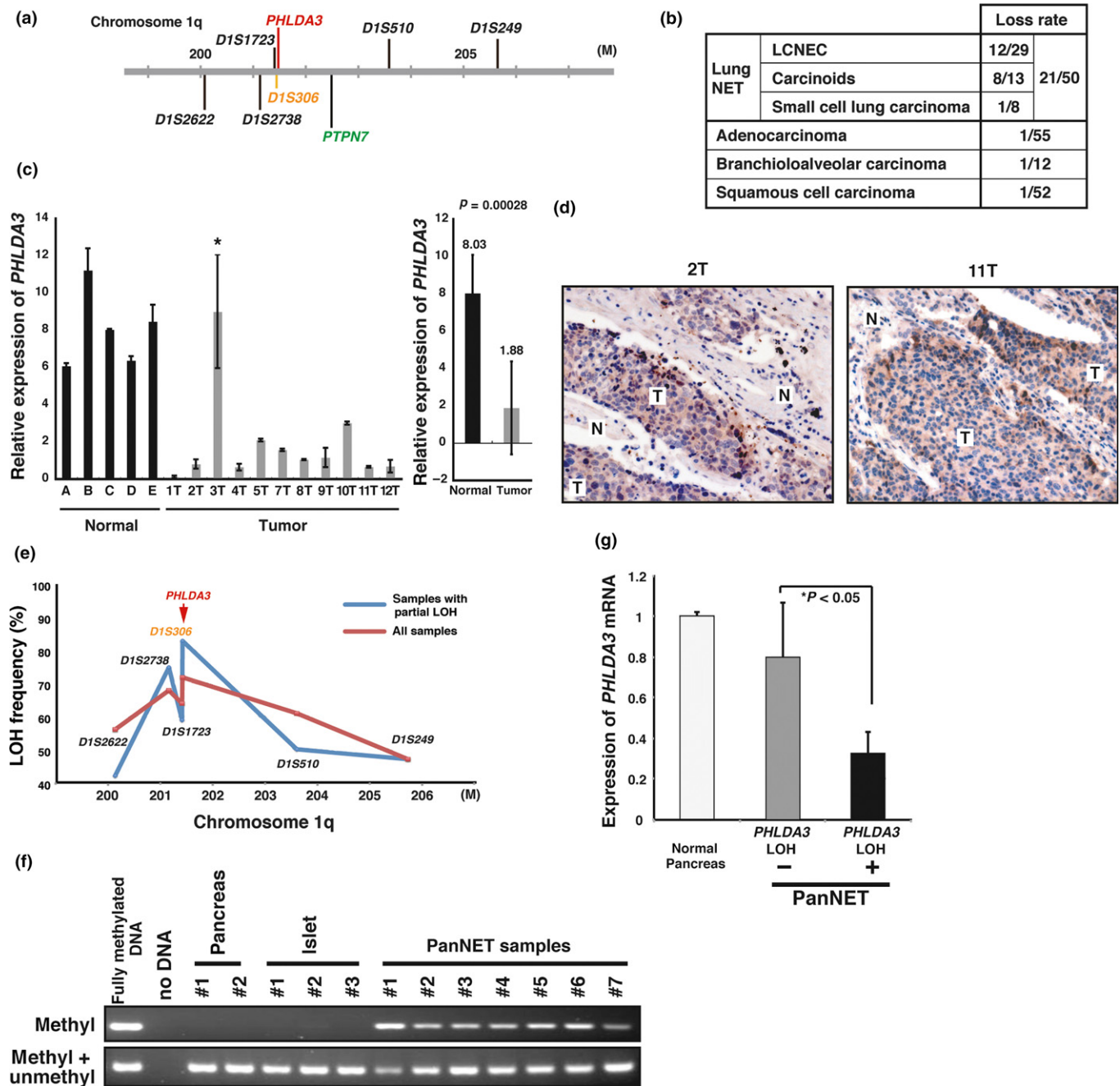


Fig. 3. *PHLDA3* locus is lost and *PHLDA3* expression is downregulated in neuroendocrine tumors (NET). (a) Chromosomal locations of the *PHLDA3* gene and microsatellite markers used in the study. *D1S306* is located just next to the *PHLDA3* gene (32 kb upstream). (b) Chromosome copy number alterations analyzed by MCG cancer array-800 CGH. (c) Expression of *PHLDA3* was analyzed by quantitative RT-PCR. Total RNA were prepared from normal lung tissues (derived from patients A–E) and LCNEC (derived from patients 1T–12T). In the right column, the mean expression \pm SD of *PHLDA3* expression in normal lungs and tumor samples is shown. (d) LCNEC tumor sections were subjected to immunohistochemistry to detect activated Akt. Stronger positive brown signals were detected in the tumor regions (T) compared to normal tissue regions (N). (e) Loss-of-heterozygosity (LOH) frequency for each microsatellite marker. Frequencies from all samples (shown by red line) and frequencies from samples showing LOH partially within the analyzed region (shown by blue line) are described. (f) Methylation status of the *PHLDA3* promoter in normal pancreas, normal isolated islets and PanNET (samples showing LOH at the *PHLDA3* locus were analyzed). Genomic DNA from the indicated samples were analyzed by methylation-specific PCR. (g) *PHLDA3* gene expression in PanNET. Total RNA were prepared from normal pancreas and PanNET. RNA was pooled from five normal pancreases for the normal controls. RNA was isolated from PanNET samples with (10 samples) or without LOH (7 samples). Gene expression was quantitated by RT-PCR and normalized to GAPDH.

cellular senescence was robustly observed in the MEF cells with p53 mutated in the first TAD. Furthermore, Kras-driven lung tumorigenesis was significantly suppressed in mice with p53 mutated in the first TAD. These results suggest that p53 mutated in the first TAD can still induce important factors that

suppress tumorigenesis. The authors identified several genes related to the inhibition of tumorigenesis, including *PHLDA3*, which was shown to efficiently suppress tumorigenicity. Over-expression of *PHLDA3* in *HrasV12;p53* null MEF or human non-small cell lung carcinoma cells inhibited cell cycle

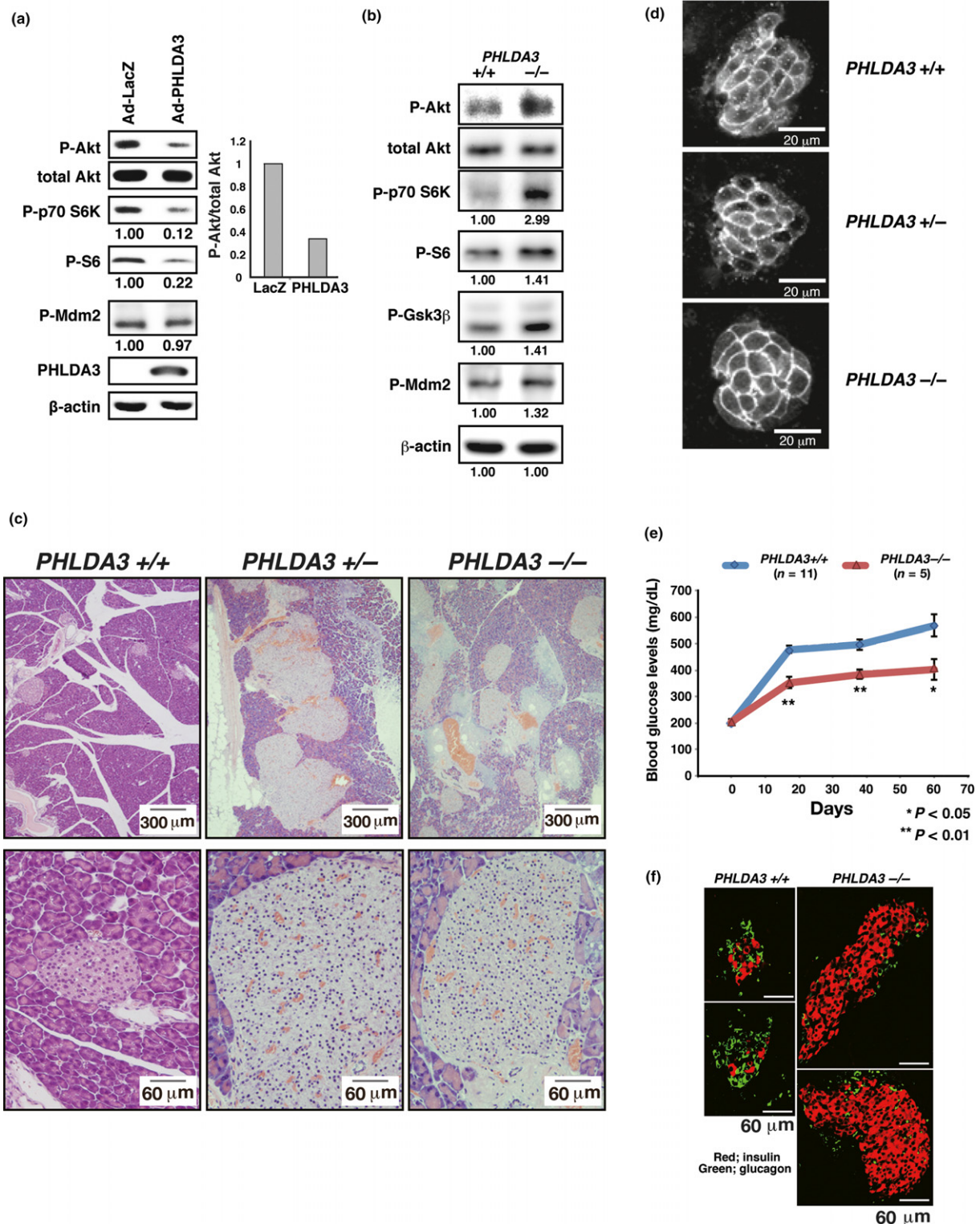


Fig. 4. PHLDA3 function in islet cells. (a) Effect of PHLDA3 expression on Akt activity in MIN6 cells. MIN6 cells were transduced with Ad-LacZ or Ad-PHLDA3 at a moi of 35, and harvested 30 h post-infection. Akt activation and phosphorylation of Akt downstream signaling molecules were analyzed by western blotting and quantified by normalization to total Akt levels (P-Akt) or by β -actin levels (P-p70 S6K, P-S6, P-GSK3 β and P-Mdm2). (b) Akt activation and phosphorylation of Akt downstream signaling molecules were analyzed by western blotting and quantified by normalization to total Akt levels (P-Akt, Right) or by β -actin levels (P-p70 S6K, P-S6, P-GSK3 β and P-Mdm2). (c) HE staining of islets from wild type, heterozygote and *PHLDA3*-deficient 10-month-old mice. (d) Islet cell size in wild type, heterozygote and *PHLDA3*-deficient mice. (e) Blood glucose levels in streptozotocin-induced diabetic mice. Indicated numbers (n) of *PHLDA3*^{+/+} or *PHLDA3*^{-/-} mice were injected i.p. with streptozotocin (STZ) for 5 consecutive days. Blood glucose levels were determined at different time points as indicated after administration of STZ. (f) Distribution of β and α cells in STZ-treated *PHLDA3*^{+/+} and *PHLDA3*^{-/-} mice. Sections were stained with antibody against insulin (β cell marker; red) and glucagon (α cell marker; green) and representative images are shown.

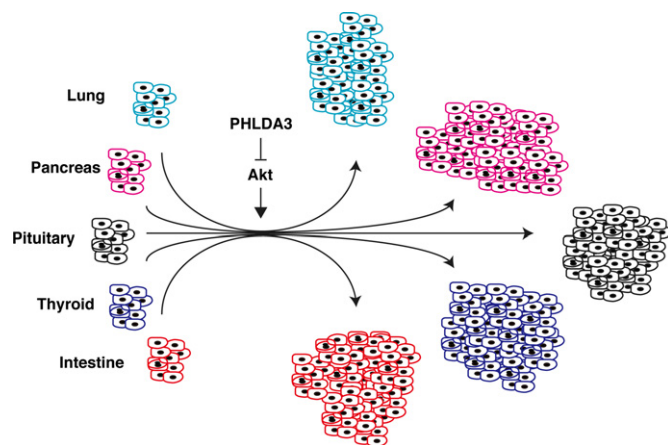


Fig. 5. *PHLDA3* is a tumor suppressor gene of neuroendocrine tumors (NET). *PHLDA3* suppresses proliferation of various types of neuroendocrine cells, such as lung, pancreas, pituitary, thyroid or intestine. Loss of *PHLDA3* in these cells results in hyperactivation of Akt and promotes NET development.

progression. Conversely, knockdown of *PHLDA3* in *E1A-HRasV12*-transformed MEF resulted in enhanced tumor growth.⁽⁴⁴⁾ This report reinforces the idea that p53 regulation of *PHLDA3* transcription functions to inhibit tumorigenesis.

PHLDA3 Function in Islet Cells

In islet β cells, cell growth and inhibition of apoptosis depend on Akt signaling.⁽⁴⁰⁾ We examined mouse MIN6 cells (an insulinoma cell line) to analyze the function of *PHLDA3* in islet β cells. We confirmed that *PHLDA3* protein levels were quite low, suggesting that *PHLDA3* function may be lost in the MIN6 cell line (Fig. 4a). We introduced *PHLDA3* into this line and analyzed its effect on the Akt pathway. We found that the phosphorylated forms of Akt, p70 S6K and S6 were significantly reduced in the *PHLDA3*-overexpressing cells (Fig. 4a). These lines of evidence indicate that the Akt pathway is suppressed by *PHLDA3* in islet cells.

We further analyzed the molecular effects of *PHLDA3* on the Akt pathway using *PHLDA3*^{-/-} mice. Consistent with our observations in MIN6 cells, the phosphorylated forms of Akt, p70 S6K and S6 were significantly increased in *PHLDA3*^{-/-} mice (Fig. 4b). Enhanced Akt activity is known to lead to cell proliferation, enlarged cell size and resistance to apoptosis.^(37,40) Compared to wild-type mice, there was a significant increase in Ki67-positive cells in *PHLDA3*^{-/-} mice, and both *PHLDA3*^{+/-} and *PHLDA3*^{-/-} mice developed islet hyperplasia (Fig. 4c). We also found that these hyperplastic islets mainly consisted of hypertrophic β cells. Islet cell size was significantly increased in both *PHLDA3*^{-/-} and *PHLDA3*^{+/-} mice (Fig. 4d). We next analyzed the effect of *PHLDA3* knockout on β cell apoptosis induced by STZ (streptozotocin), a chemical that is specifically toxic to β cells. *PHLDA3*^{-/-} β cells were found to be more resistant to STZ as judged by the elevation of blood glucose levels compared to wild-type mice (Fig. 4e). When we compared the areas of β and α cells in STZ-treated mice, we observed a significant increase in the

areas occupied by β cells in the *PHLDA3*^{-/-} mice (Fig. 4f), suggesting that *PHLDA3*-deficient β cells are relatively resistant to STZ-induced apoptosis. Collectively, these data indicate that *PHLDA3* represses Akt activity, suppresses cell proliferation and facilitates apoptosis *in vivo*.

Conclusion

In this paper we have reviewed the importance of *PHLDA3* and Akt in neuroendocrine tumors. *Akt* is an oncogene that facilitates cell proliferation and cell growth, and suppresses apoptosis. Whole exomic sequencing has revealed that 15% of PanNET specimens harbor mutations in the PI3K-Akt-mTOR pathway, demonstrating the importance of this pathway in PanNET malignancy. We found very frequent LOH of *PHLDA3* in 36 out of 50 PanNET samples (72%) and DNA methylation in 7 out of 7 samples (100%, which underwent LOH), features that are not detectable by exomic sequencing. Because *PHLDA3* is frequently inactivated in both lung and pancreatic NET, we deduce that *PHLDA3* commonly acts as a tumor suppressor gene for various types of NET (Fig. 5). *MEN1* has long been known as a tumor suppressor gene responsible for NET,^(36,45,46) and, indeed, we found that the frequency of LOH in *MEN1* and *PHLDA3* were both quite high (67% and 72%, respectively), suggesting that these two genes are equally important in PanNET development.⁽⁵⁾ Interestingly, the frequent occurrence of simultaneous LOH in *MEN1* and *PHLDA3* indicates that these pathways suppress PanNET tumorigenesis independently.

We have emphasized the molecular importance of *PHLDA3* in tumor suppressive roles for various types of NET. We suggest that examining possible genetic and epigenetic alterations in the *PHLDA3* gene contributes to a more accurate diagnosis of PanNET patients. Finally, accelerating the function of *PHLDA3* or inhibiting Akt activity could be a promising strategy for the treatment of various types of NET.

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Disclosure Statement

The authors have no conflict of interest to declare.

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