INPP4B Is a PtdIns(3,4,5)P$_3$ Phosphatase That Can Act as a Tumor Suppressor

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ABSTRACT

Inositol polyphosphate 4-phosphatase B (INPP4B) has been identified as a tumor suppressor mutated in human breast, ovary, and prostate cancers. The molecular mechanism underlying INPP4B’s tumor-suppressive role is currently unknown. Here, we demonstrate that INPP4B restrains tumor development by dephosphorylating the PtdIns(3,4,5)P$_3$ that accumulates in situations of PTEN deficiency. In vitro, INPP4B directly dephosphorylates PtdIns(3,4,5)P$_3$. In vivo, neither inactivation of $\text{Inpp4b}$ nor heterozygous deletion of $\text{Pten}$ in mice causes thyroid abnormalities, but a combination of these mutations induces malignant thyroid cancers with lung metastases. At the molecular level, simultaneous deletion of $\text{Inpp4b}$ and $\text{Pten}$ synergistically increases PtdIns(3,4,5)P$_3$ levels and activates AKT downstream signaling proteins in thyroid cells. We propose that the PtdIns(3,4,5)P$_3$ phosphatase activity of INPP4B can function as a “back-up” mechanism when PTEN is deficient, making INPP4B a potential novel therapeutic target for PTEN-deficient or PIK3CA-activated cancers.

SIGNIFICANCE: Although INPP4B expression is reduced in several types of human cancers, our work on $\text{Inpp4b}$-deficient mice provides the first evidence that INPP4B is a bona fide tumor suppressor whose function is particularly important in situations of PTEN deficiency. Our biochemical data demonstrate that INPP4B directly dephosphorylates PtdIns(3,4,5)P$_3$. Cancer Discov; 5(7); 730–9. ©2015 AACR.

See related commentary by Vo and Fruman, p. 697.

See related article by Chew et al., p. 740.

INTRODUCTION

Thyroid cancer is the most common endocrine malignancy, and its frequency is increasing dramatically in both men and women (1, 2). Indeed, thyroid cancer is predicted to be the fourth leading cancer diagnosis by 2030 (3). The histopathology of thyroid cancers is diverse. About 80% of all malignant thyroid neoplasms are papillary thyroid carcinoma (PTC), which are usually not aggressive. In contrast, about 10% to 15% of thyroid neoplasms are follicular thyroid carcinoma (FTC), which can invade blood vessels and metastasize to lung or bone. Follicular variant of papillary cancer is an especially aggressive variant that is associated with poor prognosis.

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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thyroid carcinoma (FV-PTC) has a unique histopathology characterized by the growth pattern of an FTC but the nuclear features of a PTC (4). The mechanisms driving the formation of these thyroid cancer variants are largely unknown.

Phosphoinositide (PI) signaling is a lipid second messenger cascade involving the sequential phosphorylation of phosphatidylinositol (PtdIns) to generate first PtdIns monophosphate (PtdInsP) and then phosphatidylinositol bisphosphate (PtdInsP2). Phosphorylation of PtdInsP2 by PI3Ks then generates phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3]. In mammalian cells, a total of eight PIs (three regioisomers each for PtdInsP and PtdInsP2) can be interconverted, and intracellular levels of each class of these lipids are regulated by the activities of 19 kinases and 29 phosphatases (5). PIs function as direct regulators of a broad range of intracellular proteins with diverse functions (6). To date, the list of molecules that bind to PIs includes protein kinases, phospholipases, ion channel proteins, scaffold proteins, cytoskeletal proteins, and regulators of membrane trafficking. Hence, PIs control cellular responses such as proliferation, inhibition of apoptosis, motility, secretion, and endocytosis, all of which are altered in cancer cells. Given the myriad functions of PI target proteins, it is not surprising that genetic mutations or alterations to the protein expression of PI kinases and phosphatases cause a variety of disorders. For instance, the inherited disease Cowden syndrome/multiple hamartoma syndrome is caused by either germline heterozygous inactivating mutations in the PtdIns(3,4,5)P3 phosphatase PTEN (OMIM #158350, CW51), or germline activating mutations or amplifications of the PtdIns(3,4,5)P3-synthesizing enzyme PIK3CA (OMIM #615108, CW55; ref. 7). Patients with Cowden syndrome show a predisposition to developing breast, thyroid, and endometrial cancers, and about 70% of these individuals have benign thyroid abnormalities, such as multinodular goiter, adenomatous nodules, and follicular adenoma (8). These abnormalities are presumably due to the deleterious effects of excessive intracellular PtdIns(3,4,5)P3, although formal experimental proof is lacking.

It is widely inferred that elevated PtdIns(3,4,5)P3 triggers activation of its targets in precancerous cells, including the proto-oncogene product AKT that promotes cell survival, proliferation, oncogenesis, motility, and metastasis. Recently, another phosphoinositide phosphatase called inositol polyposphate 4-phosphatase B (INPP4B; ref. 9), which dephosphorylates PtdIns(3,4)P2, has emerged as a tumor suppressor. The INPP4B gene locus (4q31.1–3) shows loss of heterozygosity in breast and ovarian carcinomas (10, 11), and expression of INPP4B protein is decreased in prostate cancer (12). Currently, however, the mechanisms underlying the putative tumor-suppressive role of INPP4B are a mystery. Intriguingly, alterations in INPP4B expression are more frequent in cancer patients who bear PTEN mutations than in those retaining wild-type (WT) PTEN function (10, 11). This dual inactivation of PTEN and INPP4B in cancers implies functional collaboration between these tumor suppressors, although how such concomitant aberrations of two lipid phosphatases would promote tumorigenesis is unclear.

Studies of PTEN-deficient mice by our laboratory and other groups have unequivocally shown that PTEN is a highly effective tumor suppressor in a wide variety of tissues (5). Pten−/− mice have a shortened lifespan, with 30% dying within 1 year of birth. Aged Pten−/− survivors develop mammary or endometrial tumors or T-cell lymphomas, among other malignancies. Interestingly, thyroid cancers, a type of tumor associated with PTEN mutations in humans, have not been detected in Pten−/− mice (13). Our mutants thus provide a unique system with which to analyze cooperation among gene mutations associated with thyroid carcinogenesis. In this study, we generated gene-targeted mice lacking the phosphatase domain of Inpp4b (Inpp4bΔΔ). Although these mutants are viable and healthy, Inpp4bΔΔ−Pten−/− compound mutant mice develop thyroid carcinomas with complete penetrance. Moreover, our initial studies in human tissues suggest that reductions in INPP4B and PTEN co-occur in human thyroid and endometrial cancers. INPP4B may therefore be an important regulator of cancer progression, especially in the context of PTEN insufficiency.

RESULTS

Loss of INPP4B Reduces the Survival of Pten+/− Mice

To gain insight into the mechanism by which INPP4B exerts its tumor-suppressive function, we scrutinized the ability of INPP4B to use each PI as a substrate. We overexpressed INPP4B in 293T cells and recovered an immunoprecipitate that was used as an enzyme source in vitro. To our surprise, in addition to PtdIns(3,4)P3, INPP4B dephosphorylated PtdIns(3,4,5)P3 (Fig. 1A). This activity toward PtdIns(3,4,5)P3 was intrinsic to INPP4B and not due to any proteins that might have bound to it in the immunoprecipitate because immunoprecipitates of the INPP4B C842S-mutant protein, which lacks hydrolase activity, did not dephosphorylate PtdIns(3,4,5)P3 (Supplementary Fig. S1A). PtdIns(3,4,5)P3 dephosphorylation catalyzed by PTEN reached a plateau at a substrate concentration of 1 mmol/L (Fig. 1B), whereas PtdIns(3,4,5)P3 dephosphorylation by INPP4B did not. These results suggest that INPP4B requires a higher PtdIns(3,4,5)P3 concentration than does PTEN to exert its phosphatase activity. Indeed, when our plots of reaction velocity versus substrate concentration were fitted to the sigmoidal Hill equation, nonlinear regression analysis revealed that the PtdIns(3,4,5)P3 concentration needed to obtain a velocity half of maximal (Km) for PTEN was 0.27 ± 0.04 mmol/L, whereas the Km for INPP4B was 0.70 ± 0.09 mmol/L. We confirmed this activity using an in-house, purified preparation of PtdIns(3,4,5)P3 (Supplementary Fig. S1B), excluding the possibility that our earlier results were due to INPP4B-mediated dephosphorylation of a contaminant [e.g., PtdIns(3,4)P2] in our commercial source of PtdIns(3,4,5)P3.

The above results raised the possibility that INPP4B might help to control PtdIns(3,4,5)P3 levels, particularly when PTEN is deficient and intracellular levels of this lipid second messenger rise as a result. To investigate this hypothesis, we generated Inpp4bΔΔ mice lacking Inpp4b exon 21, which encodes the active site motif in the phosphatase domain (Fig. 1C). Successful disruption of the Inpp4b gene was confirmed by Southern blotting and immunoblotting (Fig. 1D). Inpp4bΔΔ− mice were born at the expected Mendelian ratio, were healthy and fertile, had a normal lifespan, and showed no gross tissue abnormalities. These findings stand in sharp contrast to the
Thus, INPP4B is dispensable for PtdIns(3,4,5)P3 metabolism, as mice lacking the INPP4B phosphatase active site encoded by exon 21 (Inpp4bΔ+/Δ mice) appeared healthy at birth, they had significantly shorter lifespans than their WT (+/+ mice). Inpp4bΔ+/Δ mice had half-lifetime values of only 15 weeks (Fig. 1E), prompting us to histologically examine thyroid cells of these animals at 15 weeks of age. After hematoxylin and eosin (H&E) staining, thyroid follicles were indistinguishable from WT (Fig. 2Aa,b). However, in sharp contrast to WT, Inpp4bΔ+/Δ mice had thyroid follicles filled with irregularly arranged epithelial cells (Fig. 2Ac, closed circles; n = 40) up to 48 weeks of age.

Consistent with previous studies (13, 15), Pten−/− mice had half-lifetime values of only 15 weeks (Fig. 1E). Therefore, we hypothesized that INPP4B and PTEN have overlapping functions, and were interested in determining whether the simultaneous inactivation of INPP4B and PTEN results in premature mortality in mice. To test this hypothesis, we crossed Inpp4bΔ+/Δ;Pten−/− compound mutants. Although Inpp4bΔ+/Δ/+;Pten−/− compound mutants were born at the expected ratio and appeared healthy at birth, they had significantly shorter lifespans than Pten−/− mice, with half-lifetime values of only 26.2 weeks (Fig. 1E). Consistent with previous studies (13, 15), Pten−/− mice had a tendency to form larger follicles than WT mice, but the overall architecture and nuclear morphology of these structures were normal (Fig. 2Ab). In sharp contrast to Pten−/− follicles, Inpp4bΔ+/Δ;Pten−/− follicles were filled with irregularly arranged epithelial cells (Fig. 2Ac, e), and only a few glandular neoplasia. However, inactivation of PTEN alone is thought to be insufficient to induce thyroid cancers because these malignancies tend to affect older adults, and the increase in lifetime risk for developing thyroid cancer in Cowden syndrome patients is less than 10% (8). In mice, heterozygous loss of Pten results in thyroid follicular hyperplasia, but no tumors progress to aggressive malignancy (13, 15). Thus, genetic alterations in addition to Pten deficiency are required for benign thyroid disorders to evolve into malignant cancers.

Our Inpp4bΔ+/Δ;Pten−/− mice had larger thyroid glands than WT, Inpp4bΔ+/Δ, and Pten−/− mice, prompting us to histologically examine thyroid cells of these animals at 15 weeks of age. After hematoxylin and eosin (H&E) staining, Inpp4bΔ+/Δ thyroid follicles were indistinguishable from WT (Fig. 2Aa,b). Consistent with previous studies (13, 15), Pten−/− mice had a tendency to form larger follicles than WT mice, but the overall architecture and nuclear morphology of these structures were normal (Fig. 2Ab). In sharp contrast to Pten−/− follicles, Inpp4bΔ+/Δ;Pten−/− follicles were filled with irregularly arranged epithelial cells (Fig. 2Ac, e), and only a few glandular neoplasia.
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RESEARCH BRIEF

Figure 2. Deficiencies in INPP4B and PTEN cooperate to accelerate thyroid gland tumorigenesis in mice. A, H&E staining of thyroid glands from 26-week-old mice of the indicated genotypes. A, a and f, show magnified views of the areas surrounded by black broken-line rectangles in c and d, respectively. Scale bars, 300 μm (a–d) and 100 μm (e and f). Note the altered growth pattern of follicular epithelial cells in Inpp4bΔ/Δ;Pten+/− thyroid tissues in d and f. Arrows, thyroid follicles; *, tracheas. B, H&E staining of PTC-like nuclear phenotypes of follicular cells in Inpp4bΔ/Δ;Pten+/− thyroid tissue. a, nuclear overlapping and crowding; b, ‘ground glass’ nuclei; c, nuclear grooves; d, cytoplasmic invaginations; and e, nuclear enlargement. C and D, histologic staining to detect vascular invasion and lung metastasis. Serial sections of thyroid (C) and lung (D) from a 32-week-old Inpp4bΔ/Δ;Pten+/− mouse were stained with: a, H&E; b, Elastica-Masson; or c, anti-thyroglobulin Ab. Black arrows in C indicate vascular invasion by malignant thyroid epithelial cells. Scale bars, 100 μm.

INPP4B Is Reduced in Human Thyroid and Endometrial Cancers

Decreased expression of INPP4B protein has been documented in human breast, ovarian, and prostate malignancies (10–12), but its status in thyroid cancers has yet to be reported. To extend our findings in mice to humans, we first mined the recently published papillary thyroid cancer data in The Cancer Genome Atlas (TCGA) dataset using cBioPortal (16, 17). Using OncoPrint analysis, we found that INPP4B expression was downregulated in 30.6% (15 of 49 cases) of human FV-PTCs (Supplementary Fig. S3). Next, we generated an anti-INPP4B monoclonal antibody (Ab) suitable for human immunohistochemistry and immunostained sections
of thyroid tissues from FTC and PTC patients. Noncancerous thyroid glands from patients with goiter served as controls. High, medium, and low levels of Ab staining intensity were established such that the immunostaining of control glands fit into the medium group (Supplementary Fig. S4). Anti-INPP4B staining of native thyroid follicular epithelial cells was reduced compared with noncancerous controls in seven of eight FTC samples and seven of 39 PTC samples (Fig. 3A). These results are the first demonstration that INPP4B expression is decreased in human thyroid cancers.

Double immunostaining of our human FTC samples with anti-INPP4B and anti-PTEN Abs detected concurrent reductions in INPP4B and PTEN in three FTC cases (Fig. 3A and Fig. 3Ba–c), as has been observed for breast and ovarian carcinomas (10, 11). To extend this finding to additional thyroid cancer samples plus another tumor type, we used cBioPortal to conduct mutual exclusivity and co-occurrence analyses of the 511 cases of thyroid carcinoma and 240 cases of endometrial carcinoma listed in the TCGA database (16, 17). We identified strong tendencies toward the co-occurrence of INPP4B and PTEN mRNA downregulation in thyroid carcinoma (Supplementary Fig. S5A), and alterations to the INPP4B and PTEN genes in endometrial carcinoma (Supplementary Fig. S5B). INPP4B mutations also showed a significant positive association with PIK3CA mutations in the endometrial dataset. These profiles suggest that loss of INPP4B function may be advantageous to cancer cells when they fail to degrade, or overproduce, PtdIns(3,4,5)P3.

Loss of INPP4B Leads to Hyperactivation of AKT Signaling

A previous study has shown that genetic alterations of PTEN in thyroid cancers are associated with AKT hyperactivation (15). In line with this observation, we found that AKT phosphorylation was modestly increased in a INPP4BhiPTENlo human FTC sample compared with control thyroid gland (Fig. 3Ca,b). However, INPP4BhiPTENlo FTC samples showed dramatically increased AKT phosphorylation (Fig. 3Cc), suggesting that INPP4B is a potent negative regulator of AKT activation.

To investigate whether the reduced INPP4B in INPP4BhiPTENlo FTC actually caused the observed AKT hyperactivation, we compared the activation status of AKT and its downstream effectors in thyroid follicular cells of Inpp4B−/−Pten−/− mice and controls. Immunoblot analyses of Inpp4B−/−Pten−/− follicular cells revealed hyperphosphorylation of PDK1 (Fig. 3D), a direct target of PtdIns(3,4,5)P3, that is recruited to the plasma membrane and phosphorylates AKT. Accordingly, double-mutant thyroids exhibited increased AKT phosphorylation, just as observed in INPP4BhiPTENlo human FTC. Consistent with their activated AKT, Inpp4B−/−Pten−/− follicular cells showed enhanced phosphorylation of PRAS40, mTOR, and S6 (Fig. 3D). Histological examination of thyroid serial sections revealed positive staining for phosphorylated (phospho)-AKT at the plasma membrane of double-mutant follicular cells (Fig. 3E) and for phospho-S6 in the cytosol (Fig. 3F). Low levels of phospho-AKT and phospho-S6 were present in Pten−/− follicular cells, but staining intensities were weaker than in Inpp4B−/−Pten−/− cells. It should be noted that no activation of AKT signaling was observed in Inpp4BΔ/Δ thyroids, suggesting that INPP4B is dispensable for downregulating AKT activation in the presence of normal PTEN. These data demonstrate that INPP4B has a key role in preventing the hyperactivation of AKT and its downstream effectors that occurs in situations of PTEN deficiency.

PtdIns(3,4,5)P3 Accumulation and AKT2 Hyperactivation Drive Thyroid Tumorigenesis in Inpp4BΔ/ΔPten−/− Mice

Two AKT isozymes occur in thyroid follicular cells: AKT1 and AKT2. The enhanced activation of AKT signaling we observed in Inpp4BΔ/ΔPten−/− follicular cells prompted us to examine whether separate deletion of each AKT isozyme could ameliorate the thyroid tumorigenesis and metastasis in Inpp4BΔ/ΔPten−/− mice. We generated triple-mutant mice lacking either AKT1 or AKT2 in the Inpp4BΔ/ΔPten−/− background and found that aggressive thyroid cancers developed in Akt1−/−;Inpp4BΔ/ΔPten−/− mice (Fig. 4Aa,b), just as observed in Inpp4BΔ/ΔPten−/− animals (Fig. 2D). In contrast, Akt2−/−;Inpp4BΔ/ΔPten−/− mice had a much milder phenotype, with no cytologic evidence of malignancy or pulmonary metastasis (Fig. 4Ac,d). Consistent with these results, levels of AKT (Fig. 4B) and S6 (Fig. 4C) phosphorylation were significantly lower in mutant cells lacking AKT2 compared with those lacking AKT1. Over a 48-week observation period, 38% of Akt2−/−;Inpp4BΔ/ΔPten−/− mice remained viable, whereas only 10% of Akt1−/−;Inpp4BΔ/ΔPten−/− mice survived this long (Fig. 4D). These results suggest that AKT2 hyperactivation in thyroid follicular cells makes a greater contribution to the mortality of Inpp4BΔ/ΔPten−/− mice than AKT1 hyperactivation.

Although it has long been assumed that PTEN deficiency causes PtdIns(3,4,5)P3 accumulation in tumor cells in vivo, quantitative data validating this assumption is lacking for both mouse and human cancers. To elucidate the molecular mechanism underlying the functional collaboration between PtdIns(3,4,5)P3 and Pten in tumor suppression, we quantitated PtdIns(3,4,5)P3 levels in thyroid tissues by modifying a non radioactive method of PtdIns(3,4,5)P3 measurement developed by Clark and colleagues (18). As shown in Fig. 4E, heterozygous deletion of Pten increased cellular PtdIns(3,4,5)P3 levels in whole murine thyroid gland extracts. Consistent with the weak phosphatase activity of INPP4B toward PtdIns(3,4,5)P3, in vitro (Fig. 1A), disruption of Inpp4B alone caused only a small (but still statistically significant) increase in PtdIns(3,4,5)P3. However, PtdIns(3,4,5)P3 accumulation in the thyroid was enormous when Inpp4B was deleted in the Pten heterozygous background. These results clearly demonstrate that INPP4B is critical for dephosphorylation of the PtdIns(3,4,5)P3 that accumulates under conditions of PTEN deficiency. In contrast to PtdIns(3,4,5)P3, there was no difference in PtdIns(3,4,5)P3 levels between Pten−/− and Inpp4BΔ/ΔPten−/− thyroid tissues (Supplementary Fig. S6). Taken together, our data suggest a model (Fig. 4F) in which INPP4B’s dephosphorylation of PtdIns(3,4,5)P3 comprises a “back-up” mechanism that prevents tumorigenic accumulation of this lipid messenger when PTEN activity is insufficient.

DISCUSSION

Gene mutation, heterozygous deletion, and decreased protein expression of INPP4B have been implicated in several types of human cancers (10–12, 19). However, until
**Figure 3.** Codefi ciency of INPP4B and PTEN enhances AKT signaling in human and mouse thyroid carcinomas. 

- **A** shows the percentages of samples of human FTC (n = 8) and PTC (n = 39) showing High, Medium, or Low levels of INPP4B or PTEN expression as determined by multiplying staining intensity with the percentage of immunoreactive cells (see Methods). Scored samples were categorized into the indicated 9 (3 x 3) subgroups. Note that a substantial proportion of human FTC were classifi ed as INPP4B lo PTEN lo .

- **B** and **C** present representative fl  uorescent images of serial sections of control human goiter (a; n = 9) and two human FTC samples (b and c; n = 8) immunostained with (B) anti-INPP4B Ab and anti-PTEN Ab, or (C) anti–phospho-AKT (pAKT) Ab. DAPI, counterstaining. White arrows, PTEN-positive leukocytes. Scale bars, 10 μm.

- **D** displays immunoblot to detect the indicated total and phosphorylated (p) forms of AKT, PDK1, PRAS40, mTOR, and S6 in lysates of thyroid glands from mice of the indicated genotypes (n > 3/group). Total AKT and Tubulin levels were evaluated as loading controls. Results are representative of at least three trials.

- **E** and **F** show immunostaining of thyroid sections from mice of the indicated genotypes (n > 3/group) to detect hyperphosphorylation of AKT (E) and S6 (F) in thyroid epithelial cells. Scale bars, 50 μm.
Our study, it had not been definitively shown that INPP4B deregulation is not just a consequence of tumorigenesis but an active contributor. Here, we have used Inpp4B single, double, and triple mutant mice to establish a cause-and-effect relationship between loss of INPP4B function and tumorigenesis.

Our work is the first to demonstrate that a loss of Inpp4B predisposes mice to cancer development, providing reverse-genetics evidence for a tumor-suppressive function of INPP4B. Although mice with disruption of Inpp4B alone did not develop any tumors, all Inpp4BΔ/Δ;PtenΔ/Δ mice exhibited spontaneous and early-onset formation of metastatic thyroid cancers. These double mutants displayed a dramatically reduced lifespan compared with both PtenΔ/Δ mice (this study) and thyrocyte-specific Pten-null mice (20). Thus, INPP4B is dispensable for thyroid tumor suppression in WT mice but is essential for preventing tumorigenesis in this tissue when Pten activity is insufficient. Studies of the genomes of human thyroid cancer cells have revealed that these malignancies accumulate various genetic alterations that may promote thyroid cell dedifferentiation and drive thyroid cancer initiation and progression (1, 2). Our results are in line with this observation and indicate that loss of Inpp4B is one step of the many driving thyroid cancer progression.

Because Pten and Inpp4B are deleted in a systemic manner in our mouse model, a concurrent loss of these enzymes in non-thyroid cells may also contribute to the tumor-prone phenotype of the thyroid gland. Coincident decreases in expression of INPP4B and Pten have been observed in human breast and ovarian carcinomas (10, 11). Our mutational profiling of human thyroid and endometrial cancers using the TCGA dataset also showed that genetic alterations in INPP4B and Pten have a strong tendency to co-occur, as do mutations of INPP4B and PIK3CA. In addition, although not statistically significant, we detected coincident decreases in INPP4B and Pten expression in 38% of human FTC specimens. Our biochemical data showing that INPP4B phosphatase with a higher kcat and lower km than Pten. Thus, this activity of INPP4B is most important when intracellular PtdIns(3,4,5)P3 accumulates to high concentrations.
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which presumably cause deleterious increases in intracellular PtdIns(3,4,5)P₃. Future study should investigate the position of phosphate that INPP4B hydrolyzes.

Hyperplasia of the thyroid gland, mammary gland, and uterus are the most common manifestations of Cowden syndrome, and these patients have increased risks of developing cancers of these origins (8). For example, 66% of Cowden syndrome patients have benign thyroid abnormalities such as follicular adenomas, whereas 3% to 10% of Cowden syndrome patients develop thyroid malignancies. It is tempting to speculate that the presence or absence of INPP4B could determine whether the benign lesions in Cowden syndrome patients undergo malignant transformation. We have demonstrated in mice that concurrent PTEN and INPP4B deficiencies synergistically elevate PtdIns(3,4,5)P₃ in the thyroid gland in vivo (Fig. 4E). We therefore propose that there must be a threshold PtdIns(3,4,5)P₃ concentration above which benign hyperplastic thyroid cells acquire cancerous properties (Fig. 4F).

A provocative question arising from our work is whether INPP4A, a PI phosphatase highly related to INPP4B, also functions as a tumor suppressor. INPP4A and INPP4B share 37% amino acid sequence identity (5). Remarkably, the CxR active site motif in the phosphatase domains of these isoforms is identical (CKSAKDR). As expected, we found that INPP4A is also capable of dephosphorylating PtdIns(3,4,5)P₃ (Supplementary Fig. S1). We have previously reported that loss of the Inpp4a gene in mice leads to neurodegeneration in the central nervous system as a result of enhanced glutamate excitotoxicity (21). These mutants die within 1 month of birth, precluding an analysis of tumorigenesis. Nevertheless, we speculate that INPP4A may be a potential candidate for a novel tumor-suppressive phosphatase. Examination of genetic alterations in endometrial and lung cancers listed in the TCGA database has revealed several mutations in INPP4A, in particular a missense mutation that replaces the arginine residue in the CX₅R active site motif. Future experiments with single- and double-mutant mice bearing conditional Inpp4a deletions should answer this intriguing question.

In conclusion, our study has established a role for INPP4B in tumor suppression and demonstrated its critical function in PtdIns(3,4,5)P₃ metabolism. Our Inpp4BΔ/Δ (and parental Inpp4bΔ/Δ) mice provide useful models for studying disorders emanating from aberrant accumulation of PtdIns(3,4,5)P₃. Notably, downregulation of INPP4B has been observed in several aggressive human cancers, including in >80% of basal-like breast cancers (11), and so may be an important molecular signature of malignancy. Future studies in which the biologic properties of PtdIns(3,4,5)P₃-driven cancers are determined by reconstituting the underlying genetic alterations in mice may point toward novel clinical interventions. Because the INPP4B gene is not lost in human cancers but is only minimally transcribed, INPP4B is a plausible anticancer drug target. Our mutants may be useful for testing both PI3K inhibitors and chemical compounds capable of enhancing residual INPP4B activity in cancer cells. This approach may open up new therapeutic strategies for a wide range of hyperplasias and cancers that harbor mutations in PTEN and/or PIK3CA and accumulate deleterious levels of intracellular PtdIns(3,4,5)P₃.
chromatography (TLC) in chloroform/methanol/acetic acid/water (7:5:2:2:2). The corresponding spot was scraped off of the silica gel plate and extracted using the Bligh–Dyer method. The resultant radioactive PI(3,4,5)P$_3$ (4,000 cpm) was mixed with non-radioactive dipalmitoyl-PI(3,4,5)P$_3$ (3 mmol, Cayman) in 15-μL assay buffer [50 mmol/L Tris–HCl (pH 7.5), 0.2% Triton X-100, and 2 mmol/L dithiothreitol]. Purified recombinant FLAG-tagged phosphatase (5–10 μg/15 μL) was added to the substrate and incubated at 30°C for 20 hours. After reaction completion, lipids were extracted by the Bligh–Dyer method and isolated by TLC as described above. Radiolabeled PI(3,4,5)P$_3$ was quantitated by autoradiography using Typhoon FLA 9500 (GE Healthcare).

**Antibodies**

Anti-INPP4B antiseraum used for immunoblotting was kindly provided by Dr. Jean Vacher (Clinical Research Institute of Montreal, Canada). INPP4B-specific rat monoclonal Ab (mAb) against a purified recombinant human INPP4B fragment (2–235 amino acids) was raised for immunohistochemistry. The mAbs against PTEN, pan-AKT, phospho-AKT (S473), phospho-AKT (T308), phospho-mTOR (S2448), phospho-PDK1 (S241), phospho-PI3K (S473), and INPP4B (S235/236) were all from Cell Signaling Technology. Anti-thyroglobulin mAb was from DAKO. Anti-α-tubulin polyclonal Ab was from Medical & Biological Laboratories. Antibodies conjugated to Alexa Fluor 488 or 568 were from Vector Laboratories. Deparaffinized sections were boiled in 10 mmol/L citrate buffer (pH 6.0) at 121°C for 10 minutes. Fluorescent images were acquired on a LSM 780 microscope (Carl Zeiss) and processed with ZEN software (Carl Zeiss).

**Histologic Analyses**

Mouse thyroid glands were fixed in 10% formalin neutral buffer solution and embedded in paraffin. Sections (3–5 μm) were cut and stained with HE or Elastica-Masson in accordance with standard procedures. Immunohistochemistry, deparaffinized sections were incubated in 10 mmol/L citrate buffer (pH 6.0) at 121°C for 10 minutes, treated with 3% H$_2$O$_2$/methanol for 30 minutes, blocked with 10% BSA, and incubated overnight at 4°C with primary Ab recognizing thyroglobulin, phospho-AKT S473, phospho-S6 S235/236, and PTEN (all at 1:100 dilution). Immunostained sections were incubated with peroxidase-conjugated secondary Abs that were detected using DAB solution (WAKO). The corresponding spot was scraped off of the silica gel plate and extracted using the Bligh-Dyer method. The resultant radioactive PI(3,4,5)P$_3$ (4,000 cpm) was mixed with non-radioactive dipalmitoyl-PI(3,4,5)P$_3$ (3 mmol, Cayman) in 15-μL assay buffer [50 mmol/L Tris–HCl (pH 7.5), 0.2% Triton X-100, and 2 mmol/L dithiothreitol]. Purified recombinant FLAG-tagged phosphatase (5–10 μg/15 μL) was added to the substrate and incubated at 30°C for 20 hours. After reaction completion, lipids were extracted by the Bligh–Dyer method and isolated by TLC as described above. Radiolabeled PI(3,4,5)P$_3$ was quantitated by autoradiography using Typhoon FLA 9500 (GE Healthcare).

**Immunofluorescence Analyses**

For immunofluorescent examination of INPP4B, PTEN, and phospho-AKT S473, or with anti-INPP4B. Bind-
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