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Tumor progression locus 2 regulates signal-induced calcium release from the endoplasmic reticulum and cell migration

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Abstract

The mitogen-activated protein kinase kinase kinase (MAPKKK) tumor progression locus 2 (Tpl2) is required for the transduction of signals initiated by the thrombin-activated G protein-coupled receptor (GPCR) protease activated receptor-1 (PAR1), which promote reorganization of the actin cytoskeleton and cell migration. Here, we show that Tpl2 is activated through $G_{\alpha_{i2}}$ -transduced GPCR signals. Activated Tpl2 promotes the phosphorylation and activation of phospholipase C beta 3 (PLC β_3) and, consequently, Tpl2 is required for thrombin-dependent production of inositol 1,4,5-triphosphate (IP $_3$), the upregulation of cytoplasmic Ca^{2+} , and the activation of classical and novel members of the protein kinase C (PKC) family. A PKC feedback loop facilitates extracellular signal-regulated kinase (ERK) activation in response to Tpl2 and contributes to the coordinate regulation of the ERK and Ca^{2+} signaling pathways. Pharmacological and genetic studies revealed that stimulation of cell migration by Tpl2 depends on both of these pathways. Tpl2 also promoted Ca^{2+} signals and cell migration from G_{α_i} -coupled GPCRs other than PAR1, and from the IL-1 β receptor. Our data provide new insights into the role of Tpl2 in GPCR-mediated Ca^{2+} signaling and cell migration. In addition, they enhance our understanding of the fundamental role of Tpl2 in innate and adaptive immunity, cancer and inflammation.

INTRODUCTION

The second messenger Ca^{2+} binds various effector proteins to promote, directly or indirectly, numerous cellular functions, including cell migration (1, 2). The ability of Ca^{2+} to act as a signal depends on the maintenance of cytosolic Ca^{2+} at a very low concentration (10^{-7} M) relative to that in the extracellular space and in the endoplasmic reticulum (ER) (10^{-3} M). Various extracellular stimuli elicit the opening of Ca^{2+} channels in the plasma membrane or the ER membrane (or both), allowing Ca^{2+} to enter the cytosol very rapidly across the pre-existing steep gradients. The ensuing increase in cytoplasmic Ca^{2+} concentration can stimulate further Ca^{2+} release; however, high Ca^{2+} concentrations eventually block its further release through negative feedback loops (3). Rapid increases in cytosolic Ca^{2+} can be mediated through activation of phospholipase C (PLC), which cleaves phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P $_2$) to generate inositol trisphosphate (IP $_3$) and diacylglycerol (DAG). IP $_3$, which is water-soluble, diffuses through the cytosol to bind to and activate IP $_3$ -gated Ca^{2+} release channels in the ER membrane (4, 5). DAG, which is lipophilic, remains in the membrane where it binds to and activates classical and

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SUPPLEMENTARY MATERIALS

Supplementary Figures, Tables and Legends to the Supplementary Figures are provided as supporting material.

novel, but not atypical members of the protein kinase C (PKC) family (6). PLC comprises 13 enzyme isoforms subdivided into six major families (β , γ , δ , ϵ , ζ , and η) (7). Isoforms in the PLC β and PLC ϵ families are activated through GPCR signaling, whereas isoforms of the PLC γ family are activated by various types of receptors, including receptor tyrosine kinases and immune recognition receptors (4, 5, 8). Ca^{2+} release from the ER, which occurs during the initial response to external stimuli, diminishes the ER Ca^{2+} stores. This triggers Ca^{2+} sensors in the ER (STIM1 and STIM2; stromal cell interaction molecules 1 and 2), which activate highly-selective, low-conductance store operated calcium channels known as calcium release-activated calcium (CRAC) channels. Opening CRAC channels allows the influx of Ca^{2+} from the extracellular space and replenishes the calcium stores (9).

Ca^{2+} influx, phosphatidylinositol 3-kinase (PI3K), and the Rac small guanosine triphosphatase (GTPase), are essential components of the cell migration machinery (10–16). Ca^{2+} influx promotes the assembly of focal adhesions at the leading edge of migrating cells and the disassembly of focal adhesions in the rear (10–12). Although effector proteins activated by high concentrations of Ca^{2+} reside at the leading edge of migrating cells, the highest concentration of Ca^{2+} is in the rear (10, 11, 16). This conundrum was resolved when it was shown that, during fibroblast migration, numerous high Ca^{2+} microdomains form transiently at the leading edge and contribute to the assembly of focal adhesions. The IP_3 -mediated release of Ca^{2+} from the ER contributes to the appearance of these transient microdomains (16). The role of Ca^{2+} microdomains in other cell types, such as macrophages, which may have fundamentally different Ca^{2+} signals, has not been confirmed to-date.

Tumor progression locus-2 Tpl2, also known as Cot, is a mitogen-activated protein kinase kinase kinase (MAP3K) that is activated by provirus integration in rodent Moloney Murine Leukemia Virus (MoMuLV)-induced T cell lymphomas and Mouse Mammary Tumor Virus (MMTV)-induced mammary adenocarcinomas (17, 18). Tpl2 overexpression activates all of the mitogen-activated protein kinase (MAPK) signaling pathways and the transcription factors NFAT (nuclear factor of activated T cells) and NF- κ B (nuclear factor- κ B), and promotes cell transformation and proliferation (19–22). The C-terminally truncated, constitutively-active mutant of Tpl2, which is overexpressed in retrovirus-induced rodent neoplasms, induces T cell lymphomas in mice when overexpressed in developing thymocytes (22). Moreover, Tpl2 activation has been implicated in the emergence of resistance to B-Raf inhibitors in patients with metastatic melanomas (23). Knocking out Tpl2 in mice showed that Tpl2 is required for the transduction of Toll like Receptor (TLR), Interleukin-1 (IL-1), Tumor necrosis factor- α (TNF- α), CD40, and T cell receptor (TCR) signals and that it is implicated in cancer and inflammation (24–26).

Tpl2 has also been implicated in the transduction of signals generated by the GPCR PAR1. PAR1 signals activate Tpl2, which is required for downstream activation of the MAPKs extracellular signal-regulated kinase (ERK) 1 and 2 (ERK1/2). Tpl2-transduced PAR1 signals also promote reorganization of actin cytoskeleton and are required for the stimulation of cell migration through Rac and Focal Adhesion Kinase (FAK). Moreover, Tpl2-transduced PAR1 signals upregulate the abundance of matrix metalloproteinases (MMPs) and other secreted molecules both in fibroblasts and epithelial cells. Given the importance of GPCR signals and the importance of these molecules in cancer and inflammation, these data expand our understanding of the role of Tpl2 in these pathologies (27).

Here, we show that Tpl2 is activated by $\text{G}\alpha_{i2}$ -coupled PAR1 signals that target PLC β_3 , which catalyzes the cleavage of PtdIns-4,5-P2 into IP_3 and DAG, ultimately activating novel and classical PKCs. The stimulation of cell migration by Tpl2 depended both on the increase

in cytosolic Ca^{2+} concentration and on the activation of ERK. Tpl2 also transduced Ca^{2+} signals induced by activated GPCRs other than PAR1, or by other types of activated receptors. Overall, our data provide new insights into the regulation of Ca^{2+} and cell migration, and into the molecular pathways regulating innate and adaptive immunity and inflammation.

RESULTS

Tpl2 is activated by $\text{G}\alpha_{i2}$ -mediated PAR1 signals

One of the earliest events triggered by GPCR signaling is the dissociation of the trimeric G protein complex that is constitutively associated with the GPCR cytoplasmic domain prior to stimulation. The released α and β/γ G protein subunits bind their effector molecules and stimulate or inhibit the generation of various second messenger molecules. PAR1 is coupled to $\text{G}\alpha_i$, $\text{G}\alpha_q$, $\text{G}\alpha_{12/13}$, and members of the $\text{G}\beta/\gamma$ family (28). To determine which G protein families may be involved in GPCR signaling to Tpl2, we transiently transfected immortalized embryonic fibroblasts (MEFs) from Tpl2 knockout (*Tpl2*^{-/-} mice transiently transduced with wild type Tpl2 (Rec-WT cells) or empty vector (Rec-EV cells) with expression constructs for constitutively active members of the $\text{G}\alpha$ and $\text{G}\beta/\gamma$ protein families, including $\text{G}\alpha_{i2}\text{Q205L}$, $\text{G}\alpha_q\text{Q209L}$, $\text{G}\alpha_s\text{Q213L}$, $\text{G}\alpha_{12}\text{Q231L}$, $\text{G}\alpha_{13}\text{Q226L}$, $\text{G}\beta_1$, and $\text{G}\gamma_2$. Immunoblot analysis of the lysates of the transfected cells revealed that Tpl2 stimulated ERK phosphorylation by $\text{G}\alpha_{i2}$, but not by the other $\text{G}\alpha$, β , or γ constructs (Fig. 1A, fig. S1). Results are presented as fold induction of ERK phosphorylation (mean $\pm$ SE) in experimental samples over the Rec-EV control samples. Tpl2-mediated activation of ERK1 and 2 and c-Jun N-terminal kinase (JNK) by thrombin was inhibited by pertussis toxin (PTX) (Fig. 1B), which promotes the irreversible inactivation of $\text{G}\alpha_i$ and $\text{G}\alpha_o$ family members (29). Moreover, $\text{G}\alpha_{i2}$ knockdown with siRNA abolished thrombin-mediated phosphorylation of ERK1/2 and JNK (Fig 1C, Upper and middle panels) and the activation of Tpl2 (Fig 1C, Lower panel), whereas $\text{G}\alpha_q$ knockdown did not, supporting the hypothesis that Tpl2 activation by PAR1, and phosphorylation of its downstream targets, depends on $\text{G}\alpha_{i2}$.

Real time RT-PCR, using RNA derived from WT or EV fibroblasts, revealed that $\text{G}\alpha_{i3}$ and $\text{G}\alpha_{i1}$, were also expressed in these cells, although to a lesser degree than $\text{G}\alpha_{i2}$. Western analysis of cells transfected with control siRNA, or siRNAs directed against $\text{G}\alpha_{i1}$, $\text{G}\alpha_{i2}$, or $\text{G}\alpha_{i3}$ revealed that only $\text{G}\alpha_{i2}$ contributes to the activation of the PAR1 to Tpl2 to ERK pathway (Fig. 1D).

Immunoprecipitation analysis of human embryonic kidney (HEK) 293T cells co-transfected with HA-Tpl2 and EE-tagged $\text{G}\alpha_{i2}$ (EE- $\text{G}\alpha_{i2}$) constructs revealed that Tpl2 associates with $\text{G}\alpha_{i2}$ in unstimulated cells, and that following thrombin stimulation, the two proteins dissociate rapidly (within 2 min), and re-associate 15–30 minutes later (Fig. 1E, upper panel). Separate experiments revealed that endogenous $\text{G}\alpha_{i2}$ interacts with Tpl2 in unstimulated cells but not in cells stimulated with thrombin (Fig. 1E, lower panel). Combined, these data suggest that Tpl2 activation by GPCR occurs at the plasma membrane and depends on Tpl2 binding to $\text{G}\alpha_{i2}$. Following Tpl2 activation at the membrane, the two proteins dissociate to initiate their signaling cascades.

Tpl2 is required for the PAR1-induced increase in cytoplasmic Ca^{2+} concentration

Thrombin induces the release of Ca^{2+} from intracellular stores (30). To determine whether Ca^{2+} mobilization by thrombin was Tpl2-dependent, we loaded immortalized Tpl2^{+/+} and Tpl2^{-/-} MEFs, as well as Tpl2^{-/-} MEFs transduced with wild type (Rec-WT) or kinase-inactive forms of Tpl2 (Rec-KD) with the fluorescent Ca^{2+} indicator Fluo-4 NW and

stimulated them with thrombin. The Fluo-4 NW signal to thrombin was blocked by pre-treatment with the membrane-permeable Ca^{2+} chelator BAPTA-AM (Fig. 2B), confirming that it was indeed due to changes in cytoplasmic Ca^{2+} concentration. The PAR1-specific agonist Thr-Phe-Leu-Leu-Arg-NH₂ (TFLLRN-NH₂) also increased cytoplasmic Ca^{2+} in a Tpl2-dependent manner. These experiments confirmed that the Tpl2-dependent rapid upregulation of Ca^{2+} by thrombin is due to the activation of PAR1 (Fig. 2, Table I-II). The difference in the magnitude of the Ca^{2+} response between cells stimulated with thrombin and TFLLRN-NH₂ could be due to differences in the conformation of proteolytically-activated PAR1 (thrombin) versus peptide-activated PAR1 (31).

Tpl2-transduced GPCR signals induce IP₃ production and promote the release of Ca^{2+} from the endoplasmic reticulum

The PAR1-dependent increase in cytoplasmic Ca^{2+} concentration could result from influx of extracellular Ca^{2+} or from the Ca^{2+} release from the ER. To address this question, we stimulated Rec-WT, Rec-KD, or Rec-EV MEFs with thrombin after treating them with the Ca^{2+} chelator EGTA or the IP₃-receptor inhibitors, 2-aminoethoxydiphenyl borate (2-APB) or Xestospongin C. Cells pretreated with EGTA showed a slower increase in cytoplasmic Ca^{2+} , which we attributed to changes in cellular physiology, caused by the prolonged lack of extracellular Ca^{2+} . However, EGTA did not affect the magnitude of the Ca^{2+} response (Fig. 3A, upper panel). 2-APB and Xestospongin C both blocked the thrombin-induced increase in cytoplasmic Ca^{2+} (Fig. 3A, middle and lower panels). These data suggest that thrombin may promote IP₃-induced release of Ca^{2+} from the ER. To investigate this possibility directly, we loaded Rec-WT or Rec-EV fibroblasts with ³H-labeled myoinositol and measured the IP₃ concentration in cell extracts before, or 30 minutes after stimulation with thrombin. We found that thrombin increased IP₃ concentration and that this depends on Tpl2- (Fig. 3B, left panel). Parallel experiments in Rec-WT cells transfected with siGα_{i2} or siControl revealed that IP₃ induction by thrombin depends on Gα_{i2}- (Fig. 3B, right panel)..

Tpl2 is required for the activation of classical and novel PKCs by GPCR signals

Activation of PKC isoforms is initiated by a series of ordered phosphorylation events that stabilize these proteins and render them competent for activation, following translocation to the membrane. (32, 33). To determine whether Tpl2 controls PKC activation therefore, we examined first the phosphorylation of classical (PKCα, β_I, and β_{II}), novel (PKCδ, PKCθ, and PKCμ) and atypical PKCs (PKC ζ and λ) in Rec-WT and Rec-EV fibroblasts, before or after thrombin stimulation and we found that Tpl2 was required for the thrombin-mediated phosphorylation of classical and novel but not atypical PKCs (Fig. 3C, fig. S2A). To determine the role of Tpl2 in PKC translocation (34, 35), we probed membrane and cytosolic fractions of Rec-WT or Rec-EV cell lysates with an anti-PKCδ antibody and found that, Tpl2 also contributes to PKCδ translocation in thrombin-stimulated cells (Fig. 3D, fig. S2B). The shift of the PKCδ band observed in the Rec-WT membrane fractions (Fig. 3D) was likely caused by phosphorylation. These findings indicate that Tpl2-mediated GPCR signals promote both Ca^{2+} release from the ER and PKC phosphorylation and suggest that Tpl2 stimulates the hydrolytic cleavage of PtdIns-4,5-P₂ in response to thrombin. The specificity of Tpl2-dependent PAR1 signals toward classical and novel but not atypical PKCs, suggests that Tpl2 may regulate the binding of PKC isoforms to HSP90/Cdc37, which specifically precedes the phosphorylation of these isoforms (33). To determine whether thrombin-induced PKC phosphorylation depends on Gα_{i2}, we assessed PKCα and PKCβ_{II} phosphorylation in thrombin-stimulated Rec-WT cells, pretreated with PTX or transfected with siRNAs directed against Gα_{i2} or Gα_q and we found that whereas PTX and siGα_{i2} inhibit PKC phosphorylation, siGα_q does not. We conclude that thrombin-mediated PKC phosphorylation is Gα_{i2}-dependent (Fig. S3).

To determine whether thrombin-induced ERK activation and Ca^{2+} release from the ER depends on activation of PKC, we pretreated Rec-WT cells with GF109203X, which inhibits all the classical PKC isozymes and two novel PKCs (PKC δ and PKC ϵ), or with DMSO, and then stimulated them with thrombin. Cell lysates harvested at 0, five and 15 minutes after stimulation, were assessed by immunoblot, using an antibody specific for phospho-ERK. Parallel cultures were monitored for 140 sec from the start of the stimulation for changes in the cytoplasmic Ca^{2+} . We found that GF109203X inhibits ERK activation but not the increase in cytoplasmic Ca^{2+} concentration (Fig. S4) and we conclude that classical and novel PKCs contribute to thrombin-mediated ERK activation, but not to Ca^{2+} signaling.

Tpl2-transduced GPCR signals are required for the phosphorylation of PLC β_3 on Ser⁵³⁷

The preceding data suggested that Tpl2-mediated signals may target PLC β , an enzyme that catalyzes PtdIns-4,5-P₂ hydrolysis in response to GPCR signaling. PLC β consists of four isozymes, PLC β_1 , PLC β_2 , PLC β_3 , and PLC β_4 . PLC β_3 , which is activated by thrombin signaling (30), is the most abundant at the RNA level in spontaneously immortalized MEFs. However, the mRNA of each of the four isozymes is equally abundant in Rec-WT and Rec-EV cells (Fig. S5). PLC β_1 and PLC β_4 are activated primarily by $\text{G}\alpha_q$ (36), which appears to be the $\text{G}\alpha$ protein subunit primarily responsible for PLC β activation. PLC β_3 is activated by both $\text{G}\beta\gamma$ and $\text{G}\alpha_q$ and PLC β_2 is activated primarily by $\text{G}\beta\gamma$ (36). The activation of PLC β in response to signaling through many GPCRs, however, is PTX-sensitive (36), suggesting that PLC β may also be activated by $\text{G}\alpha_i$ signaling, perhaps through Tpl2 (Fig. 1). PLC β_3 is activated by calmodulin kinase II (CaMKII)-mediated phosphorylation of Ser⁵³⁷ (37), and inhibited by phosphorylation of Ser¹¹⁰⁵ (38). Immunoblot analysis of Rec-WT and Rec-EV fibroblasts using antibodies specific for total PLC β_3 or for PLC β_3 phosphorylated at Ser⁵³⁷ or Ser¹¹⁰⁵ showed that, whereas phosphorylation at Ser⁵³⁷ in response to thrombin was Tpl2-dependent, phosphorylation at Ser¹¹⁰⁵ was not (Fig. 4A).

To determine whether the Tpl2-dependent phosphorylation of PLC β_3 was mediated by CaMKII(38), we pretreated Rec-WT and Rec-EV fibroblasts with the CaMKII inhibitor KN-93 30 minutes before stimulation with thrombin. Immunoblot analysis, with total PLC β_3 or phospho-PLC β_3 antibodies (Fig. 4B, upper panel), indicated that KN-93 partially inhibited thrombin-dependent PLC β_3 phosphorylation. To further explore the role of Tpl2 in PLC β_3 activation, we examined PLC β_3 phosphorylation at Ser⁵³⁷ in HEK293T cells pretreated with KN-93 and transiently transfected with a Tpl2 expression construct (Tpl2) or with empty vector (EV). Tpl2 overexpression induced PLC β_3 phosphorylation at Ser⁵³⁷ in the presence or absence of KN-93 (Fig. 4B, middle panel, fig. S6), suggesting that Tpl2 may itself phosphorylate PLC β_3 . To investigate this hypothesis, we immunoprecipitated Tpl2 from lysates of Rec-WT and Rec-KD immortalized MEFs harvested at the indicated time points before and after stimulation with thrombin, and used the immunoprecipitates for *in vitro* kinase assays with PLC β_3 as the substrate. Phosphorylation of the substrate at Ser⁵³⁷ was detected with the same phospho-specific PLC β_3 antibody used in the experiment in Fig. 4A. Although we cannot exclude that a kinase that co-immunoprecipitates with Tpl2 may contribute to the *in vitro* phosphorylation of PLC β_3 , the results of this experiment strongly suggest that Tpl2 phosphorylates PLC β_3 at Ser⁵³⁷ directly (Fig. 4B, lower panel).

To validate that PLC β_3 is the PLC β isoform mainly responsible for the hydrolysis of PIP₂ and the thrombin-dependent production of IP₃, we determined how its knockdown affected IP₃ abundance. We found that PLC β_3 knockdown decreased both the thrombin-induced increase in IP₃ and in cytoplasmic Ca^{2+} concentration (Fig. 4C). To investigate the functional role of PLC β_3 phosphorylation at Ser⁵³⁷, we generated a PLC β_3 mutant (PLC β_3 S537A) in which Ser⁵³⁷ was replaced with alanine. Spontaneously immortalized lung fibroblasts from PLC β_2 and PLC β_3 double knockout mice were transduced with pBabe-puro retroviral constructs of wild type PLC β_3 , or PLC β_3 S537A, or with the empty

retroviral vector and used to measure thrombin-induced changes in IP₃ and cytoplasmic Ca²⁺. Whereas wild type PLCβ₃ restored thrombin-mediated increases in IP₃ and cytoplasmic Ca²⁺, PLCβ₃S537A only partially corrects the IP₃/Ca²⁺ defect (Fig. 4D). We conclude that the phosphorylation of PLCβ₃ via Tpl2 is required for the full functional activation of PLCβ₃ by thrombin (Fig. 4, fig. S7).

ERK activation and increased cytoplasmic Ca²⁺ in response to Gα_{i2}-transduced thrombin signals depend partially on release of Gβ/γ

Our data support the hypothesis that the activation of Tpl2 and ERK by thrombin depends on Gα_{i2}. Gα_{i2} activation is accompanied by the release of Gβ/γ which is also pertussis toxin-sensitive and contributes to the activation of ERK and of PLCβ₃ in response to GPCR stimulation. Of the five Gβ subunits, Gβ₁, Gβ₂, and Gβ₃ have been linked to the activation of PLCβ₃ (39). We therefore analyzed the effects of knocking down Gβ₁, Gβ₂, and Gβ₃ singly or in combination in Rec-WT cells on thrombin-mediated activation of ERK and increase in cytoplasmic Ca²⁺. Whereas the knockdown of Gα_{i2} substantially inhibited thrombin-mediated ERK phosphorylation (Fig. 1D) and the induction of IP₃ (Fig. 3B), knockdown of these Gβ subunits individually or in combination inhibited ERK activation and the increase in cytoplasmic Ca²⁺ only slightly (Fig. S8). We conclude that both thrombin-mediated ERK activation and the increase of cytoplasmic Ca²⁺ depend only partially on the release of the Gβ/γ subunits.

PAR1 signals engaging Gα_{i2}, Tpl2, PLCβ₃ and the IP₃ receptor sequentially promote cell migration

The preceding data provide evidence for a thrombin-initiated pathway that sequentially activates PAR1, Gα_{i2}, Tpl2, PLCβ₃, IP₃, and the IP₃ receptor, leading to release of Ca from the ER. Tpl2 and Ca²⁺ have been shown to promote cell migration (10–12, 15, 16, 27, 40). To determine whether thrombin-activated Tpl2 promotes cell migration through this pathway, we used a transwell cell migration assay to measure thrombin-induced directional cell migration in primary *Tpl2*^{+/+} and *Tpl2*^{-/-} MEFs and in the PAR1-expressing mammary adenocarcinoma (MBA-MB 231) and ovarian carcinoma (SKOV3) cell lines transfected with siTpl2 or siRNA-Control. The results confirmed earlier work (27), by showing that thrombin-induced cell migration depended on Tpl2 (Fig. 5A–5C). Knockdown of Gα_{i2} or PLCβ₃ or treatment with the IP₃-receptor inhibitor 2-APB, inhibited thrombin-induced cell migration (Fig. 5D, 5E and 5F). These data suggest that thrombin-induced cell migration is activated through the Gα_{i2}-Tpl2-PLCβ₃-IP₃ pathway. PLCβ₃ can be activated by Tpl2 in the absence of CaMKII, but CaMKII was required for its full activation (this report). Based on these findings, we hypothesized that CaMKII is activated by the initial release of cytoplasmic Ca²⁺ induced by Tpl2, and that, upon activation, it establishes a positive feedback loop that results in the full activation of PLCβ₃. An inhibitor of CaMKII should block the full activation of PLCβ₃ and should therefore inhibit thrombin-induced cell migration. The data in figure 5G confirmed this hypothesis by showing that the CaMKII inhibitor KN-93 partially inhibits cell migration. Cell migration may also be stimulated by ERK (41). The role of ERK in Tpl2-transduced migratory signals initiated by thrombin was addressed with the experiment in figure 5H, which shows that blocking ERK activation with the MEK (mitogen-activated or extracellular signal-regulated protein kinase kinase) inhibitor U0126 decreased Tpl2-dependent cell migration. We conclude that Tpl2 promotes cell migration through both ERK activation and Ca²⁺ release from the ER. Data presented in figure S4 showed that inhibition of PKC attenuates the activation of ERK and MEK, which is itself phosphorylated by Tpl2 (20). Therefore, we hypothesized that activated PKC might also promote cell migration. Indeed, pharmacological inhibition of classical and novel PKCs (with GF109203X), or classical PKCs alone (with Go6976) attenuated thrombin-induced

cell migration, consistent with the known role of Ca^{2+} -activated PKC α in cell polarization and migration (15).

Both Tpl2 and Ca^{2+} stimulate phosphorylation of FAK at Tyr³⁹⁷ and promote the extended residency of FAK at focal adhesions, focal adhesion disassembly and cell edge retraction (10–12, 27). Furthermore, FAK phosphorylation activates ERK (42, 43). Since Tpl2 is required for thrombin-induced Ca^{2+} influx via PLC β 3, we hypothesized that FAK phosphorylation in response to Tpl2 signaling might depend on Ca^{2+} , and perhaps on PLC β 3. To explore this hypothesis, we stimulated PLC β 2^{-/-}/PLC β 3^{-/-} fibroblasts transduced with pBabe-puro-based retroviral constructs of wild type PLC β 3, the PLC β 3S537A mutant, or the empty vector, with thrombin and analyzed them by immunoblot with an antibody specific for FAK phosphorylated at Tyr³⁹⁷. The results showed that phosphorylation of PLC β 3 at Ser⁵³⁷ is required for the phosphorylation of FAK at Tyr³⁹⁷ (Fig. 5K), indicating that phosphorylation of PLC β 3 by Tpl2 is indeed required for the phosphorylation of FAK at Tyr³⁹⁷.

Our data on the role of Tpl2 in PAR1 signaling and its regulation of cell migration is summarized in the model shown in Fig. 6. We propose that Tpl2 associates with G α _{i2} in resting cells. Upon GPCR activation, the two molecules dissociate, initiating to activate various signaling cascades. Activated Tpl2 phosphorylates both MEK and PLC β 3, thereby leading to the activation of ERK, the release of Ca^{2+} from the ER, and the activation of classical and novel PKCs. PKC, in turn, contributes to the activation of MEK and ERK. A positive feedback loop, initiated by the Ca^{2+} -dependent activation of CaMKII, promotes the full activation of PLC β 3 and its downstream targets and signaling pathways. The MEK-ERK and PLC β 3- Ca^{2+} pathways ultimately converge to regulate cell migration. The crosstalk between the two pathways may contribute to their coordinate regulation.

Tpl2 regulates cytoplasmic Ca^{2+} influx initiated by G α _i-coupled GPCRs other than PAR1, as well as by other types of activated receptors

G α _i-coupled signals can be initiated by many GPCRs in addition to PAR1. One family of G α _i-coupled GPCRs is the sphingosine-1-phosphate (S1P) receptor family, which is also coupled to G α _q, or G α _{12/13} (44). S1P is a bioactive lipid mediator that regulates various cellular functions, including cell migration, proliferation and differentiation (45). There are five S1P-responsive GPCRs (S1P₁₋₅), of which three (S1P₁₋₃) are found in MEFs (46). To determine whether Tpl2 contributes to GPCR-mediated S1P signals, we treated immortalized Tpl2^{-/-} MEFs transduced with wild type Tpl2 (Rec-WT) or the empty vector (Rec-EV), as well as Tpl2^{+/+} and Tpl2^{-/-} bone marrow derived macrophages (BMDM), with S1P. Western blots of cell lysates harvested before and after stimulation, revealed that Tpl2 was required for ERK and JNK activation by S1P in both fibroblasts and macrophages (Fig. S9). Measuring the cytoplasmic Ca^{2+} in the same S1P-stimulated cells, as described in the materials and methods, showed that S1P-dependent Ca^{2+} signals were mediated by Tpl2 (Fig. 7A). Similar to thrombin, S1P increased the cytoplasmic IP₃ and Ca^{2+} concentrations in a G α _{i2}- and PLC β 3-dependent manner (Fig. 7B). Furthermore, S1P-induced cell migration, like thrombin-induced migration, depends on G α _{i2}, PLC β 3, IP3 and perhaps CaMKII (Fig. 7C).

To determine whether Tpl2 increases cytoplasmic Ca^{2+} solely in response to GPCR signals, or whether it has a more global role in Ca^{2+} signaling, we measured the abundance of cytoplasmic Ca^{2+} in cells treated with Wnt5a, IL-1 β , or TNF- α . Wnt5a stimulates GPCRs of the frizzled (Fzd) family, the tyrosine kinase receptors Ror2 and Ryk and receptor complexes between Fzd and Ror2 or LRP5-LRP6 (47), while IL-1 β and TNF- α , act through receptors of the Toll-IL-1R (TIR) domain and death domain families respectively (48,49). Whereas the increase in cytoplasmic Ca^{2+} in response to Wnt5a in MEFs or IL-1 β in MEFs

or macrophages were Tpl2-dependent, the Ca^{2+} response to TNF- α was Tpl2-independent (Fig. S10).

DISCUSSION

Tpl2 transduces PAR1 signals that promote cell migration through a pathway that involves the small GTPase Rac1 and FAK and targets the actin cytoskeleton (27). This pathway can be initiated through multiple mechanisms, including cytoplasmic Ca^{2+} influx and ERK activation. Here, we showed that Tpl2-mediated GPCR signals induced by thrombin or S1P not only activate ERK as previously shown (27), but also stimulate the release of Ca^{2+} from the ER. Furthermore, we found that the ability of Tpl2 to promote cell migration depends on both ERK activation and increased cytoplasmic Ca^{2+} .

PAR1 belongs to the GPCR family of receptors, which are constitutively-associated with a trimeric G-protein complex composed of three subunits (α , β and γ). Ligand binding leads to dissociation of the trimeric complex. The released α and β/γ subunits bind their effector molecules, which include adenylyl and guanylyl cyclases, PLC, PLA2, PI-3K and phosphodiesterases. The binding of G proteins to these effectors stimulates or inhibits the generation of a variety of second messenger molecules and the influx of Ca^{2+} into the cytosol (50). GPCR-mediated stimulation of Ca^{2+} release has classically been viewed as transduced by $\text{G}\alpha_q$ (28, 50). However $\text{G}\alpha_i$ -mediated pathways, which are responsible for the activation of Tpl2 (this report), can also stimulate Ca^{2+} release (36). Tpl2 is constitutively associated with $\text{G}\alpha_{i2}$ at the plasma membrane of resting cells and the two proteins dissociate after stimulation of PAR1 with thrombin to initiate their respective signaling cascades.

Because $\text{G}\alpha_i$ activation is accompanied by the release of $\text{G}\beta/\gamma$, it has been argued that Ca^{2+} release from the ER by $\text{G}\alpha_i$ may be mediated by $\text{G}\beta/\gamma$. Our data indicate that whereas $\text{G}\beta/\gamma$ signaling is not sufficient to explain the effects of PAR1 stimulation on either Ca^{2+} release from the ER or ERK activation, $\text{G}\alpha_{i2}$ signaling is. Additionally, we found that PAR1-induced Ca^{2+} signals are transduced through Tpl2, which activates $\text{PLC}\beta_3$ by phosphorylation at Ser⁵³⁷. Classical PKCs are targeted to the leading edge of the plasma membrane of polarized cells by Ca^{2+} (15) and PtdIns-4,5-P_2 (51, 52). Our data argue that localized production of DAG at the plasma membrane by Tpl2-activated $\text{PLC}\beta_3$ may also play an important role in the targeting of PKCs to the leading edge of polarized migrating cells. Ca^{2+} and PtdIns-4,5-P_2 are both required for the targeting of the PKC C2 domain, whereas DAG may stabilize PKC targeting to the plasma membrane by binding to C1 domains (35, 51, 52).

Inhibition or knockdown of $\text{G}\alpha_{i2}$, knockdown of $\text{PLC}\beta_3$, or inhibition of IP_3 -activated Ca^{2+} channels all inhibited the PAR1-mediated Ca^{2+} signals and cell migration. These findings raised the question whether Tpl2, which phosphorylates $\text{PLC}\beta_3$ at Ser537 (this report) regulates Ca^{2+} signaling in thrombin-stimulated cells through phosphorylation of $\text{PLC}\beta_3$ at this site. Experiments designed to address this question, showed that whereas $\text{PLC}\beta_2^{-/-}$ / $\text{PLC}\beta_3^{-/-}$ cells reconstituted with wild type $\text{PLC}\beta_3$ respond to thrombin stimulation with IP_3 accumulation, rapid increase in cytoplasmic Ca^{2+} concentration and FAK phosphorylation at Tyr397, $\text{PLC}\beta_2^{-/-}$ / $\text{PLC}\beta_3^{-/-}$ cells reconstituted with $\text{PLC}\beta_3\text{S537A}$ fail to do so. These experiments formally addressed the role of phosphorylation at Ser537 in $\text{PLC}\beta_3$ activation, by showing that phosphorylation at this site is required for the functional activation of the enzyme.

Together, our data identify Tpl2 as a component of a $\text{G}\alpha_i$ -mediated pathway that regulates the release of Ca^{2+} from the ER into the cytosol in response to thrombin activation of PAR1.

In addition, they show that the cytosolic influx of Ca^{2+} through this pathway contributes to the stimulation of cell migration by PAR1.

PLC β_3 is phosphorylated by CaMKII on Ser⁵³⁷ (37). Here, we found that the phosphorylation of PLC β_3 in thrombin-stimulated cells expressing wild type Tpl2 was only partially inhibited by the CaMKII inhibitor KN-93. Furthermore, in Tpl2^{-/-} cells, PLC β_3 did not undergo phosphorylation in response to thrombin regardless of the presence of KN-93. These data suggest that PLC β_3 phosphorylation in response to thrombin may involve a combination of CaMKII-dependent and -independent Tpl2 signals. The possible contribution of CaMKII-independent Tpl2 signals was substantiated by transient transfection of HEK293 cells with a wild type Tpl2 construct, which showed that Tpl2 overexpression alone leads to phosphorylation of PLC β_3 , and by in vitro kinase assays showing that the phosphorylation of PLC β_3 by Tpl2 may be direct. These data suggest a potential explanation for the apparent biphasic nature of the Ca^{2+} response (Fig. 2). We propose that, in thrombin-stimulated cells, PLC β_3 is initially phosphorylated by Tpl2-transduced CaMKII-independent signals; an initial weak activation of PLC β_3 produces IP₃, thereby stimulating the release of Ca^{2+} from the ER. The ensuing rise in cytosolic Ca^{2+} activates CaMKII, which also phosphorylates PLC β_3 , promoting a second wave of IP₃ generation and Ca^{2+} release (Fig. 6). In the absence of Tpl2 (Rec-EV cells), there was a small initial response, which was Tpl2-independent and was not sufficient to initiate the second wave of the biphasic response.

Tpl2 is activated by various signals, including GPCR-mediated signals, to phosphorylate and activate MEK and thereby activate ERK (19–22, 24–27). Our data show that, although MEK may be activated directly through its phosphorylation by Tpl2, its activation is modulated by PLC β_3 -induced PKC signals. Given that the PKC signals do not affect PAR1-mediated Ca^{2+} influx, we conclude that they do not target Tpl2 itself. Instead, they may target MEK or other molecules, such as scaffold proteins, that regulate MEK phosphorylation by Tpl2.

Given the ubiquitous importance of Ca^{2+} signaling, we wondered whether Tpl2 mediates Ca^{2+} signals in response to stimulation of GPCRs other than PAR1, or of other types of receptors. We found that Tpl2 was required for Ca^{2+} signaling in response to S1P, Wnt5a, and IL1 β , which activate a variety of receptors, including GPCRs (45–48) but not in response to TNF- α , which activates receptors of the death receptor family (49). The ability of Tpl2 to promote Ca^{2+} signals downstream of various receptors suggests that Tpl2 ablation or inactivation may give rise to pleiotropic phenotypes associated with the partial block of these receptors. A partial block of S1P signaling, for instance, would be consistent with the resistance of Tpl2^{-/-} mice to LPS-induced endotoxin shock (24). One of the features of endotoxin shock is disseminated intravascular coagulation (DIC), which is induced through the activation of the Tpl2-dependent PAR1-S1P3 axis in dendritic cells (53).

In summary, our data indicate that Tpl2 transduces GPCR and other receptor signals that promote cytoplasmic Ca^{2+} influx and cell migration. The GPCR signals that target Tpl2 are mediated by G α_{i2} . Our data also identify PLC β_3 as a substrate for Tpl2 phosphorylation (in addition to the well-known Tpl2 substrate MEK) and they show that PLC β_3 phosphorylation by Tpl2 at Ser⁵³⁷ is required for thrombin-dependent IP₃ production, Ca^{2+} signaling, FAK phosphorylation at Tyr³⁹⁷, and cell migration. These data provide new insights into the mechanisms regulating cytoplasmic Ca^{2+} influx downstream of GPCRs and other types of receptors and into the mechanisms by which Tpl2 regulates cell migration. These insights enhance our understanding of the role of Tpl2 in innate and adaptive immunity, cancer and inflammation..

MATERIALS AND METHODS

Expression constructs, siRNAs, enzyme inhibitors, cytokines and other receptor agonists and antibodies

Wild type or kinase-inactive (K167M) Tpl2, C-terminally tagged with the HA epitope, or wild type PLC β_3 were cloned into the retroviral vector pBabe-puro, using standard procedures. The PLC β_3 mutant, in which serine residue 537 was replaced with alanine (PLC β_3 S537A), was generated using the QuickChange II Site-Directed mutagenesis kit (Agilent).

Retrovirus constructs were packaged via transient transfection of 293T cells. Spontaneously immortalized *Tpl2*^{-/-} MEFs or *PLC β_2* ^{-/-}/*PLC β_3* ^{-/-} lung fibroblasts were infected in the presence of polybrene (Sigma) and selected for 10 days in puromycin (Sigma).

Expression plasmids of the EE epitope-tagged constitutively active G α_{12} the EE epitope tagged constitutively active G α subunits G α_{12} Q205L, G α_q Q209L, G α_s Q213L, G α_{12} Q231L, G α_{13} Q226L, and expression constructs of Flag-G β_1 and HA-G γ_2 were purchased from the UMR cDNA Resource Center. To knock down mouse G α_{12} (#sc-41753), G α_q (sc-35430), and PLC β_3 (sc-36273), we used siRNAs and control siRNAs purchased from Santa Cruz Biotechnology Inc. To knock down mouse PLC β_3 (#s71798), G α_{11} (#s66786 and #s66788), G α_{12} (#s66790), G α_{13} (#s66794 and #s66792), G α_q (s66803), Gnb1, Gnb2, and Gnb3 we used siRNAs and control siRNAs purchased from Ambion (Applied Biosystems Inc).

The G α_i inhibitor pertussis toxin (PTX), the cell-permeable PKC inhibitor GF109203X, the classical PKC inhibitor Go6976, the IP $_3$ receptor inhibitors 2-aminoethyl-diphenylborinate (2-APB) and Xestospongine, and the calmodulin kinase II (CaMKII) inhibitor KN-93 were purchased from Calbiochem. Calcium chelators EGTA and BAPTA-AM were purchased from Sigma. Thrombin, sphingosine-1-phosphate (S1P) and tumor necrosis factor- α (TNF- α) were purchased from Haematologic Technologies Inc, Avanti Polar Lipids Inc. and Cell Signaling Technologies Inc., respectively. IL1- β and Wnt5a were purchased from R&D Systems. The PAR1 agonist TFLLRN-NH $_2$ was synthesized in the DNA/Protein core facility (Tufts University School of Medicine).

Polyclonal antibodies to phospho-ERK1/2, total-ERK1/2, phospho-JNKs, total-JNKs, phospho-MEK1/2, phospho-(pan)PKC (Ser⁶⁶⁰ in PKC β II or the corresponding sites in other classical or novel PKCs), phospho-PKC α/β II (Thr^{638/641}), phospho-PKC δ (Thr⁵⁰⁵), phospho-PKC δ (Ser⁶⁴³), phospho-PKC μ (Ser^{744/748}), phospho-PKC μ (Ser⁹¹⁶), phospho-PKC θ (Thr⁵³⁸), phospho-PKC ζ/λ (Thr^{410/403}), total-PKC α , total-PKC δ , total-PLC β_3 , phospho-PLC β_3 (Ser¹¹⁰⁵), and monoclonal antibodies to the HA-tag and total-PKC ζ and EGFR were purchased from Cell Signaling Technologies Inc. The monoclonal antibody to the EE-tag was purchased from Covance. Polyclonal antibodies to phospho-PLC β_3 (Ser⁵³⁷), G α_q , and the monoclonal antibody to G α_{12} were purchased from Santa Cruz Biotechnology Inc. Monoclonal antibodies to tubulin and to the Flag-tag and HRP-conjugated secondary antibodies were purchased from Sigma.

Cell culture, Retroviral Infections, Transient Transfection and immunoblotting

Primary and immortalized MEFs and bone marrow-derived macrophages (BMDMs) from *Tpl2*^{+/+} and *Tpl2*^{-/-} mice, and HEK293T cells were cultured as described (24, 27). Immortalized *Tpl2*^{-/-} MEFs transduced with pBabe-puro-based retroviral constructs of wild type Tpl2-HA (Rec-WT), kinase-dead Tpl2K167M-HA (Rec-KD), or empty vector (Rec-EV) have been described (27). MDA-MB-231 breast cancer cells and SKOV3 ovarian cancer cells were cultured in DMEM and RPMI-1640, respectively, supplemented with 10%

FBS Primary lung fibroblasts were isolated from PLC β_2 /PLC β_3 double knockout mice (a kind gift from Dr. Dianqing Wu at Yale University), cultured in DMEM supplemented with 10% FBS and immortalized via the 3T3 immortalization protocol (54). Following immortalization, they were transduced with pBabe-puro-based retroviral constructs of wild type PLC β_3 , a PLC β_3 mutant in which the phosphorylatable Ser⁵³⁷ residue is replaced with alanine (PLC β_3 S537A), or with empty vector (EV) and selected for puromycin resistance. Three cultures, independently transduced with each of the constructs, were analyzed for PLC β_3 presence and used for subsequent experiments. Transfections of expression constructs and siRNAs were carried out using Lipofectamine 2000 (Invitrogen). Immunoblotting was performed using standard procedures. Protein abundance was quantified using the ImagePC image analysis software (Scion Corporation, Frederick MD).

Quantitative real-time reverse transcriptase PCR

Purification of total cellular RNA was carried out with the RNeasy mini kit (Qiagen) and first-strand cDNA synthesis was carried out with the RETROscript kit (Ambion). PCRs were done in triplicate in a solution containing template cDNA, iQ SYBRGreen Supermix (Bio-Rad), and specific primers (final volume, 25- μ l). Data were analyzed using an Opticon2 continuous fluorescence detector (MJ Research). The relative expression of a given gene was determined by dividing the amount of its cDNA of the gene by that of the β -actin cDNA in the same sample.

Tpl2 in Vitro Kinase Assay, using GST-MEK or His₆-PLC β_3 as the substrate

Lysates of Rec-WT MEFs were harvested before thrombin (1 U/ml) stimulation or at various time points after stimulation. Cells were lysed in a lysis buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 25 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 10 μ g/ml leupeptin, and 5 μ g/ml aprotinin. Tpl2-HA was immunoprecipitated from the lysates by overnight incubation at 4° C with a mouse monoclonal anti-HA antibody. The immunoprecipitates were washed 3 times with the lysis buffer and 2 times with a kinase reaction buffer containing 20 mM Hepes, pH 7.4, 10 mM MgCl₂, 0.5 mM EGTA, 10 mM DTT, 50 mM NaF, 50 mM β -glycerophosphate and 10 μ g/ml aprotinin. The washed Tpl2 immunoprecipitates were resuspended in kinase buffer supplemented with 100 μ M ATP (Cell Signaling Technologies) and incubated with 500 ng of recombinant substrate (bacterially expressed GST-MEK or Sf9-expressed His₆-PLC β_3) at 30° C for 20 min, as previously described (27). Recombinant PLC β_3 was a kind gift provided by Dr. Alan V. Smrcka of the University of Rochester Medical Center. The reaction was terminated by adding sample loading buffer. Samples were analyzed in SDS-PAGE, transferred to PVDF membranes and probed with the phospho-MEK1/2 or phospho-PLC β_3 (S537) antibody..

Measurement of intracellular Ca²⁺ signals—Intracellular calcium signals were measured using the Fluo-4 NW Calcium Assay Kit (Molecular Probes, Invitrogen). Briefly, MEFs, HEK293T cells, or lung fibroblasts were cultured in 96-well plates at a concentration of 5000 cells per well. Macrophages were cultured in 96-well plates at a concentration of 30,000 cells per well. One day after plating, the cells were serum-starved (1% serum). Twelve hours later, they were washed with the assay buffer (20mM HEPES, 1X Hank's balanced salt solution) and incubated with the Fluo-4 loading solution for 45 min at 37° C. The agonists were robotically added and the Ca²⁺ signals were measured, using a FlexStation 3 fluorescence reader (Molecular Devices) (excitation at 494 nm and emission at 516 nm). Results are expressed as Relative Fluorescence Units. Fluorescence was given the arbitrary value of 1 in untreated cells, which was used as the basis of all comparisons.

IP₃ Assay—IP₃ concentration was measured in ³H-myoinositol-labeled cells as described by Swift *et al.* (55). Briefly, cells were plated into 12-well plates at 125,000 cells/well. ³H-labeled myoinositol was added to the cultures 24 h prior to the assay (5 µCi/ml; Perkin Elmer). Cells were washed twice with 2 ml of Dulbecco's modified MEM containing 10 mM HEPES buffer pH 7.3 and twice with 2 ml of phosphate-buffered saline containing 20 mM LiCl. Cells were incubated in 20 mM LiCl for 15 min and then stimulated with agonist for 30 min (in triplicate) and extracts were acquired using cold methanol and chloroform. Extracts were loaded onto columns containing 1 ml of the anion exchange resin AG1X8 formate form, 100–200 mesh size (Bio-Rad), while a 200 µl sample was used to estimate the total radioactivity of the sample. After loading, the columns were washed twice with 10 ml of H₂O and twice with 10 ml of 60mM ammonium formate/5mM Borax. ³H-labeled IP₃ eluted with 4 ml of 2M ammonium formate/0.1M formic acid and the radioactivity of the eluted fractions was measured using a scintillation counter. The mean of 3–5 measurements in samples of stimulated cells was compared to the mean of 3–5 measurements in samples of unstimulated cells and the results were expressed as fold induction.

Subcellular fractionation—Subcellular fractionation of lysates of Rec-WT and Rec-EV cells, harvested before and after thrombin stimulation (1 U/ml), was carried out, using a Subcellular Protein Fractionation Kit (Pierce).

Transwell filter assay—Migration assays were performed as previously described (27) using 24-well microchemotaxis chambers (Costar) with uncoated polycarbonate membranes (pore size 8 µm). Briefly, cells were preincubated with the respective inhibitor, harvested and resuspended at 5x10⁴ cells/0.1 ml in DMEM containing 0.25% BSA. The bottom chamber of each transwell unit contained 0.6 ml of DMEM supplemented with 0.25% BSA and a chemically defined chemoattractant (thrombin or S1P). The plates were incubated for 4 h at 37° C and the filters were fixed with saline-buffered formalin and stained with 0.1% crystal violet. The cells that migrated through the filter were counted, using a grid and an Optech microscope at a 20x magnification.

Statistical analysis—The significance of variability between a given group and its control was determined using the unpaired t-test. All results were obtained from at least three independent experiments, unless otherwise stated, and were expressed as mean ± SE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Tpl2 transduces PAR1-Gai2-mediated activation of MAPK signaling

A. G α_{12} promotes phosphorylation of ERK through Tpl2. ERK phosphorylation in Rec-WT and Rec-EV cells transfected with constitutively active mutants of the indicated G proteins.

B. PTX inhibits MAPK activation by thrombin.

C. G α_{12} is required for ERK and JNK phosphorylation by thrombin, while G α_{13} is not. (Upper) Knockdown efficiencies of the indicated siRNAs in Rec-WT cells. (Middle) ERK and JNK phosphorylation in Rec-WT cells transfected with the indicated siRNAs and stimulated with thrombin (Lower) In vitro kinase assay of Tpl2 immunoprecipitated from Rec-WT cells transfected with the indicated siRNAs and stimulated with thrombin.

D. ERK activation by thrombin is G α_{12} -dependent and G α_{11} -, G α_{13} -independent. (Upper) Abundance of the mRNA encoding G α_i isoforms presented as fold difference (mean $\pm$ SE) relative to that of G α_{11} in Rec-EV cells. (Lower) ERK phosphorylation in Rec-WT cells transfected with the indicated siRNAs and stimulated with thrombin.

E. Tpl2-G α_{i2} interaction. (*Upper*) HEK 293T cells were co-transfected with HA-tagged Tpl2 and EE-tagged G α_{i2} constructs. Tpl2 immunoprecipitates were probed with an anti-EE-tag antibody. UT: untransfected cells. (*Lower*) HA-Tpl2 immunoprecipitates from Rec-WT and Rec-EV cells were probed for the endogenous G α_{i2} .

Fig. 2. The thrombin-dependent increase in cytoplasmic Ca^{2+} concentration is Tpl2 -dependent
A. Ca^{2+} signals in $\text{Tpl2}^{+/+}$ or $\text{Tpl2}^{-/-}$ MEFs, and in Rec-WT, Rec-KD, or Rec-EV immortalized MEFs Cells loaded with Fluo-4 NW were stimulated with thrombin (Th) at the time indicated by the arrow. **B.** Ca^{2+} signals in $\text{Tpl2}^{+/+}$ or $\text{Tpl2}^{-/-}$ MEFs pretreated with BAPTA-AM and stimulated with thrombin. **C.** Cytoplasmic Ca^{2+} signals in $\text{Tpl2}^{+/+}$ or $\text{Tpl2}^{-/-}$ MEFs and in Rec-WT and Rec-KD cells stimulated with TFLLRN-NH₂. Cytoplasmic Ca^{2+} was monitored fluorimetrically for the indicated time period. Results (mean $\pm$ SE) from three experiments with measurements in each done in triplicate.

Fig. 3. Tpl2-mediated GPCR signals stimulate IP_3 production, Ca^{2+} signals, and PKC activation

A. Tpl2-dependent increase in cytoplasmic Ca^{2+} in response to thrombin is due to IP_3 -mediated Ca^{2+} signals. (*Upper*) Fluo-4-NW-loaded and EGTA-pretreated Rec-WT, Rec-KD and Rec-EV cells and *Tpl2*^{+/+} and *Tpl2*^{-/-} MEFs were treated with thrombin. (*Middle and Lower*) Fluo-4-NW-loaded Rec-WT cells were pretreated with 2-APB or Xestostongin C (Xesto.C) before exposure to thrombin. Results (mean±SE) from three experiments with measurement in each done in duplicate.

B. IP_3 concentration in thrombin-stimulated cells depends on Tpl2 and $G\alpha_{i2}$. IP_3 concentration (mean± SE) in ³H-myoinositol-labeled Rec-WT and Rec-EV cells stimulated with thrombin (Left panel) and Rec-WT cells transfected with si $G\alpha_{i2}$ or siControl and stimulated with thrombin (Right panel). #: indicate a statistically significant difference

between thrombin-treated Rec-WT and Rec-EV cells ($^{##}p<0.01$) or thrombin-treated cells transfected with siControl and siG α_{i2} ($^{\#}p<0.05$).

C and D. Phosphorylation and activation of classical and novel PKCs by thrombin depends on Tpl2. C. Phosphorylation of the indicated PKC isoforms in Rec-WT or Rec-EV cells, after thrombin stimulation. D. Cytoplasmic and membrane protein extracts were collected from thrombin-treated cells and they were analyzed for PKC δ . EGFR and tubulin were used as the loading controls.

Fig.4. Activation of PLCβ₃ by thrombin depends on Tpl2 and is required for Ca²⁺ influx signals
A. Thrombin-induced PLCβ₃ phosphorylation at Ser⁵³⁷ depends on Tpl2.

B. Tpl2 directly phosphorylates PLCβ₃ at Ser⁵³⁷. (*Upper*) PLCβ₃ phosphorylation in cells pretreated with KN-93 and stimulated with thrombin. (*Middle*) PLCβ₃ phosphorylation in HEK293T cells transfected with WT-Tpl2 (WT) or the empty vector (EV) and treated with KN-93. (*Lower*) In vitro kinase assay of Tpl2 immunoprecipitated from thrombin-stimulated Rec-WT cells.

C. PLCβ₃ is required for IP₃ production and Ca²⁺ signals in thrombin-stimulated cells. (*Upper Left*) Knockdown efficiency of siPLCβ₃ in Rec-WT cells. (*Upper Right*) IP₃ concentration (mean ± SE) in Rec-WT cells transfected with siPLCβ₃ or siControl and stimulated with thrombin. #: indicate a statistically significant difference between thrombin-treated cells transfected with siControl and siPLCβ₃ (##p<0.01). (*Lower*) Cytoplasmic Ca²⁺ signals (mean± SE) in Rec-WT cells transfected with siPLCβ₃ or siControl and treated with thrombin.

D. Phosphorylation of PLCβ₃ at Ser⁵³⁷ is required for the full functional activation of PLCβ₃ in response to thrombin. (*Upper Left*) Spontaneously immortalized PLCβ₂^{-/-}/PLCβ₃^{-/-} lung fibroblasts were transduced with the retroviral constructs pBabe-puro/PLCβ₃, pBabe-puro/PLCβ₃S537A, or pBabe-puro (EV). Western blot of PLCβ₃. (*Upper*

Right) IP₃ concentration (mean $\pm$ SE) after thrombin stimulation. (*Lower*) Cytoplasmic Ca²⁺ signals (mean $\pm$ SE) in cells treated with thrombin.

Fig. 5. Tpl2-transduced migratory signals initiated by thrombin depend on $G\alpha_{i2}$, PLC β_3 , ERK, and PKC activation
 (A) Migration of *Tpl2*^{+/+} and *Tpl2*^{-/-} primary MEFs in response to thrombin. (B and C) Migration of MDA-MB231 and SCO3 human cancer cell lines transfected with siTpl2 or siControl and stimulated with thrombin. (D and E) Migration of thrombin-stimulated Rec-WT and Rec-EV fibroblasts transfected with siGα_{i2}, siPLCβ₃, or the corresponding control siRNAs. (F–J) Migration of thrombin-stimulated Rec-WT and Rec-EV fibroblasts, preincubated with the indicated inhibitors (2-APB, KN-93, U0126, GF109203X, or Go6976) for 30 min. Results are expressed as mean number of migrating cells ± SE and were calculated by combining the data from three experiments with duplicate measurements in each. # indicate a statistically significant difference between cells treated with the indicated inhibitor or siRNA and thrombin and cells treated with thrombin only (#p<0.05). (K) Thrombin-induced phosphorylation of FAK at Tyr³⁹⁷, in PLCβ₂^{-/-}/PLCβ₃^{-/-} lung fibroblasts transduced with pBabe-puro-based retroviral constructs of wild-type PLCβ₃, PLCβ₃S537A, or empty vector (EV).

Fig. 6.
Schematic representation of the proposed model.

Fig. 7. Tpl2 transduces S1P-Gα_{i2} signals that target PLCβ₃, elicit Ca²⁺ signals, and promote cell migration

A. S1P-induced increase of cytoplasmic Ca²⁺ in MEFs and BMDMs is Tpl2-dependent. Changes in cytoplasmic Ca²⁺ in Fluo-4-NW-loaded *Tpl2*^{+/+} and *Tpl2*^{-/-} immortalized MEFs and BMDMs, stimulated with S1P. Combined results (mean fluorescence ± SE) from two experiments with measurements in each done in triplicate.

B. S1P-induced increase in cytoplasmic Ca²⁺ in MEFs is Gα_{i2}- and PLCβ₃-dependent. (*Upper*) IP₃ concentration (mean ± SE) in Rec-WT cells transfected with siGα_{i2} or siControl (Left) or siPLCβ₃ or siControl (Right) and stimulated with S1P. #: indicate a statistically significant difference between S1P-treated cells transfected with siGα_{i2}/siPLCβ₃ and siControl (#p<0.05). Combined data from three experiments. (*Lower*) Changes in Ca²⁺ signals in Fluo-4-NW-loaded Rec-WT cells transfected with siPLCβ₃ or siControl and

stimulated with S1P. Combined results (mean fluorescence $\pm$ SE) from three experiments with measurements in each done in duplicate.

C. Tpl2-transduced migratory signals initiated by S1P, depend on $G\alpha_{i2}$ and $PLC\beta_3$ -mediated IP3 signals. Migration (mean $\pm$ SE) of Rec-WT and Rec-EV fibroblasts in response to S1P. Cells were pretreated as indicated. Combined data from three experiments with duplicate measurements in each ($^{\#}p<0.05$).