In Vivo Role of INPP4B in Tumor and Metastasis Suppression through Regulation of PI3K–AKT Signaling at Endosomes

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ABSTRACT
The phosphatases PTEN and INPP4B have been proposed to act as tumor suppressors by antagonizing PI3K–AKT signaling and are frequently dysregulated in human cancer. Although PTEN has been extensively studied, little is known about the underlying mechanisms by which INPP4B exerts its tumor-suppressive function and its role in tumorigenesis in vivo. Here, we show that a partial or complete loss of Inpp4b morphs benign thyroid adenoma lesions in Pten heterozygous mice into lethal and metastatic follicular-like thyroid cancer (FTC). Importantly, analyses of human thyroid cancer cell lines and specimens reveal INPP4B downregulation in FTC. Mechanistically, we find that INPP4B, but not PTEN, is enriched in the early endosomes of thyroid cancer cells, where it selectively inhibits AKT2 activation and in turn tumor proliferation and anchorage-independent growth. We therefore identify INPP4B as a novel tumor suppressor in FTC oncogenesis and metastasis through localized regulation of the PI3K–AKT pathway at the endosomes.

SIGNIFICANCE: Although both PTEN and INPP4B can inhibit PI3K–AKT signaling through their lipid phosphatase activities, here we demonstrate lack of an epistatic relationship between the two tumor suppressors. Instead, the qualitative regulation of PI3K–AKT2 signaling by INPP4B provides a mechanism for their cooperation in suppressing thyroid tumorigenesis and metastasis. Cancer Discov; 5(7); 740–51. © 2015 AACR.

See related commentary by Vo and Fruman, p. 697.
See related article by Kofuji and colleagues, p. 730.

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INTRODUCTION

The PI3K–AKT signaling pathway modulates important biologic processes, such as cell cycle, survival, metabolism, and motility, which are often disrupted in cancer (1). Hyperactivation of the PI3K–AKT pathway leads to an accumulation of phosphatidylinositol (3,4,5)-trisphosphate [P(3,4,5)P3], which in turn increases the recruitment and activation of protein kinase AKT at the cytoplasmic membrane (1), a key mediator of the oncogenic effects of enhanced PI3K signaling.

The AKT protein kinase family is made up of three highly homologous isoforms, namely AKT1, AKT2, and AKT3 (2). Despite similarities in sequence and regulation, studies reveal isoform-specific functions in cancer progression. In the context of breast cancer, overexpression of AKT2 promotes metastasis (3), whereas AKT1 suppresses metastasis (4, 5). The distinct functions of the AKT isoforms could be explained, at least in part, through the association of the AKT isoforms with distinct organelles or subcellular compartments (2).

The tumor suppressor gene PTEN, encoding a lipid phosphatase, dephosphorylates P(3,4,5)P3 to P(4,5)P2 to antagonize PI3K–AKT signaling. To study the effects of Pten loss in vivo, we previously generated mouse models with constitutive and conditional Pten loss (6). We found that homozygous loss of Pten results in embryonic lethality, whereas Pten heterozygous (Pten+/−) mice develop breast, endometrium, prostate, adrenal, and pituitary tumors, and lymphoma (7). More recently, INPP4B (inositol polyphosphate-4-phosphatase, type II), another dual specificity and lipid phosphatase, has emerged as a putative tumor suppressor in the suppression of the PI3K–AKT signaling pathway. INPP4B was initially identified as an anchorage-independent growth suppressor in a shRNA-mediated genetic screen performed in HMEC cells (8), and was found to inhibit PI3K signaling and to display tumor-suppressive activity in breast tumor cell lines (9). In agreement with these findings, INPP4B expression was found to be reduced in basal-like breast cancers (9, 10), melanoma (11), nasopharyngeal carcinoma (12), and prostate cancer (13). Furthermore, expression-profiling analyses revealed that INPP4B mRNA expression is reduced in up to 47% of metastatic prostate cancer cases (14), implicating the potential role of INPP4B in metastatic progression.

INPP4B converts P(3,4)P2 to P(3)P, and because direct interaction of P(3,4)P2 with the pleckstrin homology (PH) domain of AKT is required for membrane recruitment and full activation of AKT (15), INPP4B, like PTEN, is anticipated to act as a tumor suppressor by antagonizing PI3K–AKT signaling (9). However, unlike PTEN, the underlying molecular mechanisms by which INPP4B exerts its tumor-suppressive function are poorly understood. In addition, it is not known whether INPP4B acts as a tumor suppressor in vivo.
We therefore sought to investigate the tumor-suppressive functions of INPP4B in vivo in knockout mouse models. Surprisingly, we found that INPP4B exerts a specific role in the suppression of thyroid tumorigenesis and metastasis in vivo through the inhibition of class II PI3K, α isoform (PI3K-C2α)–mediated AKT2 activation at endosomes.

RESULTS

**Inpp4b Knockout (Inpp4b−/−) Mice Do Not Develop the Tumorigenic Phenotype of Pten Haploinsufficient Mice**

To determine the in vivo tumor-suppressive function of INPP4B, we took advantage of Inpp4b knockout mice (Inpp4b−/−) generated by using a homologous recombination–targeting strategy in which the conditional targeting vector was constructed to delete exon 21 of the mouse Inpp4b gene, which encodes the phosphatase catalytic domain (Fig. 1A and B). Unlike Pten+/− mice, Inpp4b−/− mice were viable and born in accordance with Mendelian frequencies (Fig. 1C). Furthermore, Inpp4b−/− and Inpp4b−/− mice did not develop any of the tumors or the lymphoproliferative disease characteristic of Pten+/− mice in a 16- to 24-month follow-up (16). Aggressive and fatal, albeit sporadic, histiosarcomas were observed after a very long latency in Inpp4b−/− mice (data not shown). These results demonstrate unequivocally that the role of PTEN and INPP4B in tumorigenesis could be very distinct.

**Loss of Inpp4b in a Pten Heterozygous Background Leads to Metastatic Follicular-Like Thyroid Carcinoma**

We next sought to determine if loss of INPP4B cooperated with PTEN loss to promote tumor progression. We hypothesized that loss of INPP4B would accelerate overall tumor progression in Pten+/− mice, in keeping with their reported epistatic relationship in the suppression of PI3K-AKT signaling. To this end, we crossed Pten+/− mice with Inpp4b−/− mice. The resulting Pten+/−;Inpp4b−/− mice were then crossed with Inpp4b+/− littermates to generate wild-type, Pten+/−;Inpp4b+/−, and Pten+/−;Inpp4b−/− mice (Supplementary Fig. S1A). These mice were, once again, viable and born following the expected Mendelian frequencies (Fig. 1C). The lack of embryonic lethality in any of the two compound Pten+/−;Inpp4b+/− and Pten+/−;Inpp4b−/− genetic make-ups further underscores the fact that PTEN and INPP4B might exert distinct roles in signaling because the progressive PI3K-AKT elevation should result in embryonic lethality as previously reported (6). However, Pten+/−;Inpp4b−/− mice did not survive beyond 5 to 6 months of age, whereas the majority Pten+/−;Inpp4b+/− mice died between 8 and 14 months of age (Fig. 2A). Gross anatomical

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**Figure 1.** Generation and characterization of Pten+/−;Inpp4b+/− and Pten+/−;Inpp4b−/− mice. A, diagram representing the structure of INPP4B, which contains an N-terminus C2 domain and a C-terminus phosphatase domain harboring the phosphatase catalytic motif CX 5 R. B, schematic map of the wild-type [WT] INPP4B locus (top), targeting vector (upper middle), and predicted targeted allele (lower middle) and knockout allele [bottom]. C, table depicting the observed versus the expected numbers of mice of the respective genotypes from a Pten+/−;Inpp4b+/− and Inpp4b−/− cross. These values gave a χ² of 3.26, which is lower than the critical value of 11.07, which would yield an α = 0.05. Thus, we conclude that the mutants were born following Mendelian frequencies.
analyses showed that the $Pten^{+/–}$; $Inpp4b^{+/–}$ mice developed large multinodular goiters (Fig. 2B). These mice died, or were euthanized, after developing compressive airway and esophageal obstruction as a consequence of the mass effect from thyroid enlargement. Histopathologic analyses revealed that most of the thyroid tumors developed in the $Pten^{+/–}$; $Inpp4b^{−/−}$ mice had variable degrees of encapsulation (Fig. 2C and D, top left), microfollicular architecture (Fig. 2C, left middle), and many showed impressive vascular invasion closely resembling follicular thyroid carcinoma (FTC) in human. High-grade features were commonly seen, including significant mitotic activity and necrosis, whereas some portion of the tumors showed nuclear features of follicular variant papillary thyroid carcinoma (FV-PTC), such as nuclear contour irregularity, nuclear grooves, intranuclear pseudoinclusions, and chromatin pallor (Fig. 2C, right). Collectively, the majority of tumors in $Pten^{+/–}$; $Inpp4b^{+/–}$ mice displayed histologic and pathologic features of FTC and FV-PTC in human (Fig. 2C and D, top). Importantly, 50% of $Pten^{+/–}$; $Inpp4b^{+/–}$ mice developed diffuse pulmonary metastases (Fig. 2D, top right). These metastases had the histologic appearance of thyroid tissue with follicular architecture, colloid, and also stained positive for thyroglobulin, a thyroid-specific marker (Fig. 2D, middle). Necropsy did not reveal metastases in other regional and distant sites. AKT activation was observed by immunohistochemistry in both the thyroid tumors and the metastases (Fig. 2D, bottom). Serum thyroid-stimulating hormone (TSH) levels of $Pten^{+/–}$; $Inpp4b^{+/–}$ and $Pten^{+/–}$; $Inpp4b^{−/−}$ mice did not differ significantly from those in $Pten^{+/–}$ mice (Supplementary Fig. S1B). In addition, we examined the thyroids from a cohort of $Pten^{+/–}$; $Inpp4b^{−/−}$ mice.
Histopathologic analyses revealed that $\text{Pten}^{+/−}\text{Inpp4b}^{+/−}$ mice developed benign goiter and FV-PTC/FTC and presented with pulmonary metastases of thyroid carcinoma at a similar penetrance to $\text{Pten}^{+/−}\text{Inpp4b}^{−/−}$ mice (Supplementary Fig. S1C). Furthermore, $\text{Pten}^{+/−}\text{Inpp4b}^{+/−}$ and $\text{Pten}^{+/−}\text{Inpp4b}^{−/−}$ mice did not display accelerated tumorigenesis in other tissues as compared with $\text{Pten}^{+/−}$ mice by 5- to 8-month follow-up (data not shown). However, we did note a nonsignificant increased incidence of breast adenocarcinoma in $\text{Pten}^{+/−}\text{Inpp4b}^{−/−}$ mice ($P = 0.16$, 37.5% in $\text{Pten}^{+/−}\text{Inpp4b}^{−/−}$ vs. 15.5% in $\text{Pten}^{+/−}$ mice). Therefore, $\text{Inpp4b}$ loss cooperates with $\text{Pten}$ haploinsufficiency to promote thyroid cancer progression and metastasis.

**Human Follicular Thyroid Cancer Cell Lines and Tissues Display Low INPP4B Expression**

To assess the relevance of our mouse model findings to human FTC, we evaluated the status of INPP4B in human thyroid cancer cells. To this end, we utilized seven thyroid cell lines, namely Nthy-Ori 3, Htori (SV40-immortalized primary thyroid follicular epithelial cells), FTC133, FTC236, FTC238 (follicular thyroid carcinoma), TPC1 (thyroid papillary carcinoma), and 8505C (anaplastic carcinoma). We first determined the relative expression levels of INPP4B in these thyroid cell lines. We observed very low expression of INPP4B at both protein (Fig. 3A; Supplementary Fig. S2) and mRNA levels. Therefore, INPP4B is a potential therapeutic target for the treatment of follicular thyroid carcinoma.

**Figure 3.** INPP4B expression is reduced in human follicular thyroid cell lines and human surgical specimens. A, Western blot analysis of thyroid cancer cell lines for INPP4B and PTEN expression. Arrow indicates specific band. B, RT-qPCR analysis of thyroid cancer cell lines for INPP4B transcript levels. C, Western blot analysis of thyroid cancer lines for AKT activation on both Serine 473 and Threonine 308 residues. p, phosphorylated. D, RT-qPCR analysis of INPP4B in unmatched normal versus FTC patient tumor samples. E-F, thyroid cancer cell lines were treated with 3 μmol/L 5-Aza-2′-deoxycytidine (5-Aza) for 5 days. Panel shows transcript (E) and protein analysis of INPP4B expression and AKT activation levels in DMSO versus 5-Aza treated FTC cells (F). Arrows indicate specific band (see also Methods). AKT2 antibody used is 5239.
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transcript levels (Fig. 3B) in the FTC cell lines FTC133, FTC236, and FTC238 compared with SV40-immortalized human thyroid follicular cells Nthy-ori-3 and Htori or the TPC1 thyroid cancer cell line. We also noted that PTEN protein expression was not detectable in the FTC cell lines (Fig. 3A). A search using the Broad Cancer Cell Line Encyclopedia database revealed that the FTC cell lines harbor the PTEN R130* nonsense mutation. This concomitant loss of PTEN and INPP4B in our FTC human cell lines is faithfully modeled by our Pten+/−;Inpp4b+/− mice. Finally, we observed an increase in the activation of AKT in these cell lines, as indicated by higher levels of AKT phosphorylation at both Thr308 and Ser473 residues (Fig. 3C).

Importantly, INPP4B transcript expression was significantly downregulated in human FTC samples when compared with normal tissue samples (Fig. 3D). Because of the infrequency of INPP4B deletions or mutations in human cancers, we further hypothesized that its loss in FTC cell lines might be due to aberrant gene methylation. Indeed, treatment of these cell lines with 5-Aza-2′-deoxycytidine (5-Aza), which inhibits DNA methylation, increased INPP4B expression approximately 4-fold (Fig. 3E). Uregulation of INPP4B by 5-Aza decreased activation of both AKT1 and AKT2 (Serine 473 and Serine 474, respectively; Fig. 3F) with a much greater effect on phosphorylated (p) AKT2 than on pAKT1 (Fig. 3F). However, bisulfite sequencing indicated that this upregulation is potentially indirect (Supplementary Fig. S3), mediated via the upregulation of yet-to-be-defined transcription factors. Overall, the downregulation of INPP4B in human FTC strongly supports its tumor-suppressive role in this aggressive tumor type of the thyroid.

Loss of INPP4B Increases AKT Activation

We previously demonstrated that INPP4B knockdown enhanced AKT activation (9). To gain insight into the mechanisms by which INPP4B might promote thyroid tumor development, we analyzed thyroid tumor lysates from Pten+/−, Pten+/−;Inpp4b+/−, and Pten+/−;Inpp4b−/− mice for AKT activation via Western blotting. In agreement with previous findings, tumors from Pten+/−;Inpp4b−/− mice showed higher levels of AKT phosphorylation (monitored through pAKT Serine 473 and pAKT2 Serine 474) when compared with thyroid tumors obtained from Pten+/+ or Pten+/−;Jdp4b+/− mice (Fig. 4A). We also isolated primary mouse embryonic fibroblasts (MEF) of all genetic combinations—wild-type, Inpp4bflox/flox, Inpp4bflox/flx, Pten+/+, Inpp4b+/−, and Pten−/−. Inpp4b+/−. We found that MEFs with INPP4B deletion displayed increased AKT activation, monitored through the phosphorylation of both Serine 473 and Thr308 at different time points after serum stimulation (Supplementary Fig. S4). In addition, although Pten+/+, Pten−/−, and Pten+/−;Inpp4b+/− MEFs did not display much higher initial AKT activation upon serum starvation restimulation (Fig. 4B, t = 5 and 15 minutes), there was a prolonged AKT1 and AKT2 activation in Pten+/−;Inpp4b−/− and Pten+/−;Inpp4b+/− MEFs when compared with Pten+/− MEFs (Fig. 4B, t = 30 minutes). Furthermore, we found that human thyroid cancer cell lines with lower INPP4B displayed higher levels of AKT phosphorylation (Figs. 3C and 4C). Notably, both AKT1 and AKT2 were activated to a similar extent in total cell lysates (Fig. 4A–C). Therefore, loss of both lipid phosphatases, INPP4B and PTEN, resulted in an additive activation of AKT in thyroid tissue, MEFs, and cancer cell lines.

INPP4B Suppresses PI3K-C2α-Mediated AKT2 Activation at Early Endosomes in Thyroid Cancer Cells

The observation that complete inactivation of PTEN in mouse thyroid results mainly in follicular adenoma within 12 months of age (17) and that INPP4B loss does not accelerate the entire tumor spectrum of Pten+/− mice raised the possibility that loss of INPP4B specifically cooperates with PTEN loss for tumor progression and metastasis in thyroid, not through a generic elevation of PI3K-AKT signaling, but potentially by deregulating the PI3K pathway in a more selective manner. Previous studies have shown that AKT2 deficiency had little to no effect on the tumor spectrum in Pten+/− mice, and only specifically significantly decreased the incidence of thyroid tumors in Pten−/− mice (18), suggesting that AKT2 might play an oncogenic role in thyroid cancer.

Recent studies have shown that AKT2 phosphorylates its substrates on both endosomes and the plasma membrane (19). Strikingly, subcellular fractionation experiments showed that INPP4B, but not PTEN, was expressed in the Rab5-positive early endosomal (EE) fraction together with AKT2 in thyroid cancer cells (Fig. 4D and E). Although AKT1 was also localized in the EE fraction (Fig. 4D), surprisingly, knockdown of INPP4B selectively activated AKT2, but not AKT1, in the EE (Fig. 4F). Consistent with this result, thyroid cancer cell lines with lower INPP4B expression displayed higher endosomal AKT2 activation (Supplementary Fig. 5A). Furthermore, increasing INPP4B expression with 5-Aza in FTC236 cells resulted in a greater decrease in pAKT2 in the EE fraction compared with pAKT1 (Fig. 4G). Collectively, these results lend support to the selective activation of AKT2 at the early endosomes by INPP4B.

In line with PI3K-C2α producing P(3,4)P2 and being selectively activated in endocytosis as well as endocytic recycling (20, 21), we also found PI3K-C2α expression in the thyroid cancer cell lines and enriched in the EE fraction, along with INPP4B and AKT2 (Fig. 4E). The association of INPP4B, AKT2, and PI3K-C2α with EE was also confirmed by immunofluorescence, in which INPP4B, AKT2, and PI3K-C2α were colocalized with Rab5-positive punctate structures in the cells (Fig. 4H). Notably, INPP4B loss was associated with increased abundance of P(3,4)P2 (Fig. 4I and J), but not P(3,4,5)P3 (Supplementary Fig. S5B). Consequently, there was an accumulation of the P(3,4)P2-binding protein SNX9 in vesicles near the plasma membrane (Fig. 4J). Taken together, these results suggest that INPP4B negatively regulates PI3K-C2α signaling at the EE. Furthermore, loss of PI3K-C2α decreased AKT2 activation to a greater extent than that of AKT1 in total cell lysates (Supplementary Fig. S6A and S6B) and significantly inhibited cell proliferation in FTC236 cells (Supplementary Fig. S6C), underscoring the functional relevance of enhanced PI3K-C2α signaling in cells with lower INPP4B. Taken together, our results strongly suggest that INPP4B inhibits PI3K-C2α-mediated AKT2 activation in the early endosomes of thyroid cancer cells, even though they obviously do not rule out the possible involvement of other PI3K isoforms in AKT activation at the EE.
Figure 4. Loss of INPP4B in Pten−/− cells leads to increased AKT activation. A and B. Western blot analysis of lysates from Pten−/− (n = 3; 10–11 months), Pten−/−;Jnpp4b−/− (n = 2; 6–8 months), and Pten−/−;Jnpp4b−/− (n = 3; 6 months) thyroid tissues (A), lysates from immortalized MEFs after serum starvation restimulation. The MEFs were stimulated with serum for the indicated amount of time (B). C. Western blot analysis of total lysate from different thyroid cancer cells. Please note that the AKT2 antibody used in A and B (S239) is different from C (S3063), resulting in the appearance of different unspecific bands. Arrows indicate specific band (see also Methods). D and E. Western blot analysis of cell fractions from TPC1 cells. HM, cytosolic/heavy membranes; PNS, postnuclear supernatant; LE, late endosome. Arrow indicates specific band. AKT2 antibody used is 3063. F. Western blot analysis of phosphorylated AKT1 and phosphorylated AKT2 in different cell fractions derived from TPC1 cells infected with either a nontargeting shRNA or an shRNA that targets INPP4B. G. Western blot analysis of phosphorylated AKT1 and phosphorylated AKT2 in different cell fractions derived from FTC236 cells treated with either DMSO or 3 μmol/L S-Aza for 5 days. H. Immunofluorescence of PI(3,4)P2 and tubulin in TPC1 cells infected with either a nontargeting shRNA or an shRNA that targets INPP4B. Scale bars, 20 μm. I. Immunofluorescence of INPP4B, AKT2, PI3K-C2α, and RAB5 in TPC1 cells. Scale bars, 5 μm. J. Immunofluorescence of PI(3,4)P2 and SNX9 in TPC1 and FTC236 cells. Scale bars, 20 μm.
Knockdown of INPP4B in Thyroid Cell Lines Provides an Advantage for Anchorage-Independent Growth

To assess INPP4B-dependent cellular processes in vitro, we next conducted cell proliferation and soft-agar colony formation assays in two of the non-FTC thyroid cell lines, namely TPC1 and 8505C, in which we stably knocked down INPP4B (Fig. 5A and B). We found that the knockdown of INPP4B did not confer a growth advantage for thyroid cell lines in full or 1% serum growth conditions, but did in 5% serum growth conditions (Fig. 5C; Supplementary Fig. S7A), suggesting that under specific conditions of nutrients or growth factor amounts, INPP4B loss determines a growth advantage in thyroid cell lines, mirroring the in vivo phenotype. In addition, INPP4B knockdown did not result in any change in TPC1 cell morphology, nor did it alter the morphology of Pten+/−;Inpp4b−/− MEFs compared with Pten+/− MEFs (Supplementary Fig. S7B and S7C). Furthermore, INPP4B loss did not alter the distribution or arrangement of tubulin in the cytoskeleton of these cells (Supplementary Fig. S7B and S7C). Instead, INPP4B loss provided a marked advantage for anchorage-independent growth, a functional indicator of tumorigenicity and invasiveness (Fig. 5D and E).

**DISCUSSION**

This study allowed us to reach three important conclusions.

First, INPP4B is a novel tumor suppressor in FTC. Through our mouse model, we demonstrated that either a partial or complete loss of INPP4B in a Pten heterozygous background accelerates thyroid carcinoma progression, resulting...
in metastatic disease that recapitulates the hematogenous pulmonary metastases characteristic of advanced FTC in humans. The evidence that INPP4B is a relevant tumor suppressor in FTC is further supported by the observation that INPP4B expression is markedly reduced in human FTC, as compared with a normal thyroid and other subtypes of thyroid cancer.

Our findings lend further support to the notion that PI3K–AKT activation plays a central role in FTC oncogenesis. Multiple lines of evidence corroborate this notion: (i) AKT activation and expression are higher in FTC as compared with normal tissues and other thyroid tumors (22); (ii) Cowden syndrome patients, with germline mutations in PTEN, have an increased incidence of FTC (23); (iii) transgenic mice engineered to hyperactivate PI3K–AKT form FTCs (24, 25); and (iv) in a mouse model of FTC, metastatic potential is AKT-dependent (26). This evidence underscores the importance of AKT hyperactivation in the initiation and progression of FTC and also suggests that the pathogenesis of FTC is distinct from other forms of well-differentiated thyroid carcinoma, which are driven primarily via activation of the MAPK signaling pathway. Nevertheless, mutations in the RAS pathway and PAX8/PPARγ rearrangements are also found in FTC (27). Although the specific pathogenic mechanisms contributed by these alterations remain unclear, there are indications that they too converge on the PI3K–AKT pathway (28). Therefore, it will be interesting to understand in future studies whether these represent cooperative or mutually exclusive events.

Second, we observed that INPP4B is not solely epistatic to PTEN. PTEN and INPP4B are generally thought to be cooperative phosphatases that proximally regulate PI3K at the lipid level. On this basis, we anticipated that Inpp4b−/− mice would have a phenotype reminiscent of Pten−/− mice with increased susceptibility to epithelial tumors. Surprisingly, Inpp4b−/− mice were tumor free and had a tumor-free survival of 16 to 24 months. In addition, crossing Inpp4b−/− mice with Pten−/− mice did not accelerate the entire tumor spectrum of Pten−/− mice by 5 to 8 months follow-up. Rather, we observed a significant acceleration of only thyroid tumorigenesis. Due to the early mortality that occurred in the Pten−/−;Inpp4b−/− mice, further studies in conditional Pten and Inpp4b knockout mice will be needed in order to determine the potential cooperative effect between PTEN and INPP4B on tumor suppression in other tissues.

Despite the importance of PI3K–AKT signaling in the pathogenesis of FTCs, Pten−/− mice do not develop FTC. By 10 months of age, they instead develop benign adenomas that do not invade or metastasize. One might speculate that the extent of AKT activation accounts for the difference in thyroid carcinoma aggressiveness. Indeed, we observed increased AKT activation in Pten+/−;Inpp4b−/− thyroids when compared with Pten−/− or Pten+/−;Inpp4b−/− thyroids. However, in a previous study, we found that thyroid lesions in Pten knock-in mutant mice harboring specific lipid and phosphatase dead Pten mutations also demonstrated higher levels of AKT activation than Pten−/− thyroids, similar to Pten+/−;Inpp4b−/− thyroids (29). Nevertheless, only 7% to 8% of these mice developed aggressive thyroid adenocarcinoma, and, importantly, there was no occurrence of lung metastasis (29). Furthermore, conditional loss of function of both copies of Pten in the thyroid resulted mainly in follicular adenoma within 12 months of age, which progressed to invasive FTC only at advanced ages, suggesting that even in a setting of maximal activation of PI3K–AKT signaling, other events are needed to trigger FTC (17). In contrast, the Pten−/−;Inpp4b−/− mice developed aggressive, often metastatic, and always lethal follicular-like carcinoma by 5 to 6 months of age. The shorter latency to a more aggressive follicular-like carcinoma in Pten−/−;Inpp4b−/− mice further supports the notion that although increased AKT activation does play a role in follicular-like thyroid tumorigenesis, it is insufficient in mediating progression and metastases, and that the tumor-suppressive function of INPP4B therefore extends beyond its role in suppressing the overall level of PI3K–AKT pathway activation. In this respect, the striking difference between PTEN and INPP4B that emerges from our study is their differential localization at the early endosome where INPP4B but not PTEN could regulate signaling in a localized and specialized fashion.

Beyond the level of AKT activation, isoform-specific AKT signaling plays an important role in mediating cancer progression. In the context of breast cancer, AKT2 has been implicated in metastasis, whereas AKT1 suppresses metastatic dissemination (30). Interestingly, signaling through AKT2 is critical for the development of thyroid neoplasms in Pten−/− mice (18). Specifically, loss of AKT2 in Pten−/− mice rescues the development of thyroid adenomas in this model (18). Furthermore, genomic amplification of AKT2 is frequently observed in FTC, but not in anaplastic thyroid carcinoma (31). These findings indicate that AKT2 activation is particularly important in follicular thyroid carcinoma progression and metastasis. Our finding that INPP4B, but not PTEN, localizes to the early endosomes to selectively regulate AKT2 identifies a novel and specific role for INPP4B in the regulation of PI3K–AKT signaling. INPP4B loss could therefore increase the PI(3,4)P2 pool in the early endosomes, regulate endocytic trafficking, and contribute to prolonged AKT2 signaling from the endocytic membranes, through which it mediates its effects on thyroid carcinoma initiation, metastasis, and invasion. Our findings are also in line with the recent observation that activation of the endocytic trafficking pathways is critical for tumor cell migration (32). Therefore, it is possible that the aberrant activation of AKT2 at endosomes might represent the molecular mechanism underlying the characteristic metastatic propensity of FTCs. Intriguingly, although AKT1 is also localized at the early endosomes, INPP4B does not appear to regulate its activation. The exact mechanism underlying the selective regulation of AKT2 by INPP4B remains to be elucidated. Our study nevertheless represents an important first step in the understanding of the mechanism underlying isoform-specific and localized AKT regulation.

In conclusion, our study provides strong evidence that INPP4B is not epistatic to PTEN and that INPP4B loss, although insufficient to initiate cancer in the thyroid, can promote FTC progression and metastasis in the context of PTEN haploinsufficiency through the isoform-specific regulation of AKT signaling at the endosomes (Fig. 6). More generally, our findings provide compelling evidence for the critical role of a qualitative regulation of signal transduction in tumorigenesis.
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**METHODS**

**Mice and Histopathologic Analyses**

Inpp4b knockout mice were generated by Sasaki and colleagues. Total body necropsy and histopathologic analyses were performed on cohorts of male and female mice from 4 to 15 months of age. Mouse tissues were fixed in 4% paraformaldehyde and embedded in paraffin. They were then sectioned and stained with hematoxylin and eosin for pathologic analyses. The use of these mice and procedures performed were in accordance with NIH-approved guidelines, and the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center approved the studies.

**Studies with Primary Cells**

MEFs were isolated at day E13.5, immortalized with SV40 large T antigen, and maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, and 100 U/mL penicillin and streptomycin (Invitrogen).

**Cell Lines**

All cell lines were maintained in DMEM or RPMI supplemented with 10% FBS, 2 mM glutamine and 100 U/mL penicillin and streptomycin (Invitrogen). N-Tryp-Ot-3 cells were purchased from Sigma. Htori and B505C cells were kindly provided by Dr. Sarah Parangi (Massachusetts General Hospital, Boston, MA); FTC and TPC1 cells were kindly provided by Dr. Orlo H. Clark (University of California, San Francisco). Cell lines were tested for specific markers by Western blot and qRT-PCR in our laboratory, and routinely tested for Mycoplasma (MycAlert; Lonza), but not further authenticated.

**Human Tissue Collection**

The Committee for Human Research at Brigham and Women’s Hospital approved this study. Thyroid tumors and normal tissue were discarded specimens obtained from patients undergoing thyroidectomy. The specimens were snap-frozen in the operating room suite with liquid nitrogen and were maintained at −80°C until analysis. An endocrine pathologist, who confirmed the histologic diagnosis, evaluated the specimens.

**Western Blotting and Immunohistochemistry**

Cells and tissues were lysed with RIPA buffer [50 mM/L Tris (pH 8), 150 mM/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM/L EDTA, and protease and phosphatase inhibitor cocktail (Roche)]. For Western blotting, the following antibodies were used: anti-AKT (9272; 1:1,000), anti-pAKT (pSer473, 9271; 1:1,000; and pThr308, 9275; 1:1,000), anti-AKT1 (2938; 1:1,000), anti-AKT2 (3063; 1:1,000; and 5239; 1:1,000), anti-pAKT2 (pSer474, 8599; 1:1,000), anti-PTEN (9559; 1:1,000), anti-INPP4B (8450; 1:1,000), and anti–p170 (BD; 611046; 1:25) anti–γ-tubulin (Sigma; GTU-88; 1:100), anti–RAB5 (BD; 610261; 1:25) and Cell Signaling Technology; 3547; 1:50), anti–α-tubulin (Sigma; T6074; 1:100), anti–PtdIns(3,4P)2 and anti–PtdIns(3,4,5)P3 (Echelon; Z-P034b; 1:50, respectively), anti–SNX9 (Proteintech; 15721-1-AP; 1:100), and anti–AKT2 (Cell Signaling Technology; 3063; 1:100).

**Immunofluorescence**

Cells were grown on coverslips, fixed with 4% paraformaldehyde, and permeabilized with 0.3% Triton or prechilled methanol. Cells were rinsed with PBS, blocked, and then incubated with primary antibody, followed by incubation with Alexa Fluor–conjugated secondary antibodies (Life Technologies). Coverslips were mounted with Pro-Long Gold Antifade reagent with DAPI (Life Technologies). Confocal images of cells were acquired with LSM510META Confocal Laser System (Beth Israel Deaconess Medical Center) and Zeiss Observer Z1 microscope equipped with Apotome (University of Torino). Primary antibodies used were as follows: anti-INPP4B (Atlas; 37682; 1:50), anti–P13K-C2z (anti-p170, BD; 611046; 1:25) anti–γ-tubulin (Sigma; GTU-88; 1:100), anti–RAB5 (BD; 610261; 1:25) and Cell Signaling Technology; 3547; 1:50), anti–α-tubulin (Sigma; T6074; 1:100), anti–PtdIns(3,4P)2 and anti–PtdIns(3,4,5)P3 (Echelon; Z-P345; 1:50 and Z-P345; 1:50, respectively), anti–SNX9 (Proteintech; 15721-1-AP; 1:100), and anti–AKT2 (Cell Signaling Technology; 3063; 1:100).

**Early Endosome Purification**

Cells were gently homogenized in the homogenization buffer (250 mM/L sucrose, 3 mM/L imidazole, pH 7.4 with protease inhibitor cocktail). The samples were centrifuged at 3,000 rpm to remove nuclei and cell debris. Postnuclear supernatant (PNS) was subsequently separated by sucrose gradient centrifugation into different cellular fractions. In detail, the PNSs were adjusted to 40.6% sucrose using a stock solution (62% sucrose, 3 mM/L imidazole pH 7.4), loaded at the bottom of centrifugation tubes (SW55), then sequentially overlaid with 1.5 mL 35% sucrose solution (35% sucrose, 3 mM/L imidazole pH 7.4) followed by 1 mL 25% solution (25% sucrose, 3 mM/L imidazole pH 7.4) and 1 mL of homogenization buffer on top of the load. After 1 hour centrifugation, at 35,000 rpm at 4°C, early endosomes (EE) were recovered from interphase between 35% and 25% layers, late...
endosomes (LE) were recovered from the uppermost portion of 25% phase, and heavy membranes (HM), including endoplasmic reticulum, Golgi, and plasma membranes, were recovered from the lowest interphase. EE, LE, and HM were then precipitated with methanol/chloroform loaded in SDS-PAGE for Western blot analyses.

**RNA Isolation and RT-qPCR**

Total RNA was purified from cell lines and tissues using the PureLink RNA Mini Kit (Invitrogen). For qPCR analysis, 2 μg of total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). SYBR-Green qPCR analysis was then performed using Applied Biosystems StepOne-Plus in accordance to the manufacturer’s protocol. Each target was run in triplicate, and expression levels were normalized to mouse hypoxanthine-guanine phosphoribosyltransferase (Hprt) or human porphobilinogen (PBGD).

**Genotyping**

The following genotyping primers were used:

- **Inpp4b del1**: GTTTACATTTGAACGGGTTTG
- **Inpp4b del2**: TGGTCTGGCCGAAAGGAATTA
- **Inpp4b del3**: CACTGCGATGGTATAGTTCT
- **Pgen-1**: TGGGAAAACACTTAGCTTGG
- **Pgen-3**: ACTCTACCAGCCCAAGGCCCG
- **3193: CGAGACTGTGAGACGTGCTACTTCC.**

**5-Aza-2′-Deoxyctydine Treatment**

Cells were briefly treated with 3 μmol/L of 5-Aza-2′-deoxycytidine for 5 days. After that, the cells were harvested for RNA and protein analysis.

**Growth Proliferation Assay**

Cells were plated at a density of 2.5 × 10^4 cells per well in 12-well plates, and each sample was plated in triplicate. Plates were collected on days 0, 2, 4, and 6. The wells were washed with PBS, and cells were fixed with 4% paraformaldehyde (Santa Cruz Biotechnology). The plates on days 0, 2, 4, and 6 were washed with PBS, and cells were fixed with 4% paraformaldehyde (Santa Cruz Biotechnology). The wells were then solubilized with 10% acetic acid, and the fixed with 4% paraformaldehyde (Santa Cruz Biotechnology). The wells were then solubilized with 10% acetic acid, and expression levels were normalized to mouse hypoxanthine-guanine phosphoribosyltransferase (Hprt) or human porphobilinogen (PBGD).

**Soft-Agar Colony Formation Assay**

Soft-agar colony formation assay was performed by first plating 6-well tissue culture plates with 0.6% Noble agar/growth media and allowed to solidify at room temperature. Thyroid cancer cell lines (1 × 10^4) in 0.3% Noble agar/growth media were then seeded as the top layer. Each cell line was seeded in triplicate. The soft agar was allowed to solidify at room temperature, then placed in the incubator at 37°C. Fresh growth media were added every week, and colonies were counted and photographed after 2 weeks.

**Measurement of TSH Levels**

Serum was collected from **Pten+/−, Pten+/-;Jnpp4b+/-, and Pten+/-;Jnpp4b−/−** mice. The mice were between 3 and 5 months of age, and at least 4 mice in each genotype were tested. Briefly, blood was allowed to clot at 4°C for at least 2 hours. It was then centrifuged at 1,000 × g for 15 minutes. The serum was carefully removed and frozen at −20°C. For testing, we used the ultrasensitive thyroid-stimulating hormone ELISA Kit from MyBioSource (MBS042764).

**Bisulfite Sequencing**

Genomic DNA samples were collected and treated with bisulfite using the EpiTect Bisulfite Kit (Qagen) according to the manufacturer’s recommendations. PCR amplification was performed with primers specific for the methylated and unmethylated alleles, as described in Yuen and colleagues (12).

**Statistical Analysis**

For quantitative data, datasets were generally analyzed using the unpaired, two-tailed Student t tests (GraphPad Prism; GraphPad Software). P < 0.05 was considered significant.

**Disclosure of Potential Conflicts of Interest**

E. Hirsch is the founder of Kither Biotech. No potential conflicts of interest were disclosed by the other authors.

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INPP4B Inhibits Thyroid Tumorigenesis and Metastasis


In Vivo Role of INPP4B in Tumor and Metastasis Suppression through Regulation of PI3K–AKT Signaling at Endosomes

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