The Lipid Kinase PI5P4K\(\beta\) Is an Intracellular GTP Sensor for Metabolism and Tumorigenesis

**Graphical Abstract**

**Highlights**
- Cellular GTP concentration functions as a biological cue via a GTP sensor, PI5P4K\(\beta\)
- PI5P4K\(\beta\) kinase activity converts the GTP cue into PI(5)P second messenger signaling
- The GTP-sensing activity of PI5P4K\(\beta\) is critical for tumorigenesis
- GTP-sensing activity of PI5P4K\(\beta\) represents a target for cancer therapeutics

**In Brief**
Sensing cellular energy status is fundamental to all organisms. Sumita, Lo, and Takeuchi et al. identified PI5P4K\(\beta\) as an energy sensor that couples cellular GTP concentration with lipid second messenger signaling. The GTP-sensing mechanism will change the current homeostasis model and represents a target for cancer therapeutics.

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3X09
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3X0C

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The Lipid Kinase PI5P4Kβ Is an Intracellular GTP Sensor for Metabolism and Tumorigenesis

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SUMMARY

While cellular GTP concentration dramatically changes in response to an organism’s cellular status, whether it serves as a metabolic cue for biological signaling remains elusive due to the lack of molecular identification of GTP sensors. Here we report that PI5P4Kβ, a phosphoinositide kinase that regulates PI(5)P levels, detects GTP concentration and converts them into lipid second messenger signaling. Biochemical analyses show that PI5P4Kβ preferentially utilizes GTP, rather than ATP, for PI(5)P phosphorylation, and its activity reflects changes in direct proportion to the physiological GTP concentration. Structural and biological analyses reveal that the GTP-sensing activity of PI5P4Kβ is critical for metabolic adaptation and tumorigenesis. These results demonstrate that PI5P4Kβ is the missing GTP sensor and that GTP concentration functions as a metabolic cue via PI5P4Kβ. The critical role of the GTP-sensing activity of PI5P4Kβ in cancer signifies this lipid kinase as a cancer therapeutic target.

INTRODUCTION

Energy molecules such as adenosine triphosphate (ATP) and guanosine triphosphate (GTP) are evolutionarily conserved metabolites and drive numerous enzymatic reactions in cells. Concentrations of the energy molecules vary by tissue type, environment, and pathological condition. The changes in the concentrations of energy molecules affect cell status not only in a passive manner but also in an active manner, in which these concentration changes are detected by specific molecular sensors and converted into signaling for metabolic adaptations. For example, AMP-activated protein kinase (AMPK) integrates the relative ATP concentration against AMP and/or ADP concentrations for signaling (Oakhill et al., 2011; Xiao et al., 2011). Mammalian target of rapamycin (mTOR) has been suggested as another type of ATP sensor because its Michaelis constant (Km) value for ATP falls within the range of physiological cellular concentration and its kinase activity changes in direct proportion to the cellular ATP concentration (Oakhill et al., 2011; Xiao et al., 2011). Mammalian target of rapamycin (mTOR) has been suggested as another type of ATP sensor because its Michaelis constant (Km) value for ATP falls within the range of physiological cellular concentration and its kinase activity changes in direct proportion to the cellular ATP concentration (Chen et al., 2013; Dennis et al., 2001). AMPK and mTOR modulate cellular metabolism by direct phosphorylation of effector proteins (Hardie, 2008; Laplante and Sabatini, 2012; Mihaylova and Shaw, 2011). The levels of other metabolites essential for cell viability are also monitored by respective sensors. Cellular oxygen levels are detected by hypoxia inducible factor (HIF) prolyl-hydroxylases, whose...
Figure 1. PI5P4K\(\beta\) is a GTP-Binding Phosphatidylinositol Kinase (A) A list for kinases that were identified in a proteome screening with GTP-conjugated agarose beads. (B) Enzymatic functions of PI5P4K/Type II PIPK and PI4P5K/Type I PIPK. (C) Cellular PI5P4K\(\beta\) binds to GTP-conjugated agarose beads more than ATP- and m7GTP-conjugated agarose beads. After incubated with HEK293T cell lysates, PI5P4K\(\beta\) as well as control proteins that bound to the indicated control or nucleotide conjugated beads were immunoblotted. (D) Direct binding of GTP to PI5P4K\(\beta\) assessed by solution-state NMR. The signal intensity of GTP (200 \(\mu\)M, H6 position) was reduced by addition of PI5P4K\(\beta\) (10 \(\mu\)M, middle), which was partially recovered by addition of physiological amount of ATP (1 \(mM\), right). See also Figure S1A. (E) PI5P4K\(\beta\) possesses superior GTP hydrolysis activity in vitro. The real-time reduction of GTP- and ATP-specific signals was monitored by NMR. See also Figure S1B.

\(K_m\) for oxygen is in the range of the atmospheric oxygen concentration (Schofield and Ratcliffe, 2004; Stiehl et al., 2006). Recently an amino acid sensor, SLC38A9, has been reported to have a \(K_m\) value for arginine corresponding to its physiological concentration and transmits the amino acid concentration to cellular signaling (Wang et al., 2015). These studies reveal that molecular sensors for metabolites must have the following three fundamental features: (1) an ability to bind directly to the metabolite; (2) appropriate \(K_m\) value so that its activity is regulated by physiological changes of the concentration of the target metabolite; and (3) an ability to evoke a signal for cellular functions.

GTP is particularly important for protein synthesis where two GTP molecules are consumed per every one amino acid incorporation into a polypeptide (Ertel et al., 1968; Green and Noller, 1997; Haselkorn and Rothman-Denes, 1973). Therefore, a large amount of GTP is required in rapidly dividing cells, such as tumor cells, and tissues producing serum proteins, such as the liver, pancreas, and adipose tissue (Caro and Palade, 1964; Havel, 2002; Ma and Blenis, 2009; Miller et al., 1951; Weiss, 1953). Cellular GTP concentration changes independently of the concentrations of ATP, ADP, and AMP and the ratios of ATP/AMP and GTP/GDP in bacterial, yeast, and mammalian cells (Lopez, 1982; Matsushika et al., 2013; Neidhardt et al., 1990; Traut, 1994). Regulatory links between GTP concentration and metabolic adaptation have been found; GTP concentration actively controls rRNA synthesis rates in bacterial and mammalian cells (Gaal et al., 1997; Grummt and Grummt, 1976; Krášny and Gourse, 2004). In gram-positive bacteria, a transcriptional factor, CodY, controls the expression of genes involved in branched-chain amino acid biosynthesis in response to changes in GTP concentration (Ratnayake-Lecamwasam et al., 2001). These studies indicated a fundamental requirement for cells to sense the GTP concentration in an active manner and alter their metabolisms with a GTP sensor acting as a gauge. G-proteins have some aspects of being a GTP sensor. However, G-proteins are unlikely to respond to the changes in GTP concentration because their \(K_m\) values for GTP and GDP are, in most cases, in the pM to nM range, which is far below cellular GTP concentration (~0.1 to 1 \(mM\)) (Traut, 1994). Until now, no molecular sensor for cellular GTP concentration has been identified in higher organisms. For this reason, molecular mechanisms of GTP-responsive signaling remain unclear. In the present study, we set out to identify a protein that would meet the requirements for the GTP sensor, and we discovered a GTP sensor, Phosphatidylinositol 5-phosphate 4-kinase \(\beta\) (PI5P4K\(\beta\)), which converts GTP concentration cues into phosphatidylinositol 5-phosphate (PI(5)P) second messenger signaling and tumorigenesis.

RESULTS

Proteomic Screen Identifies PI5P4Ks as GTP-Binding Proteins

To identify a candidate for the GTP sensor, we performed a proteomic screening for signaling proteins that have an ability to bind to GTP. The GTP-conjugated agarose beads were incubated with dialyzed cell lysates, and proteins bound to the agarose beads were eluted with GTP. The eluted proteins were precipitated, digested with trypsin, and identified by mass spectrometry. As expected, the GTP-eluted fraction contained several G-proteins and two types of protein kinases, Src-family tyrosine-protein kinases and Casein kinase II (CKII), which have been shown to utilize GTP with less or equal efficiency as compared to ATP (Feder and Bishop, 1990; Niefind et al., 1999). Notably, all three isoforms of PI5P4K/Type II PIPK were also found in the GTP-eluted fraction (Figures 1A and 1B), while other families of phosphoinositide kinases (PI4P5K/Type I PIPK and PI3P5K/Type III PIPK/PIKfyve) were not detected. PI5P4Ks, an emerging target for cancer therapy, control the levels of lipid second messenger, PI(5)P (Clarke and Irvine, 2013; Emerling et al., 2013; Jude et al., 2015; Keune et al., 2013). These results raised the possibility that PI5P4Ks have an intrinsic ability to bind to GTP.
PI5P4Kβ Directly Binds to GTP
To validate the binding of PI5P4Ks to GTP, dialyzed lysates from HEK293T cells were incubated with ATP-, GTP-, or 7-methyl-GTP (m7GTP)-conjugated agarose beads, and proteins bound to those beads were analyzed by SDS-PAGE followed by western blotting (Figure 1C). Immunoblotting of extracellular signal-regulated kinase-2 (ERK2), phosphoenolpyruvate carboxykinase (PEPCK), and eukaryotic initiation factor-4E (eIF4E) indicated selective binding of these proteins to ATP-, GTP-, and m7GTP-conjugated agarose beads, respectively. We found that PI5P4Kβ in cell extracts bound to the GTP-conjugated agarose beads more strongly than to the ATP-conjugated agarose beads, which verified the result of the proteomic screening. Thus, we purified recombinant PI5P4Kβ and assessed its direct binding to GTP in vitro by solution-state nuclear magnetic resonance (NMR) spectroscopy (Figure 1D). The NMR signal of GTP was reduced by the addition of PI5P4Kβ (Figure 1D, middle). The addition of excess ATP competed with the GTP binding to PI5P4Kβ (Figure 1D, right). These results indicated that PI5P4Kβ directly binds to GTP and that GTP and ATP shared the binding site in PI5P4Kβ. Given that PI5P4Kβ has only one nucleotide-binding site, we hypothesized that PI5P4Kβ binds to GTP through its catalytic site for its kinase reaction.

PI5P4Ks Can Hydrolyze GTP In Vitro
Recently, it has been shown that PI5P4Kβ hydrolyzes ATP in vitro (Clarke and Irvine, 2013). ATP-dependent kinases often possess intrinsic ATP hydrolysis activity (phosphoryl transfer to water), because of their resemblance to kinase reactions (Kashem et al., 2007). To test if PI5P4Kβ can utilize GTP as a phosphodonor, we performed a real-time GTP hydrolysis assay using NMR (Figures 1E; Figure S1A). PI5P4Kβ hydrolyzed not only ATP but also GTP, and the rate of GTP hydrolysis was five faster than its ATP hydrolysis. In contrast, PI5P4Kα showed 0.6-fold slower GTP hydrolysis than ATP hydrolysis (Figure S1B). The PI5P4Kγ isoform displayed marginal ATP and GTP hydrolysis activities (Figure S1A, bottom). These results indicated that each PI5P4K possesses a different degree of intrinsic activity toward GTP and raised the possibility that PI5P4Ks use GTP for their kinase reactions.

PI5P4Kβ Is a GTP-Dependent Kinase and Its KM Value Is within Physiological Variation of GTP Concentration
To examine whether the PI5P4K isoforms use GTP to phosphorylate their substrate PI(5)P, we performed in vitro kinase assays. We found that all PI5P4K isoforms possess GTP-dependent kinase activities (Figures 2A; Figure S2A). Their ATP-dependent kinase activities were also confirmed, as previously reported (Demian et al., 2009; Kunz et al., 2002). Interestingly, the Michaelis-Menten kinetics analysis showed that PI5P4K isoforms displayed distinct activities and KM values. PI5P4Kα showed the highest activity and the lowest KM values, which are 5 and 3 μM for ATP and GTP, respectively (Figure S2B, upper). This suggests that both the ATP- and GTP-dependent kinase activities of PI5P4Kα are always near their maximal levels at cellular ATP (1–5 mM) and GTP (~0.1–1 mM) concentrations (Traut, 1994). Thus, PI5P4Kα is not suitable for the GTP sensor. PI5P4Kγ displayed weak ATP- and GTP-dependent kinase activities (Figures S2A and S2B, bottom); two orders of magnitude lower than PI5P4Kα and PI5P4Kβ. PI5P4Kγ could be a GTP sensor, because of its higher GTP-dependent kinase activity as compared to the ATP-dependent kinase activity and the KM value within the physiological variation of GTP concentration. However, it was difficult to analyze the functions of PI5P4Kγ as the first candidate GTP sensor due to its low enzyme activity.

Based on its kinetic property, PI5P4Kβ is a good candidate for a functional GTP sensor. PI5P4Kβ displayed a higher GTP-dependent kinase activity than ATP-dependent kinase activity (Figure 2B), regardless of PI(5)P levels and the types of divalent metal ions (Figure 2C; Figure S2C). The GTP-dependent kinase activity can change in the range of physiological GTP concentration due to its relatively high KM value for GTP (Figure 2B, red). On the other hand, the ATP-dependent kinase activity is always near saturated level at physiological ATP concentration (Figure 2B, blue). Even in the presence of physiological concentration of ATP (1 and 2 mM), PI5P4Kβ still responded to the changes in GTP concentration (Figure 2D). In addition, relative contribution of ATP and GTP for PI(4,5)P₂ production was assessed under physiologically relevant conditions (Figure 2E), which revealed that nearly or over 50% of PI(4,5)P₂ was produced by using GTP at 0.5 mM concentration. These results suggest that concentration of cellular GTP (~0.1–1 mM) has substantial impact on total PI5P4Kβ activity. The results also revealed that PI5P4Kβ has the appropriate characteristics of a GTP sensor.

PI5P4Kβ Recognizes Guanine Nucleotides via a “Tetris Spin”
To further analyze the GTP-dependent activity of PI5P4Kβ at the atomic level, we carried out the structural analyses by X-ray crystallography. Crystal structures of human PI5P4Kβ in complex with non-hydrolysable GTP and ATP analogs (GMPNP and AMPNP, respectively) were determined at 2.6- and 2.7-Å resolutions, respectively, under physiological pH and Mg²⁺ concentration (Table 1; Figure S3). The bases of both the ATP and GTP analogs are placed in a hydrophobic groove formed by Phe-139, Val-148, Val-204, Phe-205, Leu-282, and Ile-368 (Figures 3A and 3B). The positions of the nucleotide bases are restrained by the sidechain of Ile-368 that protrudes out into the hydrophobic groove, and the I368A mutation altered the position of guanine and adenine nucleotides in the binding pocket (Figures S3I–S3L; Table 2).

In the deep end of the nucleotide-binding pocket, the guanine nucleotide base is secured by four hydrogen bonds with Thr-201, Arg-202, Asn-203, and Val-204 (Figure 3D). In contrast, the adenine nucleotide base forms two hydrogen bonds with Arg-202 and Val-204 (Figure 3E). These include newly formed indirect hydrogen bonds via water between the O6 position of the guanine nucleotide base and Thr-201 sidechain, as well as direct and indirect hydrogen bonding from the N1 and N2 positions of the guanine nucleotide base, respectively, to the side chain of Asn-203. In addition, the main chainHN of Val-204 coordinates to the O6 position of the guanine nucleotide base instead of the N1 nitrogen in the adenine nucleotide base. These characteristic interactions for the GTP analog are enabled by ~15° rotation of the base moiety and ~90° rotation of the ribose group relative to the ATP analog within the hydrophobic groove.
PI5P4K-dependent kinase activity is critical, since methods such as GTP-dependent kinase activity without perturbing the ATP-generating of a PI5P4K mutant that specifically lacks the kinase activity changes within the physiological variations of GTP concentration. PI5P4K kinase activity was assessed with the indicated levels of GTP and ATP. PI5P4K retains its preference to GTP at any ATP concentration. PI5P4K efficiently utilizes GTP in the presence of physiological amount of ATP. GTP- and ATP-dependent kinase activities of PI5P4K were assessed with mixtures of cold and radiolabeled GTP or ATP and 20 μM of PI(5)P. PI5P4K retains GTP responsiveness in the presence of physiological amount of ATP. PI5P4K kinase activity with the indicated amount of radiolabeled GTP was assessed in the presence of 1 and 2 mM of cold (non-radiolabeled) ATP. PI5P4K retains its ability to sense GTP concentration.

(Figures 3C and 3F). These rotations induce a shift of the guanine nucleotide base by ~1.5 Å toward Phe-205, promoting the formation of aromatic-aromatic interactions with Phe-139 and Phe-205 (Figure 3C). In contrast, the aromatic-aromatic interaction of the adenine nucleotide base to Phe-205 is less important due to its separation from the phenyl ring compared to the guanine nucleotide base. The present crystallographic analysis revealed that GTP binds to its designated position in PI5P4K by rotating and shifting within the hydrophobic groove relative to ATP, which could be analogous to the “Tetris” spin in the tile matching video game Tetris (Figures 3G and 3H).

Structure-Based Development of the GTP-Insensitive Mutant of PI5P4K
To analyze the GTP-sensing function of PI5P4K in vivo, generation of a PI5P4K mutant that specifically lacks the GTP-dependent kinase activity without perturbing the ATP-dependent kinase activity is critical, since methods such as PI5P4K knockout, gene silencing, and production of kinase-dead mutations would disrupt both GTP- and ATP-dependent PI5P4K activities. Therefore, we engineered PI5P4K based on structural data to specifically reduce its GTP preference, so that the role of PI5P4K in GTP sensing can be directly tested.

The crystal structures indicated that Thr-201 and Phe-205 are critical for guanine base recognition in PI5P4K (Figure 3). Thr-201 is conserved in PI5P4K and PI4P5K, while it is substituted to methionine in PI5P4KY and PI4P5K/Type-I PIPKs. Phe-205 is conserved among PI5P4Ks, while it is leucine in PI4P5K/Type-I PIPKs. Thr-201 and Phe-205 in PI5P4K were mutated to methionine and leucine, respectively, and the direct interactions between nucleotides and PI5P4K were analyzed. A saturation transfer difference (STD) NMR experiment revealed that PI5P4K and PI5P4K specifically reduced binding to the GTP analog (Figure 4B). The real-time GTP hydrolysis and in vitro kinase assays verify that PI5P4K and PI5P4K mutants...
showed a decrease in both GTP hydrolysis and GTP-dependent kinase activity (Figure 4C; Figures S4A–S4C). While PI5P4KβF205L retained ATP-dependent activity comparable to the wild-type (WT), PI5P4KβT201M showed higher ATP-dependent activity in physiological nucleotide concentrations (Figures S4A and S4B). The nucleotide specificities of the two mutants were analyzed by crystal structures (Figure S4D; Table 2).

The decreased GTP-dependent kinase activity of PI5P4KβT201M could be explained by the loss of indirect hydrogen bonding by the T201M mutation, and its higher ATP-dependent activity could arise from an additional contact between the substituted methionine and ATP (Figure S4D). The reduction of the GTP-dependent activity of PI5P4KβF205L could be explained by the loss of the aromatic-aromatic interaction between guanine nucleotide base and Phe-205. The reduced binding affinity to the guanine nucleotide is supported by weak electron density for GMP in PI5P4KβF205L (Figure S4D). In contrast, no significant differences were observed in AMP-binding mode in PI5P4KβF205L (Figure S4D). Taken together, we selected PI5P4KβF205L to analyze the in vivo significance of the GTP-sensing activity.

The GTP-Sensing Activity of PI5P4Kβ Is Required for Metabolic Adaptation and PI(5)P Accumulation under a GTP-Energy Crisis

Previously identified molecular sensors, such as AMPK, mTOR, and HIF prolyl-hydroxylases, alter metabolism via cellular signaling in response to changes in their respective metabolite concentrations. We therefore investigated whether the GTP-sensing activity of PI5P4Kβ is involved in metabolic regulation. WT-PI5P4Kβ or PI5P4KβF205L was stably expressed in PI5P4Kβ-deficient SV40-transformed mouse embryonic fibroblasts (MEFs) (Figure 5A). The metabolic states of PI5P4KβF205L cells are compared with those of WT-PI5P4Kβ cells in the presence and absence of mycophenolic acid (MPA), an inhibitor for inosine monophosphate dehydrogenase (Hedstrom, 2009; Weigel et al., 2001). Treatment with MPA decreased cellular GTP concentration within 4 hr without significantly altering ATP concentration (Figure 5B). LC-MS/MS analysis revealed that metabolites can be categorized into two classes, passive and active classes (Figure 5C; Table S1). The passive metabolites showed only a marginal difference between WT-PI5P4Kβ and PI5P4KβF205L cells in response to the change in

### Table 1. Crystallographic Summary of WT-PI5P4Kβ

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Each structure was determined from single-crystal diffraction. PDB, Protein Data Bank; RMS, root-mean-square.

*Highest resolution shell is shown in parentheses.*
GTP concentration (changes in PI5P4KβF205L cells are between 0.5- and 2-fold compared to that in WT-PI5P4Kβ cells). This class of metabolites would not be directly regulated by the GTP-sensing activity of PI5P4Kβ, yet they appear to be affected by changes in GTP concentration in a passive manner. On the other hand, there are metabolites showing distinct responses to GTP concentration changes in PI5P4KβF205L cells as compared to WT-PI5P4Kβ cells (changes in PI5P4KβF205L cells are < 0.5-fold or > 2-fold compared to that in WT-PI5P4Kβ cells). It appears reasonable to consider that these differences in “active-class metabolites” arise from the GTP-sensing activity of PI5P4Kβ.

It has been suggested that PI5P4Ks control the levels of PI(5)P, a second lipid messenger, for downstream signaling.

Figure 3. Recognition of GTP and ATP by PI5P4Kβ.

(A–F) Hydrophobic and hydrogen-bond interactions in the human PI5P4Kβ-nucleotide complexes. (A) Guanine and (B) adenine nucleotides are tightly fitted into the slit formed by the hydrophobic residues shown in the stick representation. Aromatic interactions between the protein and nucleotide bases are highlighted by orange dotted lines. (D and E) The observed hydrogen-bonding network between the protein and nucleotides are highlighted by red dotted lines. (C and F) The displacement of GMPPNP relative to AMPPNP is shown. The GMPPNP (red) in PI5P4Kβ complex is superimposed against AMPPNP (blue). The side chains of Phe-139 and Phe-205, which form aromatic interactions against the guanine base, are shown in green. (G and H) Schematic representation of (G) GTP and (H) ATP recognition by PI5P4Kβ. The red and blue circles indicate the positions of proton donors and acceptors in the nucleotides, respectively. The red and blue squares indicate those in PI5P4Kβ. Hydrogen bonds are depicted as dashed green lines. For comparison, the superimposed ATP structure is represented as light gray in the GTP scheme. See also Figure S3 and Table 1.
Thus, we assessed the levels of PI(5)P in WT-PI5P4K\textsubscript{b} and PI5P4K\textsubscript{b}F205L cells under normal and the MPA-induced GTP-depleted conditions. The MPA treatment increased the levels of PI(5)P by 50% in WT-PI5P4K\textsubscript{b} cells. In contrast, PI5P4K\textsubscript{b}F205L cells showed higher basal levels of PI(5)P under normal conditions, and the PI(5)P level did not change upon treatment with MPA (Figure 5D). These results suggest that cellular GTP concentration is reflected in the levels of PI(5)P lipid second messenger via the GTP-sensing activity of PI5P4K\textsubscript{b}.

Taken together, PI5P4K\textsubscript{b} can be considered to be a GTP sensor in mammalian cells; PI5P4K\textsubscript{b} binds to GTP, changes its activity depending on the cellular GTP concentration, and transfers biological signals presumably through PI(5)P to alter the metabolism of cells.

GTP Sensing by PI5P4K\textsubscript{b} Is Critical for Tumorigenesis

We next asked the biological significance of the GTP-sensing activity. It has been shown that solid tumor cells need to adapt their metabolism in order to cope with nutrient and energy stresses during tumorigenesis (Cantor and Sabatini, 2012; Jones and Thompson, 2009; Laderoute et al., 2006; Schafer et al., 2009). PI5P4Ks have been shown to promote tumorigenesis in several types of cancers (Emerling et al., 2013; Jude et al., 2015; Keune et al., 2013; Luoh et al., 2004), raising the possibility that the GTP-sensing activity of PI5P4K\textsubscript{b} is involved in its tumorigenic activity. Thus, we compared anchorage-independent soft-agar colony formation of WT-PI5P4K\textsubscript{b} cells to that of PI5P4K\textsubscript{b}F205L cells, where cells are exposed to similar nutrient depleted conditions as cancer cells in tissues (Paoli et al., 2013; Schafer et al., 2009). In the soft-agar assay, PI5P4K\textsubscript{b}F205L cells produced significantly fewer colonies than WT-PI5P4K\textsubscript{b} cells (Figure 6A), while the cell proliferation rates of WT-PI5P4K\textsubscript{b} and PI5P4K\textsubscript{b}F205L cells were indistinguishable on plastic plates (Figure 6A). These results suggest the possibility that the GTP-sensing activity of PI5P4K\textsubscript{b} is important for withstanding metabolic stress during tumorigenesis. To test this possibility in vivo, we performed an

### Table 2. Crystallographic Summary of PI5P4K\textsubscript{b} Mutants in Complex with Nucleotides.

<table>
<thead>
<tr>
<th></th>
<th>T201M- AMP</th>
<th>T201M- GMP</th>
<th>F205L- AMP</th>
<th>F205L- GMP</th>
<th>I368A- AMP</th>
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<td>C 222\textsubscript{1}</td>
<td>C 222\textsubscript{1}</td>
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<td>108.3, 183.4, 107.3</td>
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<td>90.0, 90.0, 90.0</td>
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<td>Resolution (Å)\textsuperscript{a}</td>
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<td>91.44–2.65 (2.79–2.65)</td>
<td>70.37–2.70 (2.85–2.70)</td>
<td>48.18–2.60 (2.74–2.60)</td>
<td>93.27–2.60 (2.74–2.60)</td>
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Each structure was determined from single-crystal diffraction. PDB, Protein Data Bank; RMS, root-mean-square. \textsuperscript{a}Highest resolution shell is shown in parentheses.
allograft study using WT-PI5P4Kβ and PI5P4KβF205L cells. Immuno-compromised mice were injected with WT-PI5P4Kβ and PI5P4KβF205L cells, and tumor growth was monitored over an 11-week period. As shown in Figure 6B, 50% of mice injected with the WT-PI5P4Kβ cells developed tumors; however, no tumor formation was observed in mice injected with PI5P4KβF205L cells. These results indicate that the GTP-sensing activity of PI5P4Kβ provides an advantage in tumorigenesis in vivo.

Figure 4. Development of the GTP-Insensitive PI5P4Kβ Mutant
(A) A sequence alignment of PI5P4K and PI4P5K family proteins. The positions Thr-201 and Phe-205 in human PI5P4Kβ are indicated by asterisks (*).
(B) STD-NMR experiments using PI5P4Kβ mutants. The smaller STD values of GMPPNP relative to WT indicate that PI5P4KβF205L and PI5P4KβT201M mutants specifically decreased the affinity to the GTP analog. The STD values are not proportional to the reduction in affinity to nucleotides, but reflect the population of nucleotides in complex with PI5P4Ks under experimental condition. A decrease in the STD values for GMPPNP from 0.3 to 0.2 observed here for mutants would correspond to a 3-fold decrease in affinity. Data are displayed as mean ± SD, n = 3. See Supplemental Experimental Procedures for calculation of STD value.
(C) PI5P4KβF205L specifically decreases GTP-dependent kinase activity, yet retains full ATP-dependent kinase activity. See also Figure S4 and Table 2.

DISCUSSION
PI5P4Kβ Is a GTP Sensor that Couples GTP Concentration to Phosphoinositide Signaling
Detecting and responding to changes in the levels of high energy metabolites are of fundamental importance for survival. Failure to respond to such change is detrimental to cells and predisposes organisms for systemic dysfunction or metabolic disease. While regulatory links between GTP concentration and metabolism have been found in unicellular organisms (Gaal et al., 1997; Grummt and Grummt, 1976; Krášny and Gourse, 2004; Ratnayake-Lecamwasam et al., 2001), the molecular identity of a GTP sensor has been missing in higher eukaryotes. Our data show that (1) PI5P4Kβ directly binds to GTP; (2) PI5P4Kβ activity is regulated by the GTP concentration because its Kₘ value is within the range of physiological variations of GTP
For GTP concentration (Figure 6C). These results demonstrate that PI5P4K activity in PI5P4K MEFs that stably express WT-PI5P4K favors the evolutional mechanism of the GTP-dependent kinase preference of PI5P4Ks in the future, which will be a key to decipher the molecular mechanism. Acquisition of the GTP preference, however, seems to be not simple. Our crystal structures demonstrate that Thr-201 and Phe-205 are responsible for the GTP-dependent kinase activity of PI5P4K. While sequence alignment of PI5P4K/Type II PIPKs shows two patterns in combination of residues 201 and 205 (T-F and M-F types) (Figure 4A), it is difficult to deduce nucleotide selectivity of individual PI5P4Ks. Indeed, Clarke and Irvine indicated that the amino acid sequence difference in Thr-201 of PI5P4K seems to be evolved from an ATP-dependent kinase. Acquisition of the GTP preference, however, seems to be not simple. Our crystal structures demonstrate that Thr-201 and Phe-205 are responsible for the GTP-dependent kinase activity of PI5P4K. While sequence alignment of PI5P4K/Type II PIPKs shows two patterns in combination of residues 201 and 205 (T-F and M-F types) (Figure 4A), it is difficult to deduce nucleotide preference only from the two residues. For example, PI5P4K has methionine (Met-203) in the position corresponding to Thr-201 in PI5P4K, yet possesses a strong GTP-preference. Since there are additional structural differences in the nucleotide binding residues in PI5P4K and PI5P4K compared to PI5P4K, different set of residues seem to contribute to nucleotide selectivity of individual PI5P4Ks. Indeed, Clarke and Irvine (2013) indicated that the amino acid sequence difference in Thr-201 of PI5P4K is critical for determining the GTP-dependent activity of PI5P4Ks. Therefore, structures of PI5P4Kx and PI5P4Kγ in complex with ATP or GTP are needed to clarify the mechanism for nucleotide preference of PI5P4Ks in the future, which will be a key to decipher the evolutionary mechanism of the GTP-dependent kinase activity.

Given the higher GTP-dependent activity over ATP-dependent activity in PI5P4Kγ and the existence of its Km value within the range of physiological variation in GTP concentration, it is certainly a possibility that PI5P4Kγ plays a role in GTP sensing. There is a recent report that PI5P4Kγ is involved in the development and maintenance of epithelial cell functional polarity (Clarke et al., 2015). The role of the PI5P4Kγ isoform in GTP sensing would be an important next step to explore.

The GTP-Sensing Activity of PI5P4Kγ Fills a Gap in the PI5P4K Paradox

Mammalian genes encode three isoforms of PI5P4Ks (α, β, and γ). Among the isoforms, PI5P4Kγ has the highest kinase activity. The kinase activity of PI5P4Kx is reported to be 100-fold higher than that of PI5P4Kβ and 2,000-fold higher than that of PI5P4Kγ; therefore, it has been considered that PI5P4Kx has a dominant role in cells and PI5P4Kβ and PI5P4Kγ may act as scaffolds to recruit PI5P4Kx by heterodimerization (Bultsma et al., 2010; Clarke and Irvine, 2013). Paradoxically, PI5P4K knockout mice do not exhibit any obvious phenotypes under normal conditions (Emerling et al., 2013), while the knockout mice of the less active PI5P4Kβ display the following dramatic phenotypes: reduced body weight, resistance to obesity induced by high-fat diets, and increased insulin sensitivity (Lamia et al., 2004). In the present study, we established that the GTP-dependent activity of PI5P4Kβ is of the same order of magnitude as PI5P4Kx, indicating that the PI5P4Kβ activity is strong enough to vary the PI5P4K activity. In addition, PI5P4Kβ has the additional feature of its kinase activity being regulated by the GTP concentration. The GTP-sensing activity of PI5P4Kβ could affect a PI(5)P population and might play a role in the different signal pathway from PI5P4Kx with distinct cellular localization. These observations would fill the gap in the PI5P4K paradox and explain why the PI5P4Kβ knockout shows a more severe phenotype than the PI5P4Kγ knockout.

PI5P4Kβ Converts Metabolic Cue from GTP into PI(5)P Signaling

PI5P4Kβ regulates two lipid second messengers, PI(5)P and PI(4,5)P2. It has been suggested that a major role of PI5P4K is to regulate the levels of PI(5)P, since the majority of PI(4,5)P2 is produced by another pathway by PI4PSK/Type-I PIPKs from PI(4,5)P2. It has been suggested that a major role of PI5P4K is to regulate the levels of PI(5)P, since the majority of PI(4,5)P2 is produced by another pathway by PI4PSK/Type-I PIPKs from PI(4,5)P2. It has been suggested that a major role of PI5P4K is to regulate the levels of PI(5)P, since the majority of PI(4,5)P2 is produced by another pathway by PI4PSK/Type-I PIPKs from PI(4,5)P2.
PI(5)P (Clarke and Irvine, 2012, 2013). Thus far, a series of effector molecules of PI(5)P have been reported. Transcriptional regulators, ING2, UHRF1, IRF3, and TAF3, have been shown to bind to PI(5)P to change its subcellular localization and/or activity (Bua et al., 2013; Gelato et al., 2014; Kawasaki et al., 2013; Stijf-Bultsma et al., 2015). Binding of PI(5)P to WIP12, an autophagy effector, has been shown to induce Vps34-independent non-canonical autophagy (Vicinanza et al., 2015). PI(5)P’s role in regulating membrane trafficking and cytoskeletal rearrangement, via TOM1, TIAM1, and the Doc family, has been reported (Boal et al., 2015; Guitard et al., 2010; Viaud et al., 2014). Our data suggest that there may be unidentified effectors of PI(5)P that play a role in regulating metabolism, and that the changes of GTP concentration would induce systemic responses in cells through these PI(5)P effectors.

One critical question is by how much the amount of GTP concentration changes could be sensed by this signaling system. The results of the present study using MPA suggest that an approximately 85% reduction of GTP concentration, which leads to a 1.5-fold increase in PI(5)P level, is enough to evoke the GTP-dependent PI5P4K activity (Figure 5). Indeed, with the 85% reduction of GTP concentration, cells harboring the GTP-insensitive PI5P4Kb mutant (PI5P4KbF205L) showed no variation of the PI(5)P concentration and significant differences in metabolic responses as compared to WT cells (Figure 5). In addition, PI5P4KbF205L cells decrease the tumorigenic activities in vitro and in vivo (Figure 6). Thus, it is reasonable to suggest that the reduction of GTP-dependent kinase activities of PI5P4K and the resulting changes in PI(5)P level are sufficient for trigger functional signaling. Presumably, there may be a mechanism that amplifies the changes in PI5P4K activity. In support of this notion, many biological systems are not linear and signaling activities are dynamically propagated in a spatiotemporal manner through feedback amplification and site-specific localization (Kholodenko, 2006). It would also be possible to consider contribution of another GTP sensor.

The GTP-Sensing Activity of PI5P4Kb May Be the Achilles’ Heel for Human Diseases

Receiving biological cue from GTP concentration may be important in pathological and metabolic conditions that lead to GTP depletion, because rapidly dividing tumor cells as well as metabolic tissues, including the liver, pancreas, and adipose tissue, consume massive amounts of GTP to maintain tumorigenesis and/or to produce serum proteins (Cao and Palade, 1964; Havel, 2002; Ma and Blenis, 2009; Miller et al., 1951; Weiss, 1953). In line with this notion, it is intriguing that the PI5P4Kb knockout mice display the phenotypic link to tumorigenesis as well as whole-body metabolism (Emerling et al., 2013; Lamia et al., 2004). Taken into consideration with the present work, these observations argue that biological cue from GTP concentration need to be integrated into the current energy model as an independent and crucial benchmark for tumorigenesis and metabolic diseases. In addition, increased dependence of those pathological states on GTP makes the GTP-sensing activity of PI5P4Kb an Achilles’ heel for those human diseases. Our crystal structures reveal the unpredicted recognition mode of GTP by PI5P4Kb, and importantly, we were able to modulate GTP-dependent activity by introducing rational mutations. We believe that the structural data presented in this work will benefit pharmaceutical targeting of the GTP-sensing activity of PI5P4Kb that provides an opportunity to develop unique cancer therapeutics.

EXPERIMENTAL PROCEDURES

A Proteomic Screening for GTP-Binding Proteins

A proteomic screening for GTP-binding proteins was carried out by pulling down by GTP-conjugated agarose beads and the successive liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Details are described in the Supplemental Experimental Procedures.

The PI5P4K Kinase Assay

The in vitro kinase assay was carried out using 32P-radiolabeled ATP or GTP (Perkin Elmer), synthetic PI(5)P dC16 (Echelon Biosciences), and recombinant PI5P4Ks. The radiolabeled PI(4,5)P2 was quantified with a phosphorimager (Molecular Dynamics, STORM840, GE Healthcare). Details of in vitro kinase assay are described in the Supplemental Experimental Procedures. Non-tagged recombinant PI5P4Ks were used otherwise as mentioned.

Crystallization, Data Collection, and Structure Determination

Diffraction data were collected at PF in KEK. Crystallographic calculations were performed by PHENIX (Tables 1 and 2) (Adams et al., 2010). Details of the crystallographic work are described in the Supplemental Experimental Procedures.

NMR Spectroscopy

STD experiment and real-time GTP- and ATP-hydrolysis assays were performed on a Bruker Avance 700 MHz spectrometer equipped with triple resonance probe at 25 °C. Details of the NMR experiments are described in the Supplemental Experimental Procedures.

Analysis of Metabolites by Targeted Mass Spectrometry

The metabolomics study was carried out by the targeted mass spectrometry using the SV40-transformed PI5P4Kb-deficient MEFs stably expressing WT-PI5P4Kb or PI5P4KbF205L. Details of the sample preparation, data acquisition, and processing are described in the Supplemental Experimental Procedures.

Colony Formation Assays

Anchorage-independent colony formation assay for assessing the transforming activity and cell proliferation assays on a plastic plate were carried out. The details are described in the Supplemental Experimental Procedures.

Allograft Assay

SV40-transformed PI5P4Kb-deficient MEFs stably expressing WT-PI5P4Kb or PI5P4KbF205L were subcutaneously injected into the flank of 8- to 10-week-old four athymic NCr nu/nu mice. Tumors were harvested at 76 days. Details of allograft assay are described in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

All atomic coordinates used in this manuscript have been deposited in the Protein Data Bank under accession codes PDB: 3ZZZ, 3X01, 3X02, 3X03, 3X04, 3X05, 3X06, 3X09, 3X0A, 3X0B, and 3X0C.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.12.011.

AUTHOR CONTRIBUTIONS

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