

Supplementary Materials for

Tumor Progression Locus 2 Mediates Signal-Induced Increases in Cytoplasmic Calcium and Cell Migration

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SUPPLEMENTARY MATERIALS

Table S1. Statistical differences (p values) between samples 25 s after treatment with thrombin, using the unpaired t test (data presented in Figure 2A). The measurements from $Tpl2^{-/-}$ cells were used as the basis of all comparisons.

	<i>Tpl2</i> ^{-/-}	<i>Tpl2</i> ^{+/+}	Rec-WT	Rec-KD	Rec-EV
<i>Tpl2</i> ^{-/-}	-	0.0046	0.0125	N.S.	N.S.
<i>Tpl2</i> ^{+/+}		-	N.S.	0.0018	0.0087
Rec-WT			-	0.0051	0.03
Rec-KD				-	N.S.
Rec-EV					-

N.S., Non Significant.

Table S2. Statistical differences (p values) between samples 25 s after treatment with PAR1 agonist, using the unpaired t test (data presented in Figure 2C). The measurements from $Tpl2^{-/-}$ cells were used as the basis of all comparisons.

	<i>Tpl2</i> ^{-/-}	<i>Tpl2</i> ^{+/+}	Rec-WT	Rec-KD
<i>Tpl2</i> ^{-/-}	-	0.0031	0.0023	N.S.
<i>Tpl2</i> ^{+/+}		-	N.S.	0.0006
Rec-WT			-	0.0004
Rec-KD				-

N.S., Non Significant.

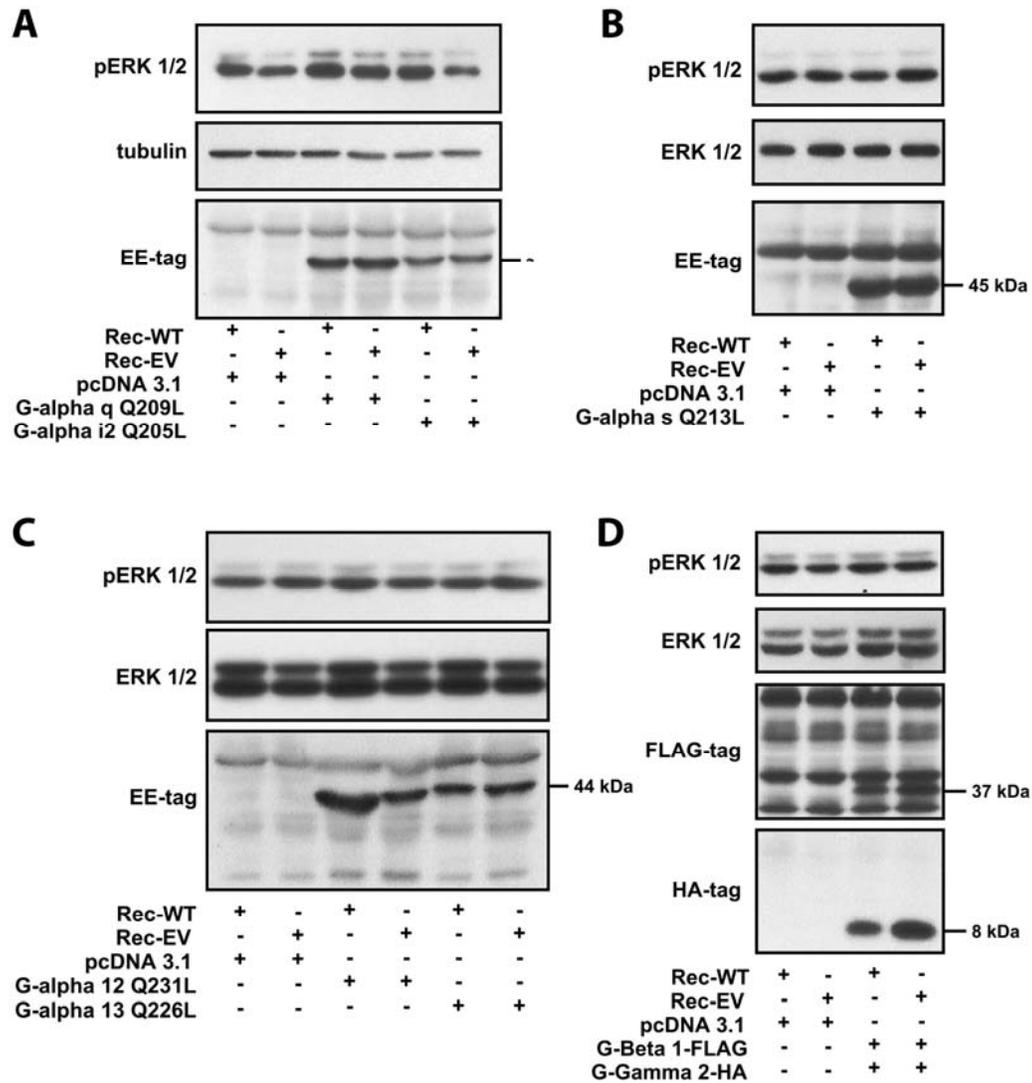


Figure S1. $G\alpha_{i2}$ promotes phosphorylation of ERK1/2 through Tpl2. ERK phosphorylation in Rec-WT and Rec-EV cells transfected with constitutively active mutants of the indicated G proteins or the empty vector (pcDNA 3.1). Representative blot from one of three independent experiments.

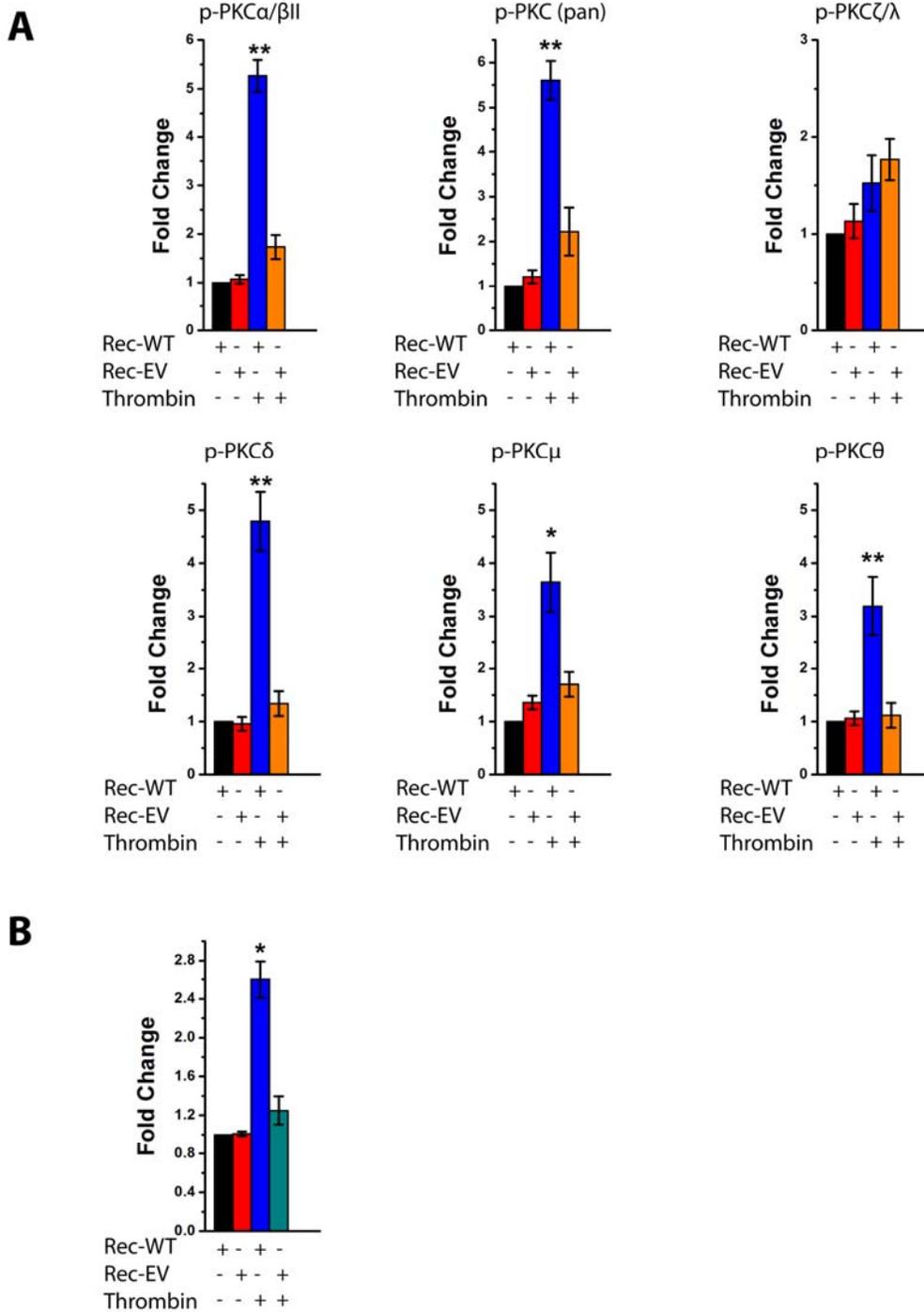


Figure S2. Phosphorylation and activation of classical and novel PKCs by thrombin depends on Tpl2. **A.** Phosphorylation of the indicated PKC isoforms in Rec-WT or Rec-EV cells, after thrombin stimulation. The amounts of phosphorylated PKC isoforms were

quantitated by densitometric analyses and results were expressed as mean fold induction of phosphorylation, calculated from the combined results of three independent experiments (* $p < 0.05$, ** $p < 0.01$). The phosphorylation of all tested PKC isoforms was given the arbitrary value of 1 in untreated Rec-WT cells, which were used as the basis of all comparisons. **B.** Cytoplasmic and membrane protein extracts were collected from thrombin-treated cells and they were analyzed for PKC δ . The amounts of PKC δ were quantitated and results were expressed as mean fold induction, calculated from the combined results of three independent experiments (* $p < 0.05$). The amounts of PKC δ isoform were given the arbitrary value of 1 in untreated Rec-WT cells.

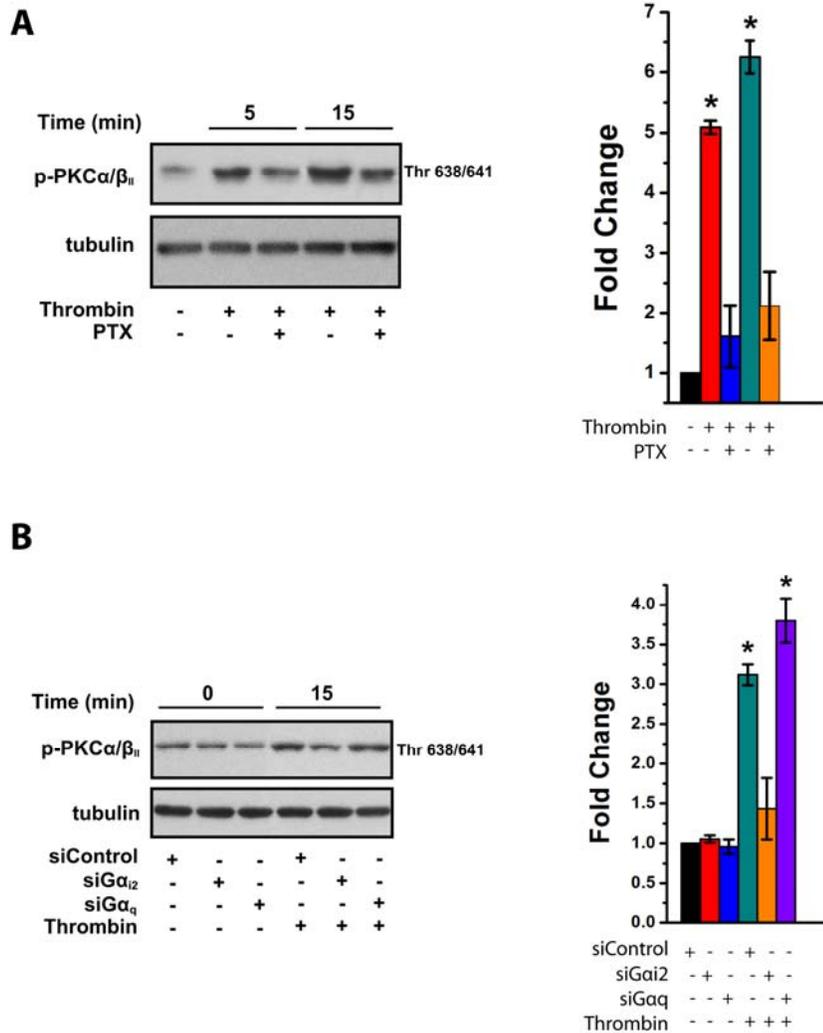


Figure S3. The phosphorylation of classical PKCs after thrombin stimulation depends on $G\alpha_{i2}$. **A.** Rec-WT cells incubated with pertussis toxin (PTX) (100 ng/ml) for 24 hours were stimulated with thrombin (1U/ml). Western blots of cell lysates harvested at the indicated time points after thrombin stimulation, were probed with an antibody against PKC α and PKC β_{II} phosphorylated at Ser638/641. Tubulin was used as the loading control. The amounts of phosphorylated PKC isoforms were quantitated by densitometric analyses and results were expressed as mean fold induction of

phosphorylation, calculated from the combined results of two independent experiments (* $p < 0.05$). The phosphorylation of PKC α/β_{II} was given the arbitrary value of 1 in untreated cells and was used as the basis in all comparisons. **B.** Rec-WT cells were transfected with G α_{i2} siRNA (50 nM), G α_q siRNA (50 nM), or control siRNA (siControl) (50 nM). Knockdown efficiency for siG α_{i2} #s66790 (Ambion) and siG α_q #sc-35430 (Santa Cruz Biotechnology) was estimated at ~80 and 75%, respectively. After 36 hours cells were treated with thrombin for 15 min and total cell lysates were analyzed by Western blotting. Western blots were probed with an antibody against the phosphorylated forms of PKC α and PKC β_{II} . Tubulin was used as the loading control. The amounts of phosphorylated PKC isoforms were quantitated by densitometric analyses and results were expressed as mean fold induction of phosphorylation, calculated from the combined results of two independent experiments (* $p < 0.05$). The phosphorylation of PKC α/β_{II} was given the arbitrary value of 1 in cells transfected with the siControl and was used as the basis in all comparisons.

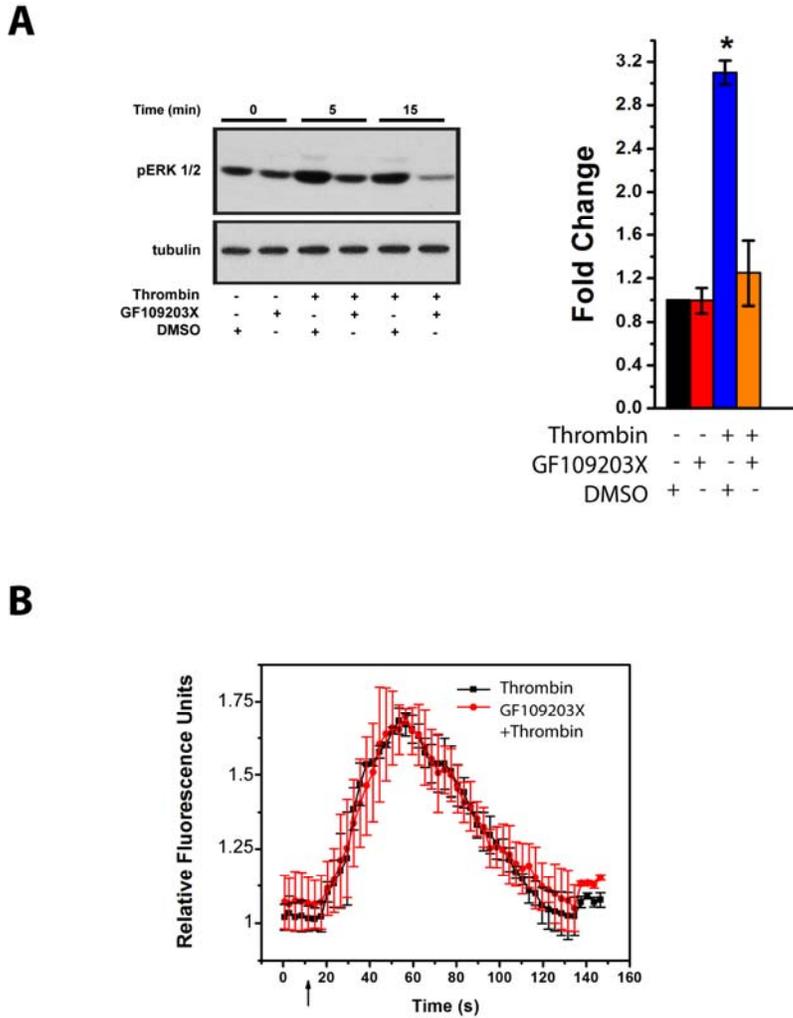


Figure S4. PKC inhibition attenuates thrombin-induced ERK1/2 phosphorylation but not Ca^{2+} signals. **A.** Rec-WT cells were incubated with the PKC inhibitor GF109203X (10 nM) for 30 min and Western blots of lysates harvested at the indicated time points after thrombin stimulation (1U/ml), were probed with an antibody against the phosphorylated forms of ERK1/2. Tubulin was used as the loading control. The amounts of phosphorylated ERK1/2 were quantitated by densitometric analyses and results were expressed as mean fold induction of phosphorylation, calculated from the combined

results of two independent experiments (* $p < 0.05$). The phosphorylation of ERK1/2 was given the arbitrary value of 1 in cells treated with the vehicle (DMSO) and was used as the basis in all comparisons. **B.** Rec-WT cells loaded with Fluo-4 NW, were pretreated with the PKC inhibitor GF109203X (10 nM). Pretreated cells were stimulated with thrombin (1U/ml). Changes in cytoplasmic Ca^{2+} were measured as described in the Materials and Methods. Results are expressed as mean fluorescence \pm SEM and were calculated by combining the results of three independent experiments. In each experiment, duplicate measurements were made per condition tested.

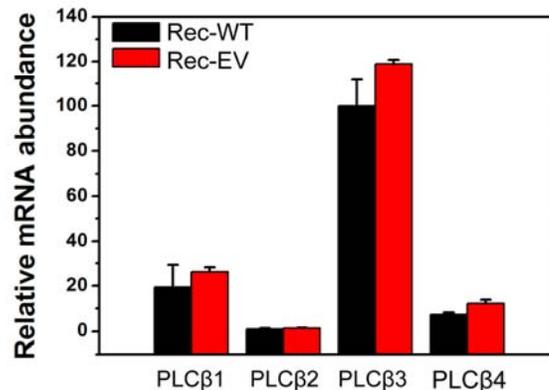


Figure S5. Expression profile of PLC β isoforms in Rec-WT and Rec-EV cells. mRNA abundance was measured by quantitative real time RT-PCR. The expression of each PLC β isoform in Rec-WT and Rec-EV cells was given as the fold difference (mean difference \pm SE of the mean) between the abundance of the mRNA encoding this isoform

and that of PLC β_2 in Rec-EV cells. The results represent two independent experiments. In each experiment, we made triplicate measurements per PLC isoform in each cell type.

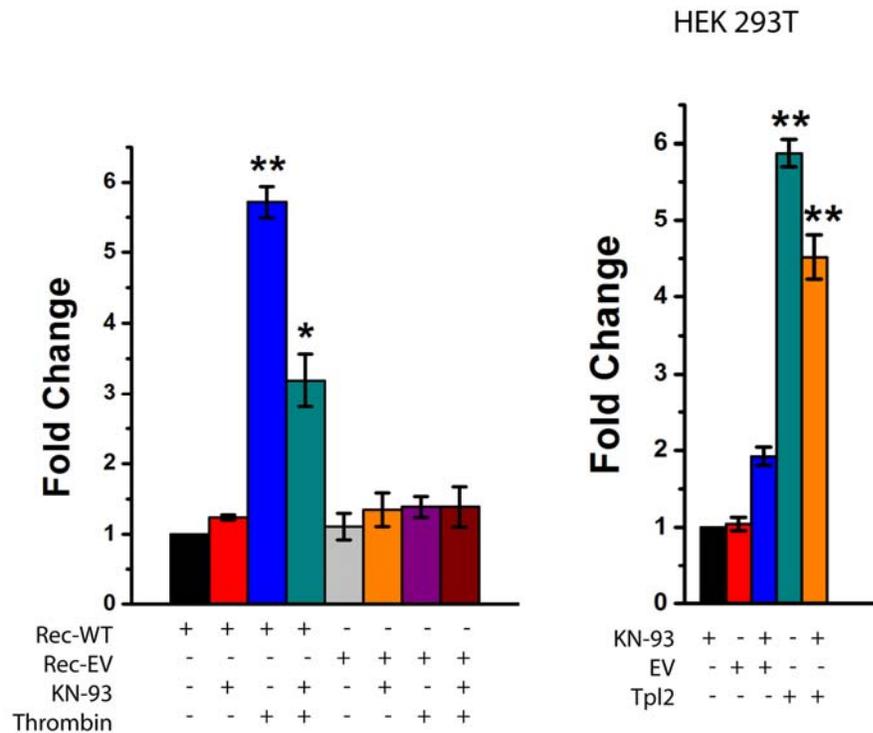


Figure S6. Thrombin-induced PLC β_3 phosphorylation at Ser⁵³⁷ depends on Tpl2. *(Left)* PLC β_3 phosphorylation in Rec-WT and Rec-EV cells pretreated with KN-93 and stimulated with thrombin. *(Right)* PLC β_3 phosphorylation in HEK293T cells transfected with WT-Tpl2 (WT) or the empty vector (EV) and treated with KN-93. The amounts of phosphorylated PLC β_3 at Ser⁵³⁷ were quantitated by densitometric analyses and results were expressed as mean fold induction of phosphorylation, calculated from the combined results of three independent experiments (* $p < 0.05$, ** $p < 0.01$). The phosphorylation

of PLC β_3 at Ser⁵³⁷ was given the arbitrary value of 1 in untreated Rec-WT cells and was used as the basis in all comparisons.

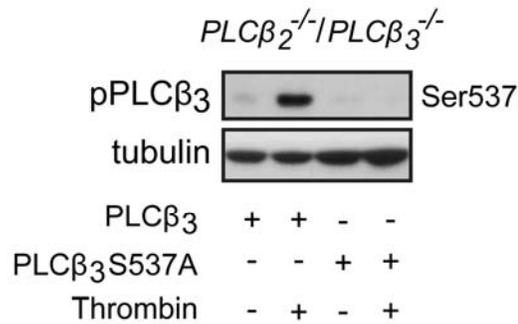
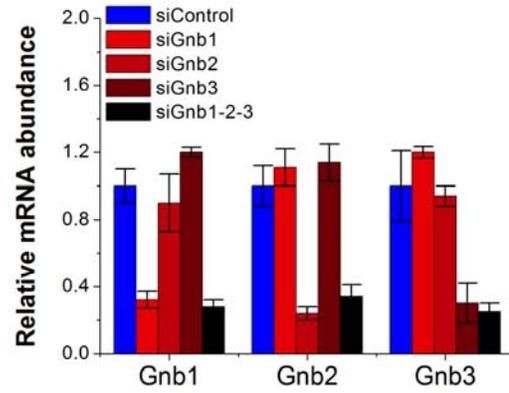
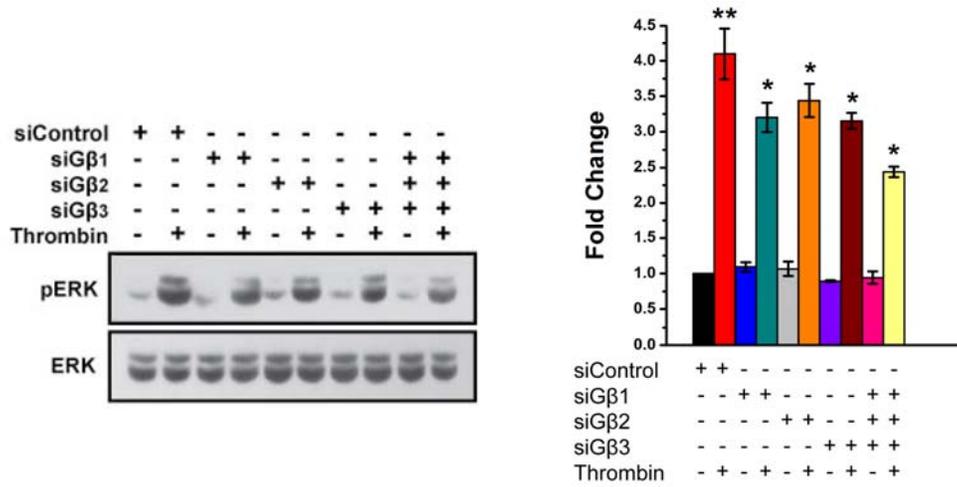


Figure S7. Specificity of the antibody that recognizes PLC β_3 phosphorylated at Ser⁵³⁷. *PLC β_2 ^{-/-}/PLC β_3 ^{-/-}* fibroblasts transduced with pBabe-puro-based retroviral constructs of wild type PLC β_3 , or PLC β_3 S537A, were stimulated with thrombin following serum starvation overnight. Cell lysates were harvested 2 min after stimulation and Western blot was probed with the PLC β_3 phospho-antibody recognizing PLC β_3 phosphorylated at Ser⁵³⁷.

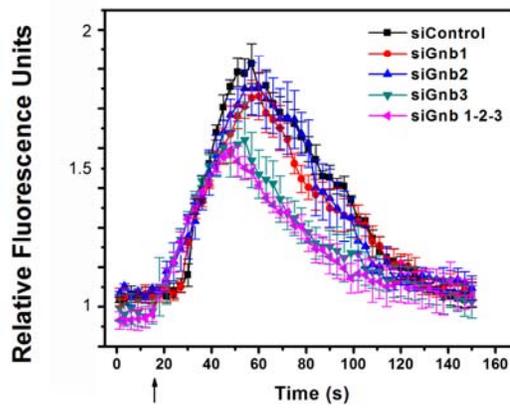
A



B



C



	siGnb1	siGnb2	siGnb3	siGnb1-2-3
siControl	N.S.	N.S.	N.S.	0.015

Figure S8. The knockdown of $G\beta_1$, $G\beta_2$, or $G\beta_3$, singly or in combination, interferes only partially with thrombin-induced ERK phosphorylation and Ca^{2+} signals. (A). Efficiency of $G\beta_1$, $G\beta_2$ and $G\beta_3$ knockdown in Rec-WT cells. For the knockdown of each gene tested, two different siRNAs were used: siGnb1 #s66815 and #s66814 (Ambion), siGnb2 #s66816 and #s66817 (Ambion) and siGnb3 #s66822 and #s66824 (Ambion). Knockdown efficiency for siGnb1 #s66815, siGnb2 #s66816, siGnb3 #s66822 was estimated at ~70%, 75% and 75% respectively. (B). Knocking down $G\beta_1$, $G\beta_2$, or $G\beta_3$, singly or in combination, partially inhibited the activation of ERK by thrombin. The amounts of phosphorylated ERK1/2 were quantitated by densitometric analyses and results were expressed as mean fold induction of phosphorylation, calculated from the combined results of two independent experiments (* $p < 0.05$, ** $p < 0.01$). The phosphorylation of ERK1/2 was given the arbitrary value of 1 in cells transfected with the siControl and was used as the basis in all comparisons. (C). Knocking down of $G\beta_1$, $G\beta_2$, and $G\beta_3$, singly or in combination, partially inhibited thrombin-induced Ca^{2+} signals. Statistical differences between samples 30 sec after thrombin stimulation. Measurements in cells transfected with the siControl were used as the basis in all comparisons. N.S: Non significant.

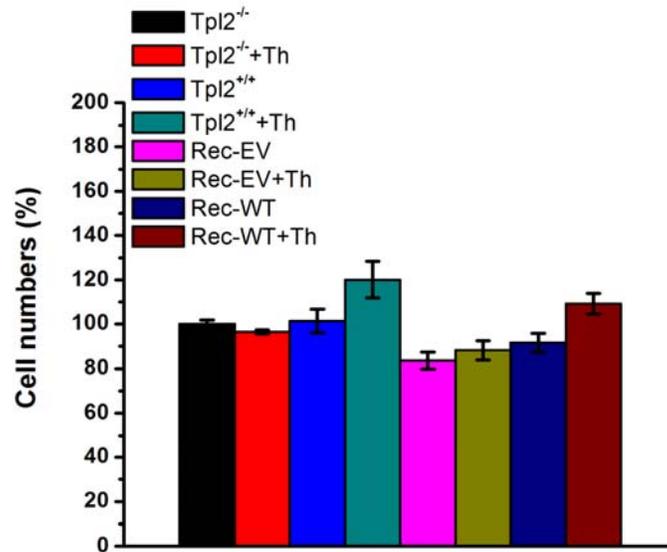


Figure S9. Number of cells before and after thrombin stimulation. Cell number was evaluated 48h after plating (with or without thrombin).

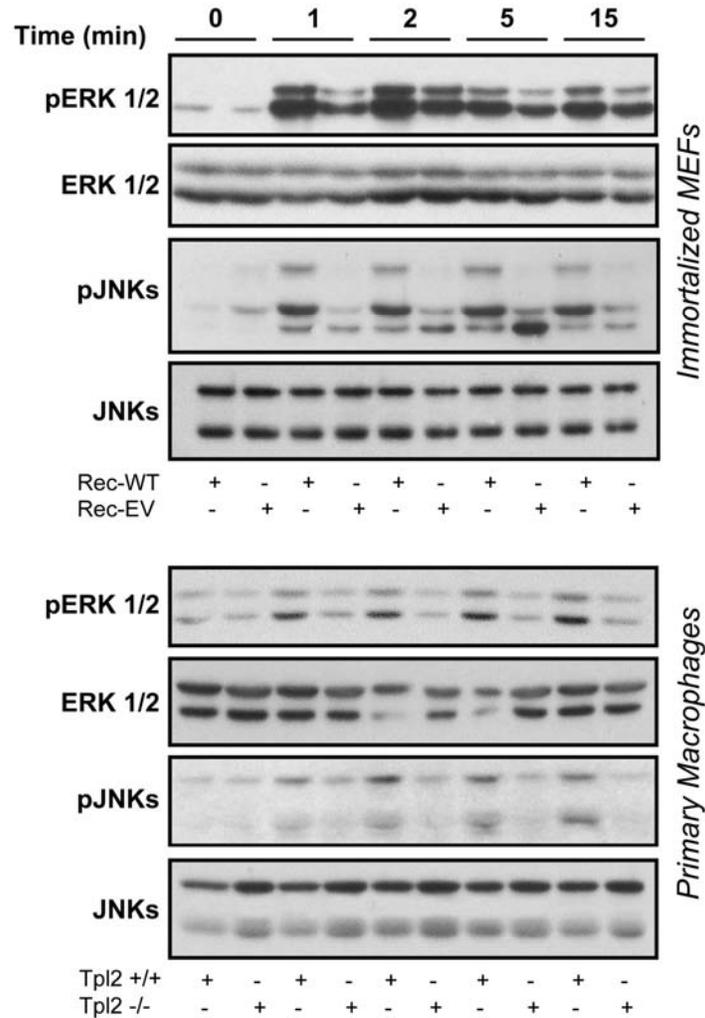


Figure S10. ERK and JNK phosphorylation by S1P in immortalized MEFs and BMDMs is Tpl2-dependent. Rec-WT and Rec-EV fibroblasts, or *Tpl2*^{+/+} and *Tpl2*^{-/-} macrophages were stimulated with S1P (2 μ M). Western blots of cell lysates harvested at the indicated time points were probed with antibodies against total ERK1/2 and JNK or their phosphorylated forms. Representative blot from one of two independent experiments.

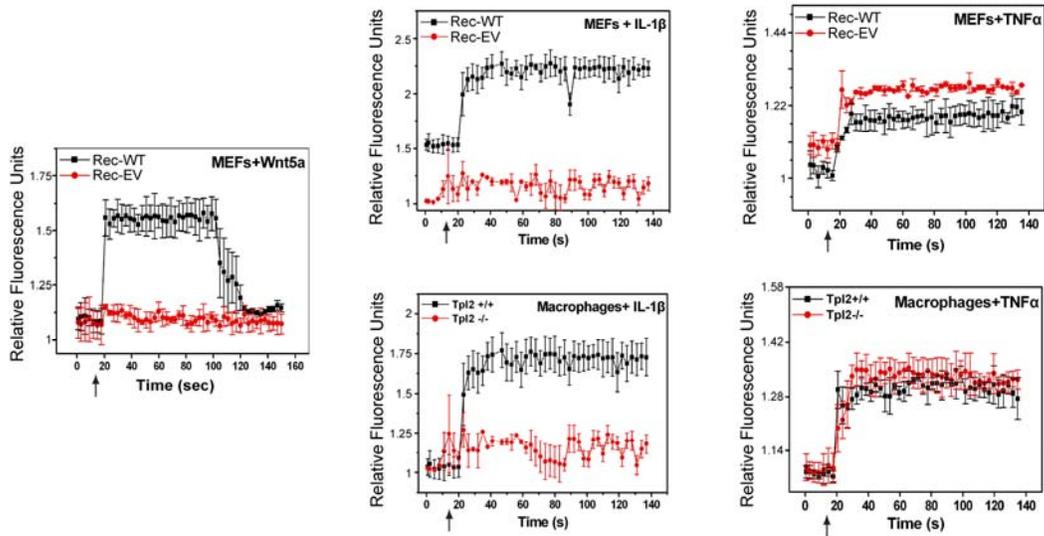


Figure S11. The increase in cytoplasmic Ca^{2+} , induced by Wnt5a and IL-1 β , but not TNF α , depends on Tpl2. Rec-WT or Rec-EV-transduced *Tpl2*^{-/-} immortalized MEFs and *Tpl2*^{+/+} and *Tpl2*^{-/-} BMDMs were loaded with Fluo-4 NW and stimulated with the indicated agents: Wnt5a (200 ng/ml), IL-1 β (10 ng/ml) and TNF α (20 ng/ml). Changes in cytoplasmic Ca^{2+} were measured as described in the Materials and Methods. Results are expressed as mean fluorescence \pm SEM and were calculated by combining the results of three independent experiments. In each experiment, duplicate measurements were made per time point.