# Supplementary Material for

**Phospholipase C γ and Phosphoinositide 3-Kinase Link Cytokines to ERK Activation in Hematopoietic Cells With Normal and Oncogenic *Kras***

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**The PDF file includes:**

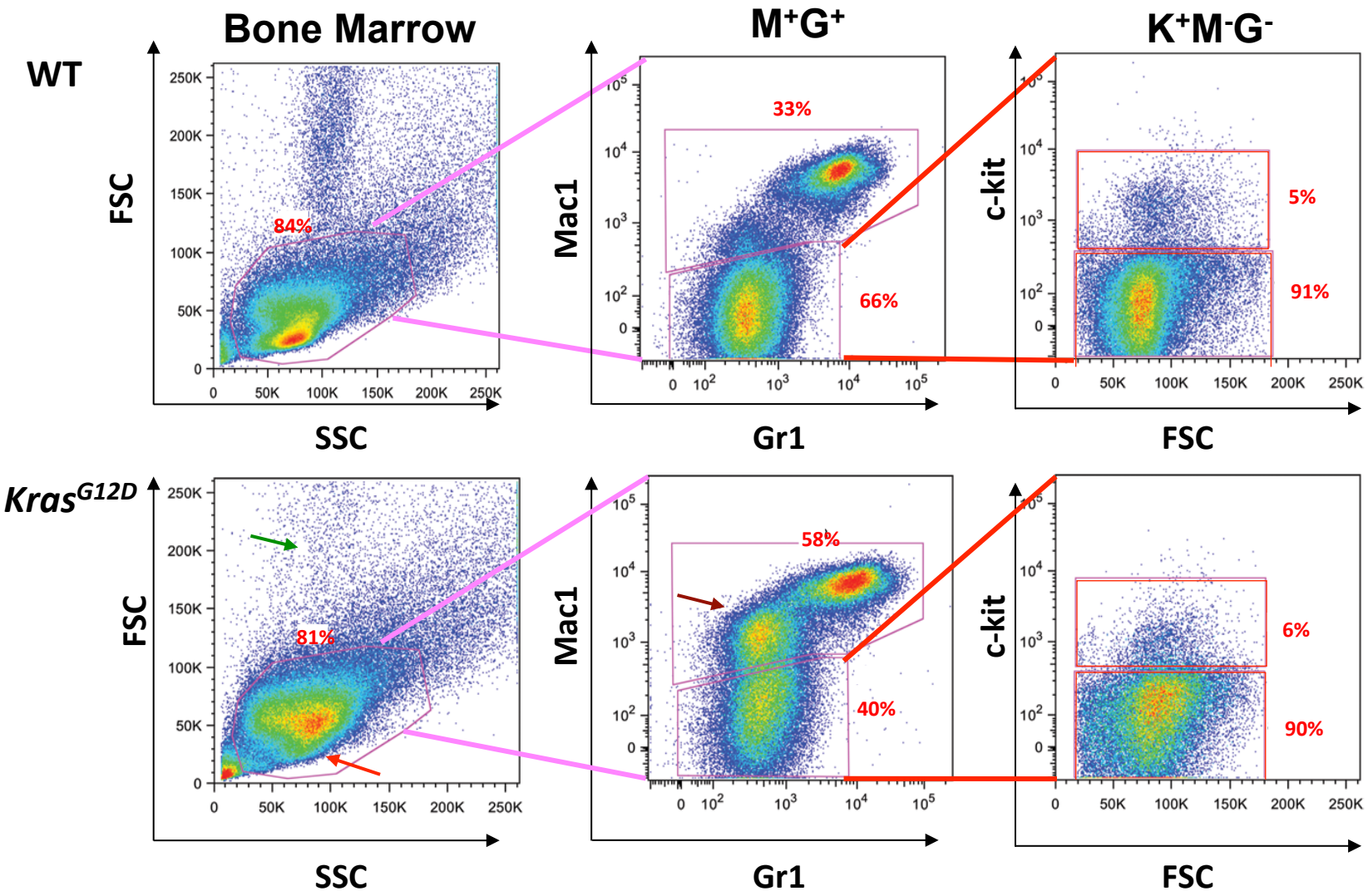



**Fig. S1.** Characterization of the $K^+M^-G^-$ and $M^+G^+$ cell populations. Live, non-neutrophil cells were first gated based on forward and side scatter (FSC and SSC) properties and then gating cells that expressed the c-kit lineage marker and were negative for the mature lineage markers Mac1 and Gr1 ($K^+M^-G^-$) or cells expressing Mac1 and Gr1 surface markers and negative for c-kit ($M^+G^+$). Note that neutrophils (green arrow) and lymphocytes (red arrow) are reduced in *Kras*$^{G12D}$ bone marrow while immature monocytic cells ($Mac1^+/Gr1^-$) are greatly increased (brown arrow). These plots are representative of multiple independent experiments. Percentages refer to the frequency of gated cells within each plot.

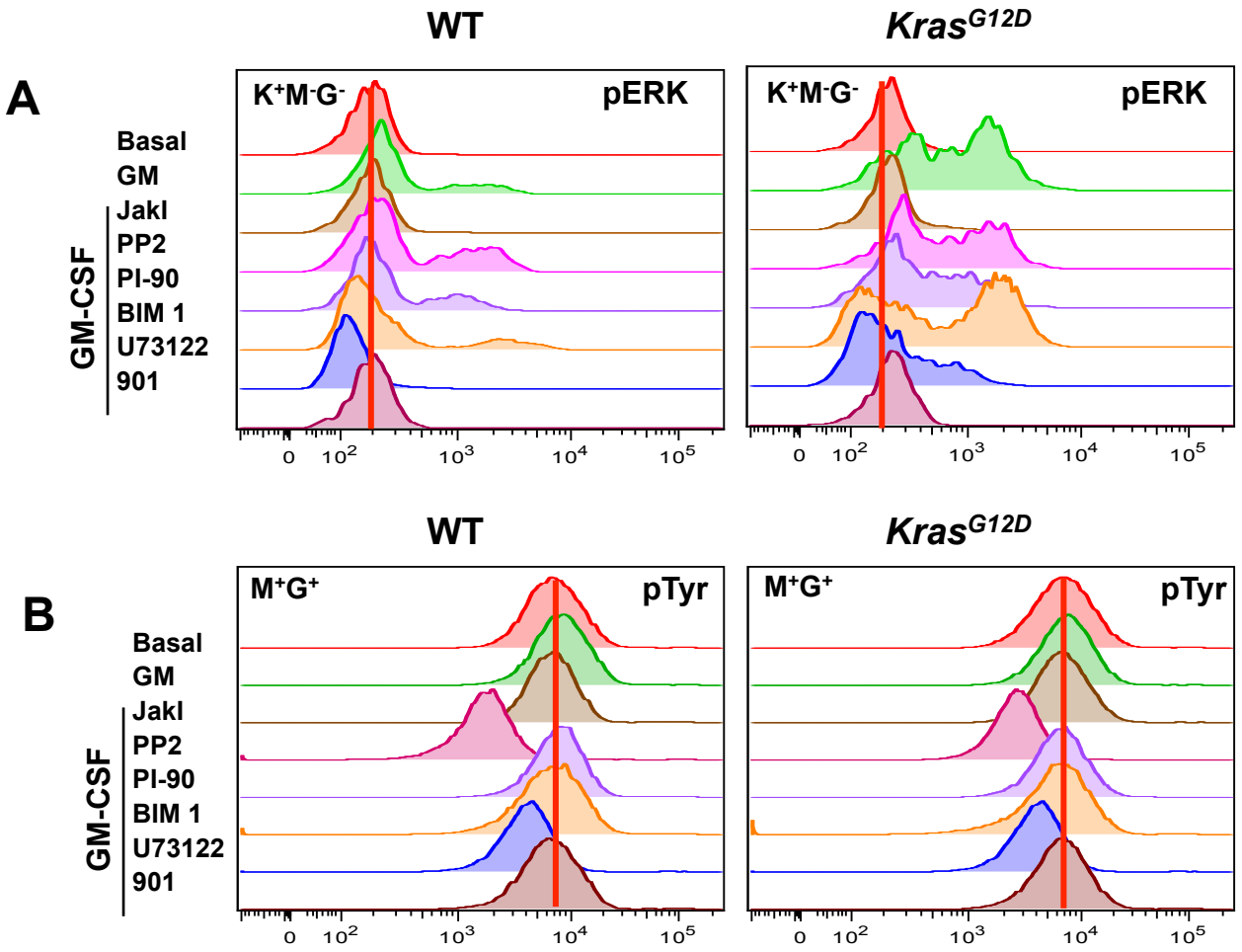



**Fig. S2.** Effects of various inhibitors on phosphorylated ERK (pERK) and phopshorylated tyrosine (pTyr) abundances in wild-type (WT) and *Mx1-Cre, Kras*$^{G12D}$ bone marrow cells. (A) pERK levels in $K^+M^-G^-$ cells stimulated with GM-CSF. (B) PP2 profoundly reduces tyrosine phosphorylation in WT and *Kras*$^{G12D}$ $M^+G^+$ cells stimulated with GM-CSF.

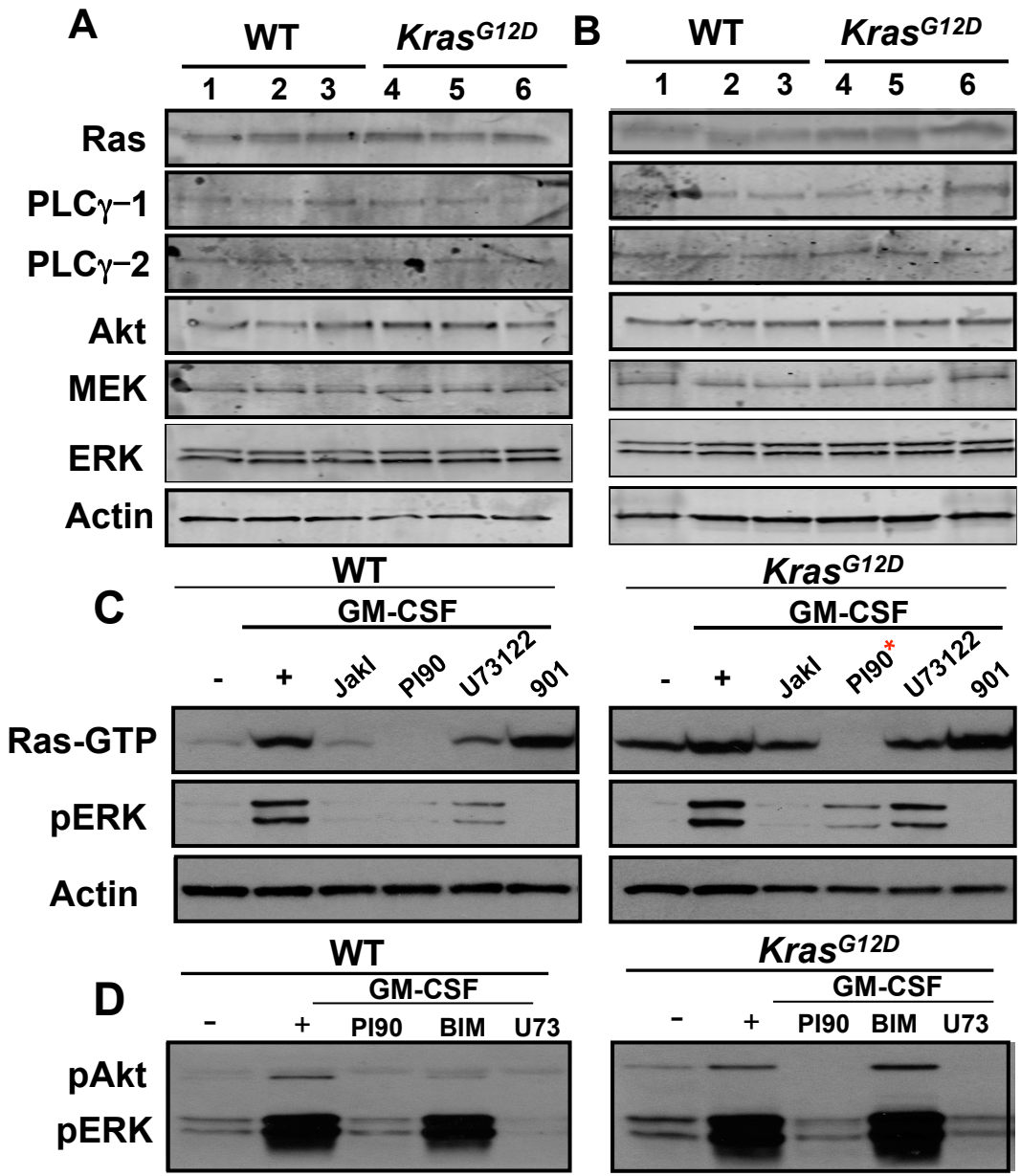



**Fig. S3.** Abundance of signaling molecules in hematopoietic cells. (A, B) Bone marrow cells (panel A) and bone marrow macrophage progenitor cells (panel B) were isolated from 3 independent wild-type (WT) and *Mx1-Cre, Kras*$^{G12D}$ mice and lysed. Western blotting was performed to measure the abundance of the indicated proteins. (C) Ras-GTP and pERK abundance were measured in bone marrow cells from mice of each genotype treated with the indicated inhibitors. The red asterisk (*) denotes absence of the indicated Ras-RBD pulldown specimen due to a loading error. (D) pAkt and pERK abundance was measured in bone marrow cells from mice treated with the indicated inhibitors.

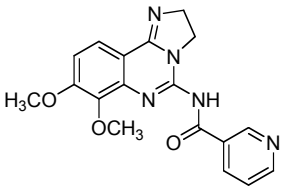
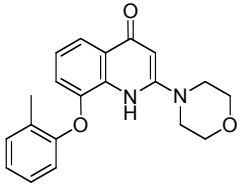
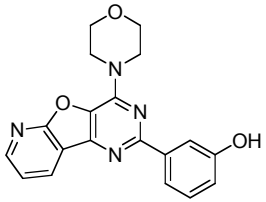
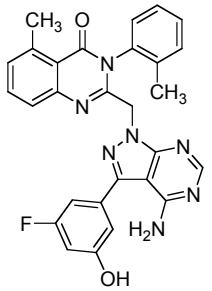
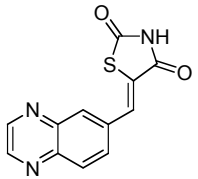
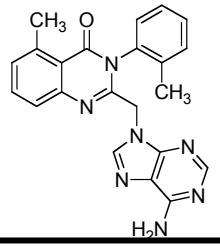
| Name | Structure | Name | Structure |
|---|---|---|---|
| PIK-90 (PI3K type I) |  | TGX-221(p 110β specific) |  |
| PI-103 (PI3K type I/ mTOR) |  | SW13 (p110δ specific) |  |
| AS605240(p 110γ/α specific) |  | IC87114(p 110δ specific) |  |

**Fig. S4. Names and structures of the PI3K inhibitors used in this study**.

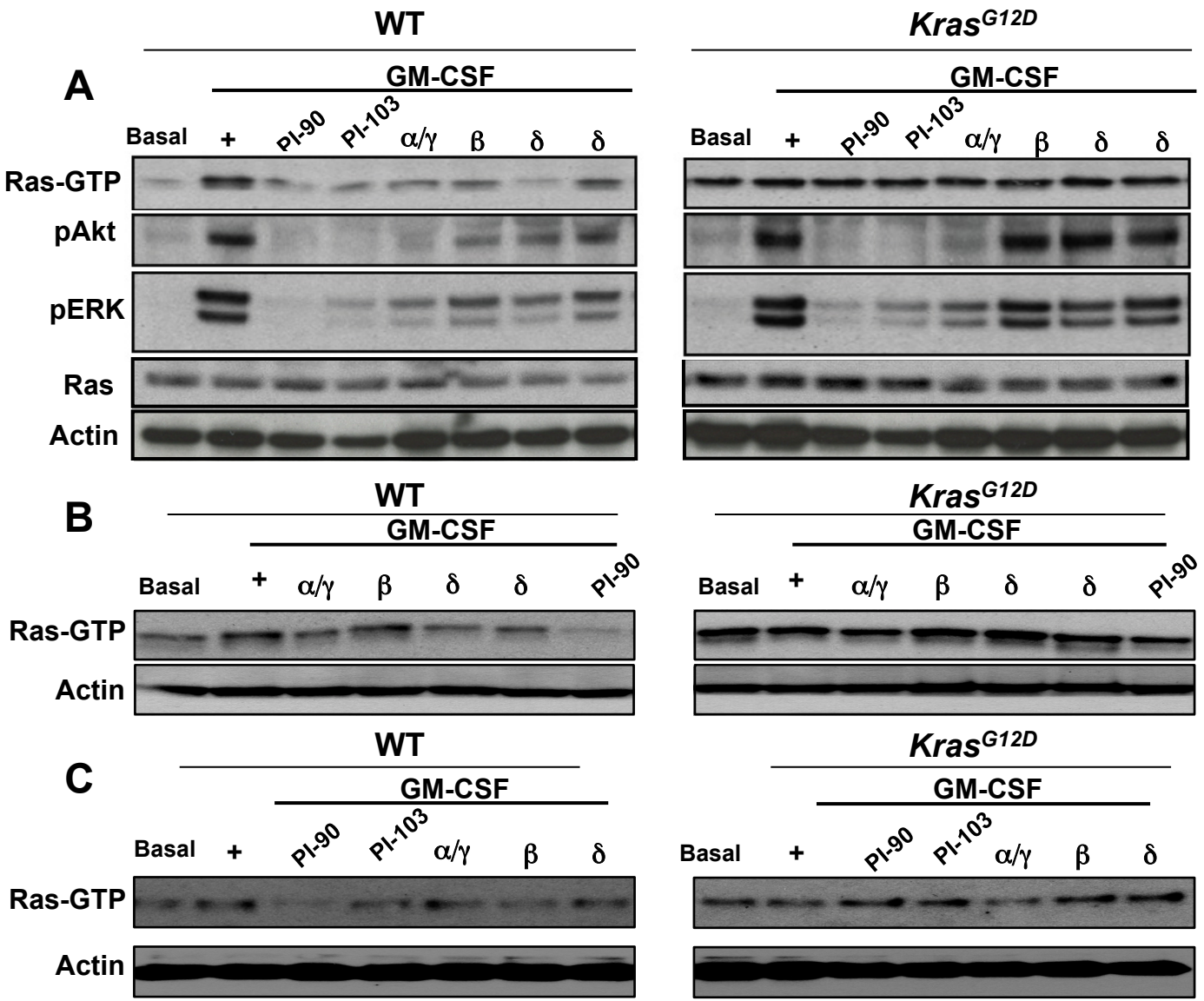



**Fig. S5.** Effects of PI3K inhibitors on Ras-GTP abundance and on ERK and Akt phosphorylation in bone marrow cells stimulated with GM-CSF. (A) Abundance of Ras-GTP, pERK, pAkt, and total Ras was measured in the presence of each of the PI3K inhibitors shown in fig. S4. Basal Ras-GTP abundance is elevated in bone marrow cells from *Mx1-Cre, Kras*$^{G12D}$ mice, but pERK abundance is low and increases after GM-CSF stimulation. PI-90, PI-103, and a p110$\alpha/\gamma$ selective inhibitor (AS 605240) reduced ERK phosphorylation in response to GM-CSF and abrogated pAkt induction. Ras and Actin are used as loading controls. Panels B and C show Ras-GTP abundance from independent experiments.

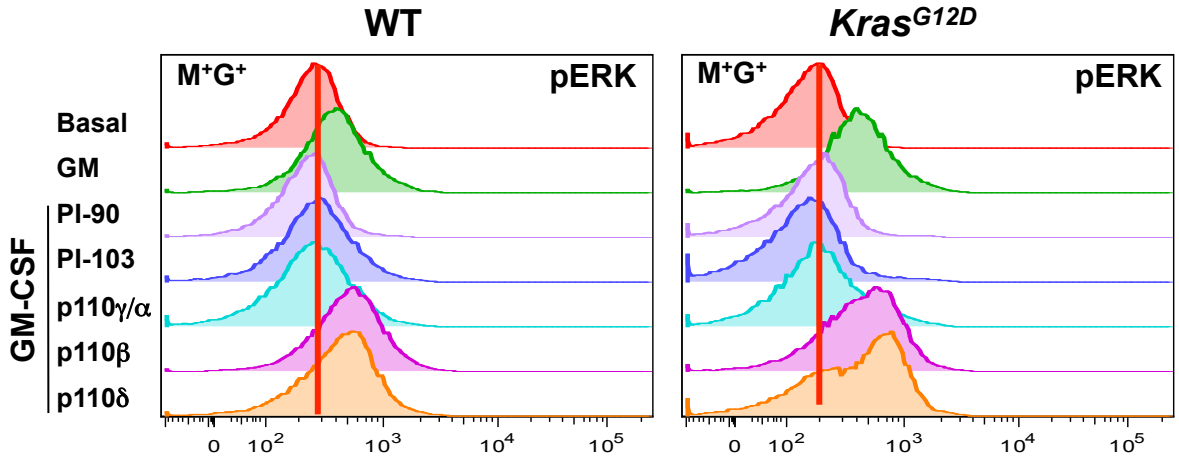



**Fig. S6.** Effects of PI3K inhibitors on pERK abundance in bone marrow M+G+ cells stimulated with GM-CSF. pERK abundance was measured in the presence of each of the PI3K inhibitors shown in Supplementary Figure 4. PI-90, PI-103 and a p110$\alpha/\gamma$ selective inhibitor (AS 605240) reduced ERK phosphorylation in response to GM-CSF.

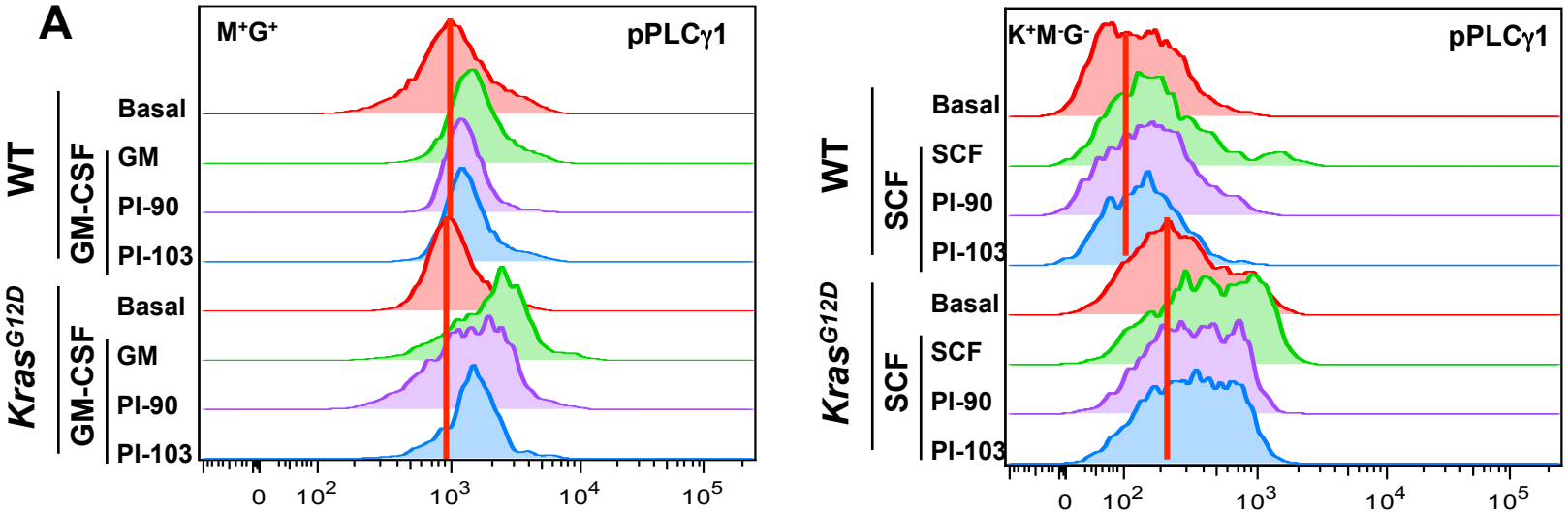



**Fig. S7.** Effects of PI3K inhibitors on PLC-γ1 phosphorylation. (A) M+G+ cells from WT and *Mx1-Cre, Kras*$^{G12D}$ mice were exposed to the PI3K inhibitors PI-90 and PI-103, then stimulated with GM-CSF. (B) Effects of PI-90 and PI-103 on PLC-γ1 phosphorylation in K+M-G- cells stimulated with SCF.

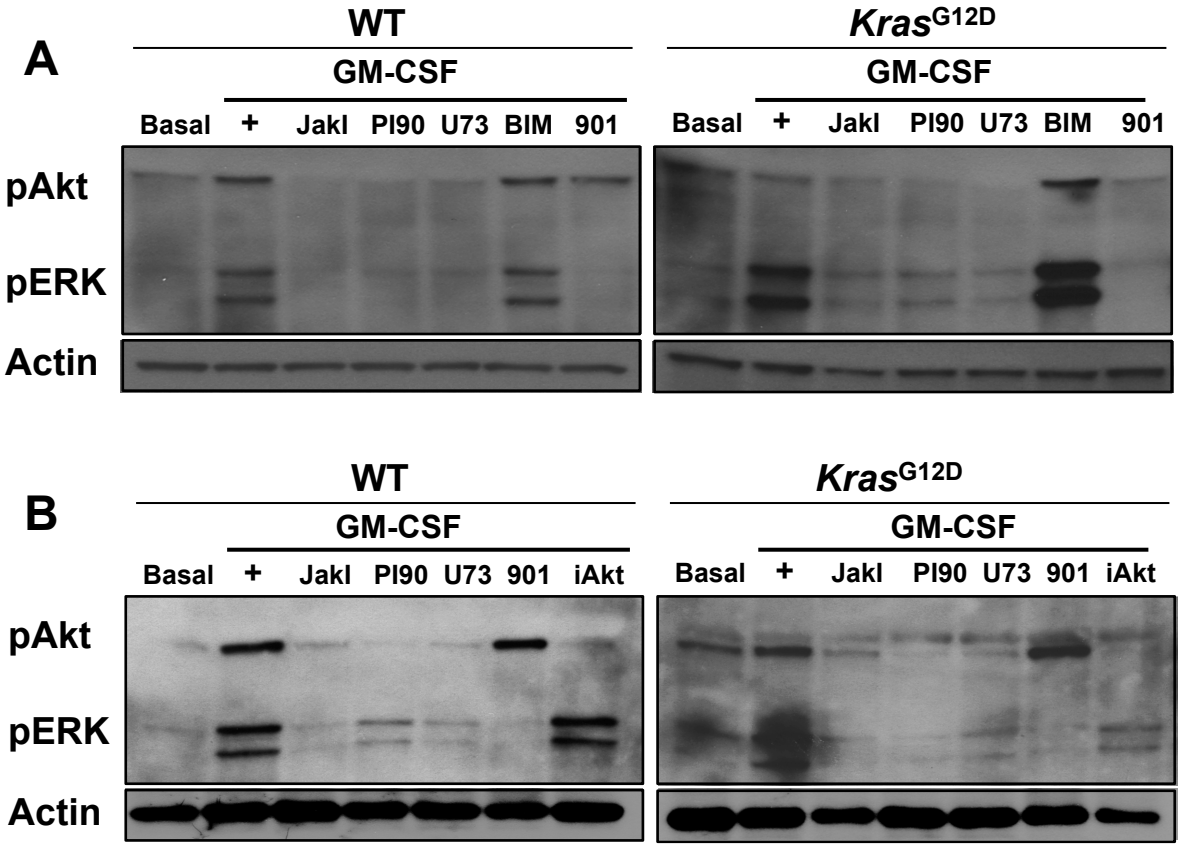



**Fig. S8.** JAK2, PLC-γ, and PI3K mediate GM-CSF-induced ERK phosphorylation in BMMPC. (A) WT and *Kras*$^{G12D}$ BMMPC that were incubated with 5 μM of JakI, PI-90, U73122, or PD0325901 were stimulated with GM-CSF, and pAkt and pERK abundance was measured by Western blot. Studies of JakI, PI-90, U73122, or PD0325901 were performed three times with panels A and B presenting data from independent experiments.

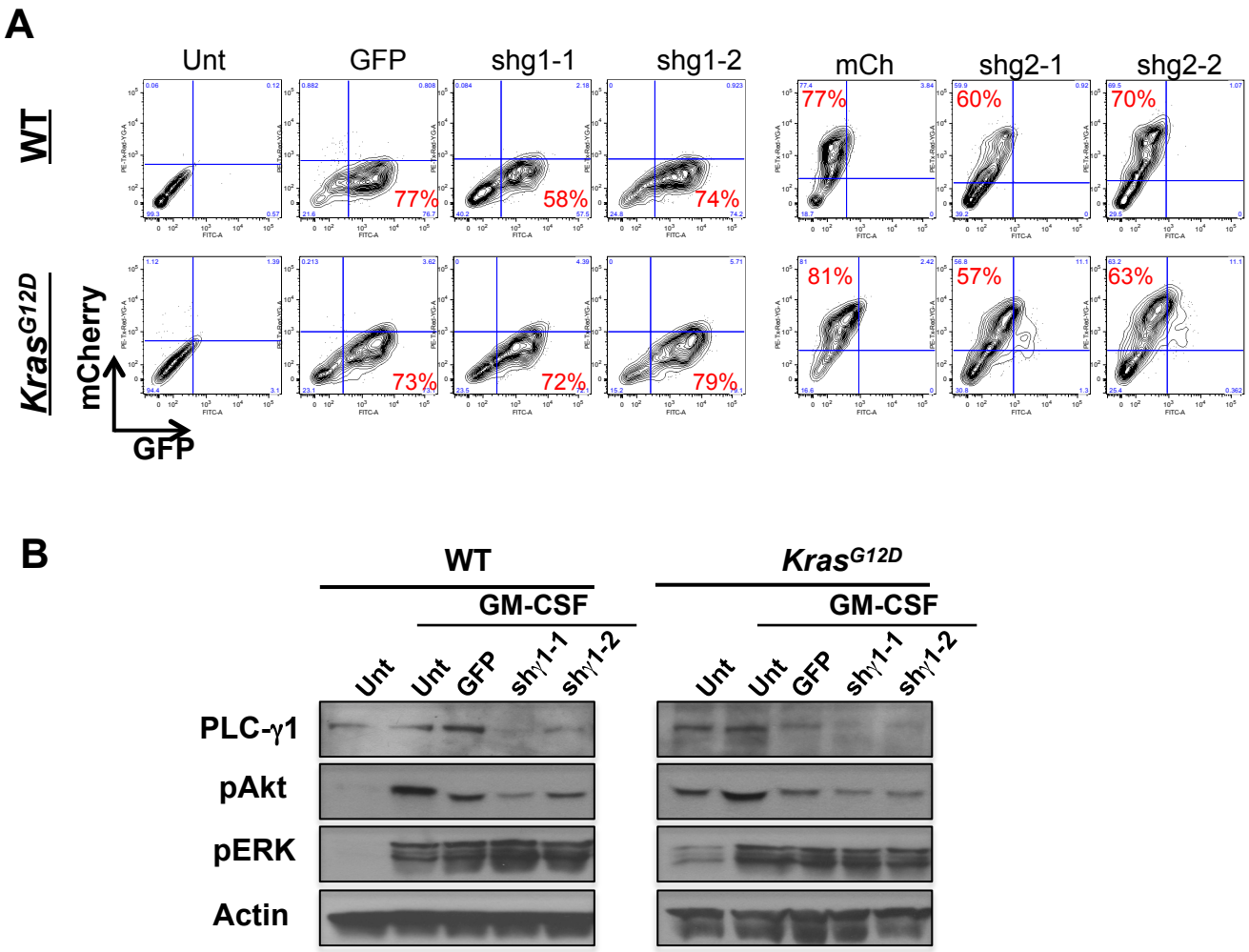



**Fig. S9.** Transduction efficiencies in BMMC and knock down with shRNA constructs. (A) WT and *Kras*$^{G12D}$ Bone marrow cells were retrovirally transduced with vectors containing a *neomycin* selectable marker expressing GFP only (control), GFP and a shRNA against PLC-γ1, or mCherry (mCh) and a shRNA against PLC-γ2. BMMPC were generated from bone marrow cells and G418 was used to select cells expressing the GFP or mCherry containing constructs, which were grown for an additional week. Expression of GFP or mCherry containing constructs was measured by flow cytometry. (B) Abundance of PLC-γ1 and pAkt, pERK and Actin was measured by Western blot in BMMPC expressing GFP empty vector or GFP and shRNA against PLCγ1 (shγ1-1, shγ1-2).

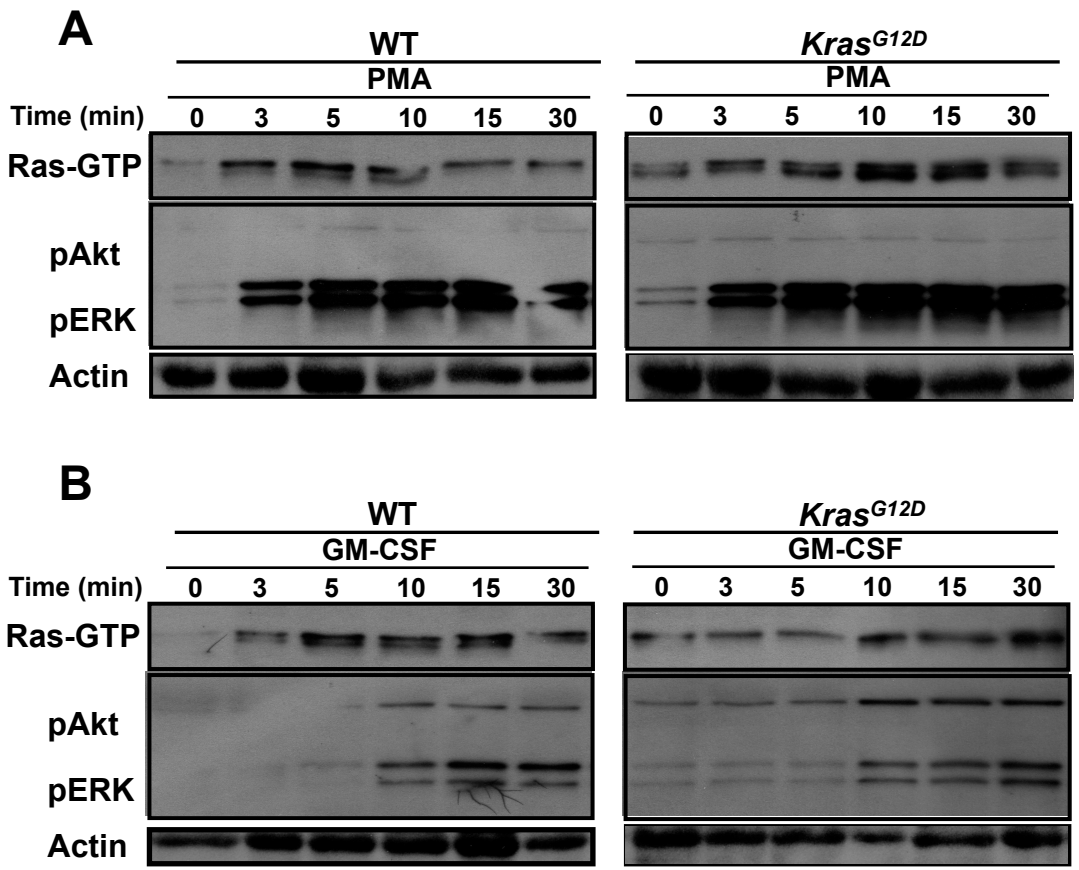



**Fig. S10.** PMA and GM-CSF induce Ras-GTP loading at different time points in wild-type (WT) and *Kras*$^{G12D}$ BMMPC. (A and B) WT and *Kras*$^{G12D}$ BMMPC were stimulated with PMA 100 nM or GM-CSF (10 μg/ml), and samples were taken at the indicated time points. Ras-GTP, pAkt and pERK abundance was measured by Western blot. The two independent experiments shown above are consistent with the data shown in Fig. 2B.

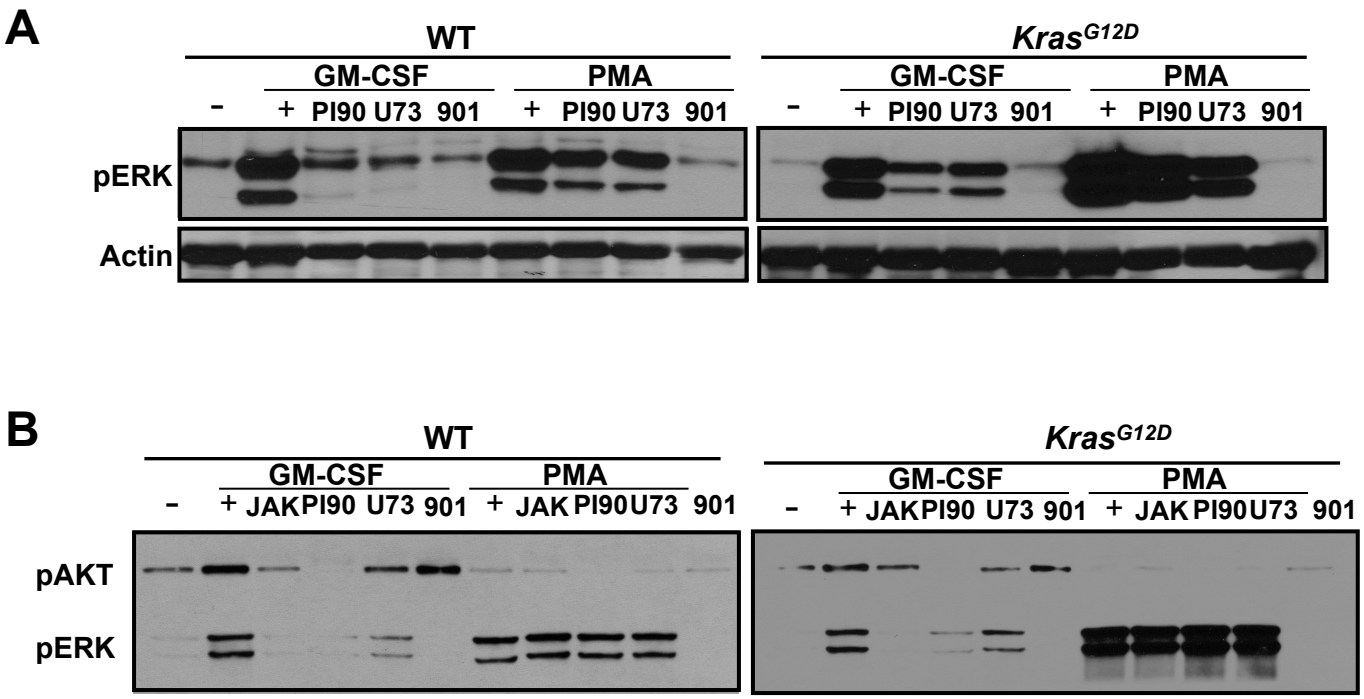



**Fig. S11.** PMA activation of pERK is insensitive to PI3K and PLC-γ inhibition, but not MEK inhibition. (A, B) BMMPCs were pretreated with PI-90, U73122 (U73), or PD0325901 (901) and then either left unstimulated or were stimulated with GM-CSF or PMA for 15 min. Cells were then analyzed by Western blotting for the abundances of pERK and Actin was used as loading control. The two independent experiments shown above are consistent with data shown in Fig. 2C.

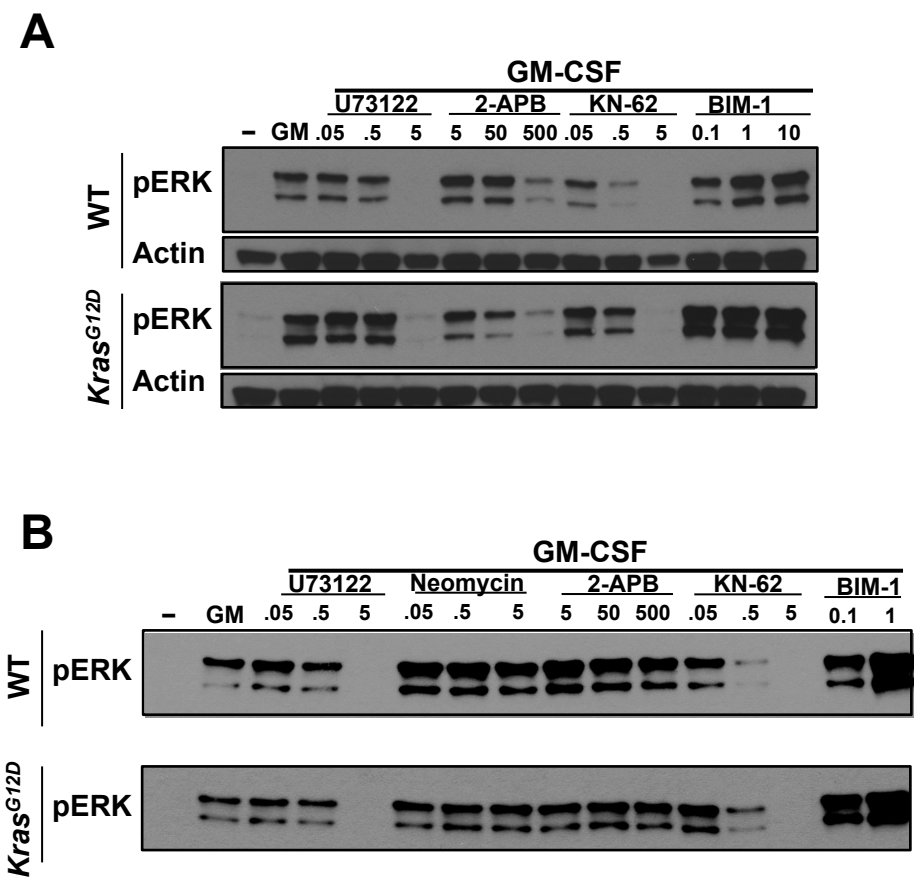



**Fig. S12.** PLC-γ and CaMKII inhibitors, but not PKC inhibitors, block ERK phosphorylation. (A) Dose-dependent inhibition of ERK phosphorylation at increasing concentrations of U73122, 2-APB, and KN-62 (0.05 – 5μM). BMMPC were incubated for 30 min with each compound and stimulated with GM-CSF for 15 min. This experiment was repeated as shown in (B) with the inclusion of Neomycin sulfate (a calcium channel inhibitor).

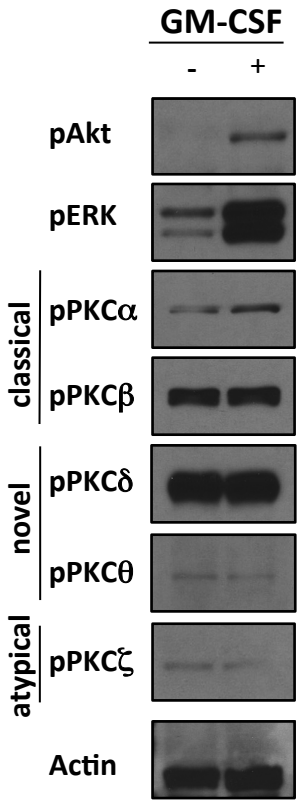



**Fig. S13.** Basal levels of phosphorylated PKC isoforms in wild-type (WT) BMMPC and response to GM-CSF stimulation. Cells were stimulated with GM-CSF (10 ng/mL) for 15 min. The cells were lysed and PKC isoform abundances were assessed by Western blotting. pAkt and pErk were also measured to control for GM-CSF stimulation. Proteins were detected by bioluminescence using ECL plus and developing for 1-5 min. This experiment was performed twice.

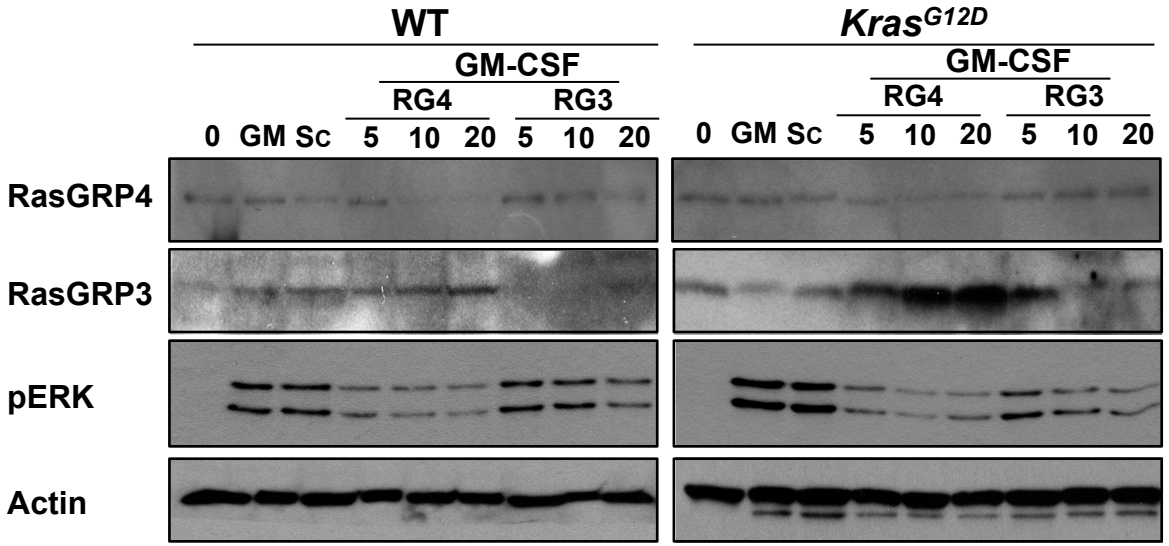



**Fig. S14.** Effects of siRNA knockdown of RasGRP3 and RasGRP4 on ERK phosphorylation. BMMPCs expressing siRNAs targeting *Rasgrp3* (RG3) or *RasGrp4* (RG4) or a control scrambled siRNA (Sc) were stimulated with GM-CSF for 15 min. The cells were lysed and Western blot analysis was performed to determine the amounts of RasGRP3, RasGRP4, and pERK, with Actin used as a loading control.

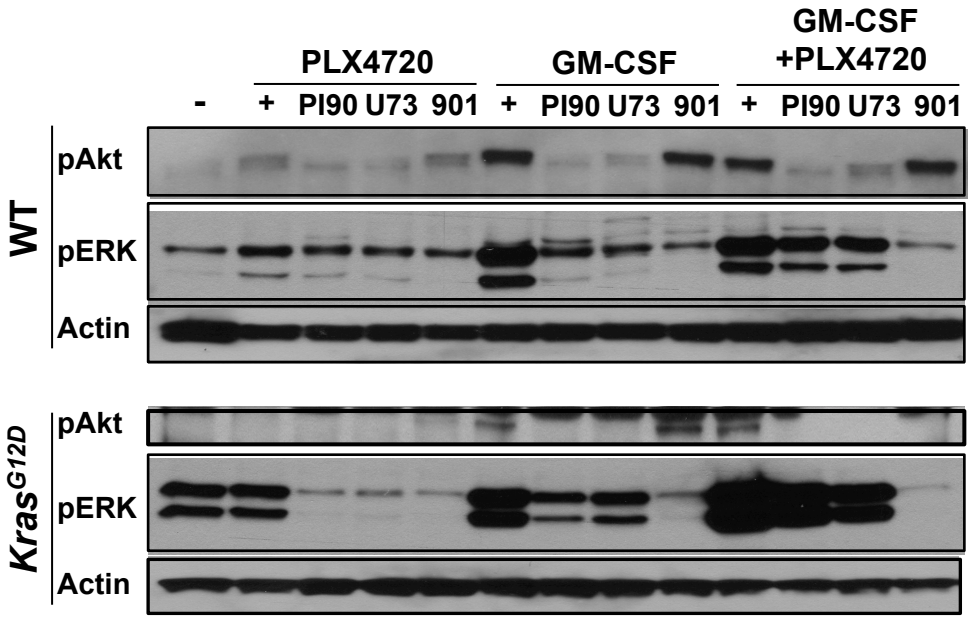



**Fig. S15.** Pharmacological activation of Raf largely overcomes the inhibitory effects of PI3K and PLC-γ inhibition. BMMPCs were starved of serum and cytokines overnight, incubated with PI-90, U73122, KN-62, or PD0325901 for 30 min, and then exposed to PLX4720 (10 μM), GM-CSF, PLX4720 + GM-CSF. The cells were then lysed and analyzed by Western blot to determine the abundances of pAkt and pERK, with Actin used as a loading control. Data from an independent experiment are presented in fig.3D.

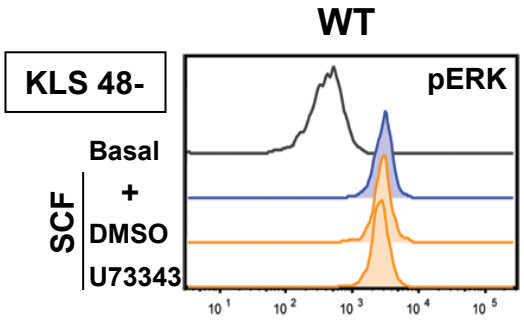



**Fig. S16.** DMSO and U73343 do not alter pERK levels in KLS CD48$^-$ cells. U73343, an structural analog of the PLC-γ inhibitor U73122 with no activity on PLC-γ does not affect pERK levels in response to SCF. The experiment was performed twice.

## Table 1

## Sequences of PLC-γ1 and PLC-γ2–specific shRNAs

shRNA for PLCγ1 sequence

| ShRNA | Sequence |
|---|---|
| shγ1-1: | TGCTGTTGACAGTGAgcgAcagcaagatctactactctgaTAGTGAAGCCACAGATG<br>TAtcagagtagtagatcttgctgcTGCCTACTGCCTCGGA |
| shγ1-2: | TGCTGTTGACAGTGAgcgCcactctcaccaccaagatcaaTAGTGAAGCCACAGATG<br>TAttgatcttggtggtgagagtgtTGCCTACTGCCTCGGA |
| shγ2-1: | TGCTGTTGACAGTGAgcgCacctgtcgatctattgcgataTAGTGAAGCCACAGATG<br>TAtatcgcaatagatcgacaggttTGCCTACTGCCTCGGA |
| shγ2-2: | TGCTGTTGACAGTGAgcgCaaagcagattattgaagacaaTAGTGAAGCCACAGAT<br>GTAttgtcttcaataatctgcttttTGCCTACTGCCTCGGA |

**Legend**. Oligonucleotides were cloned into the EcoRI / XhoI site in MSCV/LTRmiR30-Neo-IRES-GFP (LMN GFP) or MSCV/LTRmiR30-Neo-IRES-mCherry (LMN mCherry) with an shRNA targeting Renilla Luciferase (Renilla.713) serving as a negative control. Both vectors were kindly provided by Drs. Johannes Züber and Scott Lowe at CSHL. A complete description of these vectors was published in: *Nat Genet. 2005 Nov;37(11):1289-95.*