

## **ABSTRACT**

WANG, CHUN-CHAO. Systematic Analysis of Crosstalk in the PDGF Receptor Signal Transduction Network. (Under the direction of Jason M. Haugh.)

Intracellular signal transduction is traditionally characterized in terms of pathways, comprised of serial activation processes. Although it is appreciated that canonical signaling pathways are simply dominant routes of regulation embedded in larger interaction networks, relatively little has been done to quantify pathway crosstalk in such networks. Through quantitative measurements that systematically canvas an array of stimulation and molecular perturbation conditions, together with computational modeling and analysis, we have elucidated crosstalk mechanisms in the platelet-derived growth factor (PDGF) receptor signaling network, in which phosphoinositide 3-kinase (PI3K) and Ras/extracellular signal-regulated kinase (Erk) pathways are prominently activated. We show that, while PI3K signaling is insulated from crosstalk, PI3K enhances Erk activation in multiple ways. Whereas simultaneously blocking Ras and PI3K abolishes PDGF-stimulated Erk phosphorylation, each pathway makes an independent contribution to Erk activation, and PI3K affects Ras activation as well. The magnitudes of these effects depend strongly on the stimulation conditions, subject to saturation effects in the respective pathways and negative feedback loops. Motivated by those dynamics, a kinetic model of the network was formulated and used to precisely quantify the relative contributions of PI3K-dependent and -independent modes of Ras/Erk activation.

# Systematic Analysis of Crosstalk in the PDGF Receptor Signal Transduction Network

by  
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## **DEDICATION**

This work is dedicated to my parents.

獻給我的父母王安雄先生與陳麗珠女士。

## **BIOGRAPHY**

Chun-Chao Wang was born on May 9<sup>th</sup>, 1975 in Taipei, Taiwan. He grew up with his grandparents Chun-Kun and Kan, his parents An-Hsiung and Li-Chu, and his sisters Chun-Min, Yi-Hsin and Yen-Wen. During his early schooling, he was fascinated by mathematics, science and arts. With the desire to pursue science, Chun-Chao enrolled at National Taiwan University (NTU) where he studied to become a chemical engineer. Biochemistry and mathematics deeply interested him when he was an undergraduate in NTU and he decided to study Biochemical Engineering in the future. Chun-Chao Wang was admitted into North Carolina State University in 2003 and is doing his research on signal transduction networks, under the direction of Professor Haugh in the department of Chemical and Biomolecular Engineering.

## **ACKNOWLEDGMENTS**

My experience here at North Carolina State University has been wonderful and truly memorable. Successfully pursuing a Ph.D. in the Department of Chemical and Biomolecular Engineering is a great challenge, and it required the help and support from many people in my life. I would especially like to thank my advisor, Dr. Jason Haugh for his guidance, understanding and support throughout my graduate studies. He taught me a lot about research as well as how to run a successful laboratory. I am also very grateful for having a great doctoral committee and wish to thank Dr. Robert Kelly, Dr. Carla Mattos and Dr. Balaji Rao for their encouragement and support.

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# CHAPTER 1. INTRODUCTION

## 1.1. Background

In multicellular organisms, intercellular communication is required for cell growth and differentiation. Cells synthesize and release extracellular signaling molecules, such as growth factors. Growth factors are high-affinity ligands for transmembrane receptors belonging to the family of receptor tyrosine kinases (RTKs). Binding of a growth factor to the extracellular domain of the receptor influences the target cell by triggering signaling cascades inside the cytoplasm. This process of converting extracellular signals into cellular responses is termed *signal transduction* (Fedi and Aaronson, 2000; Medico and Comoglio, 2000).

Cells control their behavior by maintaining a balance between signal transduction pathways and the disruption of this balance has been implicated in the evolution of normal cells into cancer cells (Elledge, 1996). The cell signaling pathways are usually woven together through so-called crosstalk interaction. Crosstalk interaction allows cellular signals to be distributed across multiple pathways, and perturbations that target specific signaling molecules might therefore have unexpected effects. Thus, the fundamental understanding of crosstalk interaction is relevant to medicine and biotechnology.

In this study, we aim to systematically analyze a signaling network, comprised of Ras/extracellular signal-regulated kinase (Erk) and phosphoinositide 3-kinases (PI3K)/Akt pathways, stimulated by platelet-derived growth factor (PDGF). Molecular level control over the signaling network, through genetic and pharmacological intervention, will allow

these complex regulatory interactions to be isolated. Constitutively active G12V H-Ras and dominant-negative S17N H-Ras was transfected into NIH 3T3 cells to modulate the levels of Ras-GTP. PI3K inhibitor, LY294002, and MEK inhibitor, PD098059, are commonly used to block cellulate functions of PI3K and MEK, respectively. We accessed the phosphorylation of Akt and Erk as the outputs of PI3K/Akt and Ras/Erk pathways respectively, and detected Ras-GTP level and MAPK phosphatase 1 (MKP-1) concentration to analyze the effect of two negative feedback interactions on Ras/Erk pathway.

### **Objectives:**

- Acquire a high-density data set monitoring readouts in the PDGF receptor signaling network leading to activation of Erk and Akt.
- Perturb key interactions in the network to characterize the underlying crosstalk and feedback interactions.
- Formulate a mathematical model to more precisely quantify the contributions of Ras and PI3K to the Erk signaling circuit.

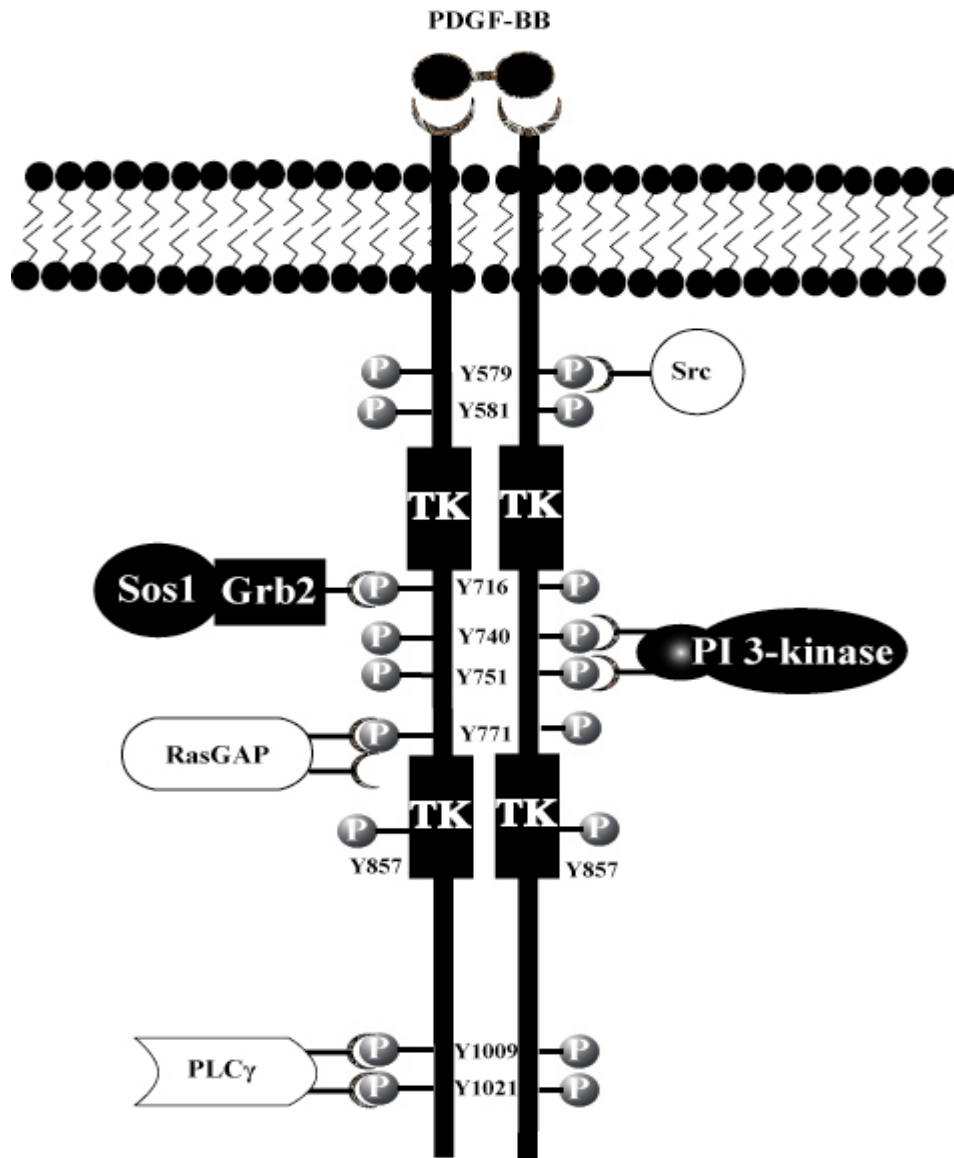
### **1.2. Platelet-derived Growth Factor Receptor (PDGFR)**

PDGF is a potent stimulator of cell growth. Four PDGF polypeptide chains, A, B, C, and D, have been identified, and these polypeptide chains are linked by disulfide bonds to form five known dimeric isoforms: PDGF-AA, -AB, -BB, -CC, and -DD (Heldin et al., 1998; Heldin et al., 2002; Pietras et al., 2003). The PDGF isoforms bind to PDGF receptors (PDGFRs), which are RTKs consisting of an extracellular ligand-binding domain, a single

transmembrane region, and an intracellular intrinsic kinase domain. There are two structurally related PDGF receptors, PDGF  $\alpha$ - and  $\beta$ -receptor. Binding of PDGF induces receptor dimerization on the cell surface, and PDGF-BB can induce  $\alpha\alpha$ ,  $\beta\beta$ , and  $\alpha\beta$  receptor dimers (Heldin and Westermark, 1999; Li et al., 2000; Bergsten et al., 2001; Gilbertson et al., 2001; LaRochelle et al., 2001). PDGFR dimerization causes transphosphorylation of specific tyrosine residues (Tyr849 in the  $\alpha$ -receptor and Tyr857 in the  $\beta$ -receptor) in the intracellular portions, leading to an increase in the catalytic efficiencies of the intrinsic kinases (Kaziauskas and Cooper, 1989) and creating docking sites for signal transduction molecules containing Src homology 2 (SH2) domains (Pawson and Scott, 2005).

SH2 domains are conserved motifs that regulate the function of the kinase domain and its interactions with targets (Sadowski et al., 1986). A large number of SH2 domain-containing signal transduction proteins have been reported to bind to different autophosphorylation sites in PDGF receptors (Fig. 1.1). Some of these are enzymes, such as PI3K, phospholipase C (PLC)- $\gamma$ , Src family tyrosine kinases, and Ras GTPase activating protein (RasGAP). Others, such as Grb2 (growth factor receptor-bound 2) and Shc (Src-homology-2-containing), have adaptor functions, linking the receptor with downstream catalytic molecules. For example, Grb2 forms a complex with Sos, a guanine nucleotide exchange factor (GEF) for Ras (Heldin and Westermark, 1999; Pawson et al., 2001).

The different SH2 domain-containing signaling proteins initiate different signal pathways. Two pathways stimulated by PDGF thought to play a central role in the regulation of cell survival and proliferation are PI3K/Akt and Ras/Erk pathways.



**Figure 1.1. Interaction between PDGF β-receptor and SH2 domain-containing signal transduction molecules.** The intracellular proteins of homodimeric PDGF β-receptor complexes are depicted, each containing dual tyrosine kinase (TK) domains and several autophosphorylation sites. Signaling proteins interact with these phosphotyrosine motifs, most often through modular SH2 domains (Pawson and Scott, 2005). Among the proteins that associate with PDGF receptors directly are: Src, a non-receptor tyrosine kinase; Grb2, an adaptor that recruits Sos for elevation of Ras-GTP; RasGAP, which accelerates the hydrolysis of Ras-GTP back to Ras-GDP; the regulator subunit of PI3K; and phospholipase C-γ (PLC-γ), which catalyzes lipid hydrolysis (leading to a rise in cytosolic  $\text{Ca}^{2+}$ ). Many of these proteins are themselves tyrosine phosphorylated, often impacting protein function.

### 1.3. PI3K/Akt Pathway and Cell Survival

Phosphatidylinositol (PI) is a major phospholipid component of the plasma membrane. The acute phosphorylation of PI at the 3' position of the inositol head group puts in motion a coordinated set of events leading to cell growth, cell migration, and cell survival. PI3K phosphorylates PI at the 3' position in response to growth factor stimulation (Fruman et al., 1998). It was recognized that PDGF receptor-mediated activation of PI3K provides cells with a survival signal that prevents programmed cell death, or apoptosis (Fig. 1.2) (Yao and Cooper, 1995; Cantley, 2002). Indeed, more recent evidence has shown that the contributions of other PDGF  $\beta$ -receptor-associated signaling molecules, such as Src, PLC-  $\gamma$ , and RasGAP, are far less important for PDGF-stimulated survival (Vantler et al., 2005).

#### 1.3.1. PI3K

Type I PI3Ks are heterodimers consisting of catalytic and regulatory subunit. In the case of type I<sub>A</sub> PI3Ks, three mammalian catalytic subunits p110 associate with a regulatory subunit p85 that has two SH2 domains and one Src homology 3 (SH3) domain (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1999).

Two tyrosines, Tyr740 and Tyr751, in the PDGF  $\beta$ -receptor have been shown to be autophosphorylation sites that bind p85 SH2 domains (Fig. 1.1) (Fantl et al., 1992; Kazlauskas et al., 1992; Kashishian et al., 1992). The binding of the p85 to PDGF receptor induces a conformational change, which is transmitted to the p110 and regulates enzymatic activity by an allosteric mechanism (Shoelson et al., 1993). Active type I PI3K converts phosphatidylinositol-4,5-bisphosphate (PI(4,5)P<sub>2</sub>) to phosphatidylinositol-3,4,5-trisphosphate

(PI(3,4,5)P<sub>3</sub>), the second messenger. The typically delayed generation of phosphatidylinositol-3,4-bisphosphate (PI(3,4)P<sub>2</sub>) may be produced in part by the action of inositol 5-phosphatases, like SHIP-1 and SHIP-2 (SH2 domain-containing inositol phosphatase-1 and -2), which dephosphorylate the 5' position of PI(3,4,5)P<sub>3</sub>, yielding PI(3,4)P<sub>2</sub> (Stephens et al., 1991; Damen et al., 1996); PI(3,4,5)P<sub>3</sub> and PI(3,4)P<sub>2</sub> largely overlap with respect to their intracellular functions. The lipid phosphatase phosphatase and tensin homologue (PTEN) counteracts PI3K activity by dephosphorylating the 3' position of the inositol ring, producing PI(4,5)P<sub>2</sub> (Maehama and Dixon, 1998).

LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one] is a cell-permeable, low molecular weight compound that was designed as a PI3K inhibitor, and is commonly used to elucidate cellular functions of PI3K (Vlahos et al., 1994; Djordjevic and Driscoll, 2002). LY294002 is a competitive inhibitor of ATP binding and effects no significant change on the conformation of the binding-site residues (Djordjevic and Driscoll, 2002). Although the reported IC<sub>50</sub> of LY294002 (around 1 μM) is much higher than that of wortmannin, LY294002 is widely used as a specific PI3K inhibitor because it is much more stable in solution than wortmannin and thus better suited for long-term studies (Jones et al., 1999).

### **1.3.2. Akt and PDK-1**

Signaling proteins with pleckstrin-homology (PH) domains accumulate at sites of PI3K activation by directly binding to PI(3,4,5)P<sub>3</sub> (Pawson and Scott, 1997; Klarlund et al., 2000; Pawson et al., 2002; Pawson and Scott, 2005). Of particular interest in this study are the

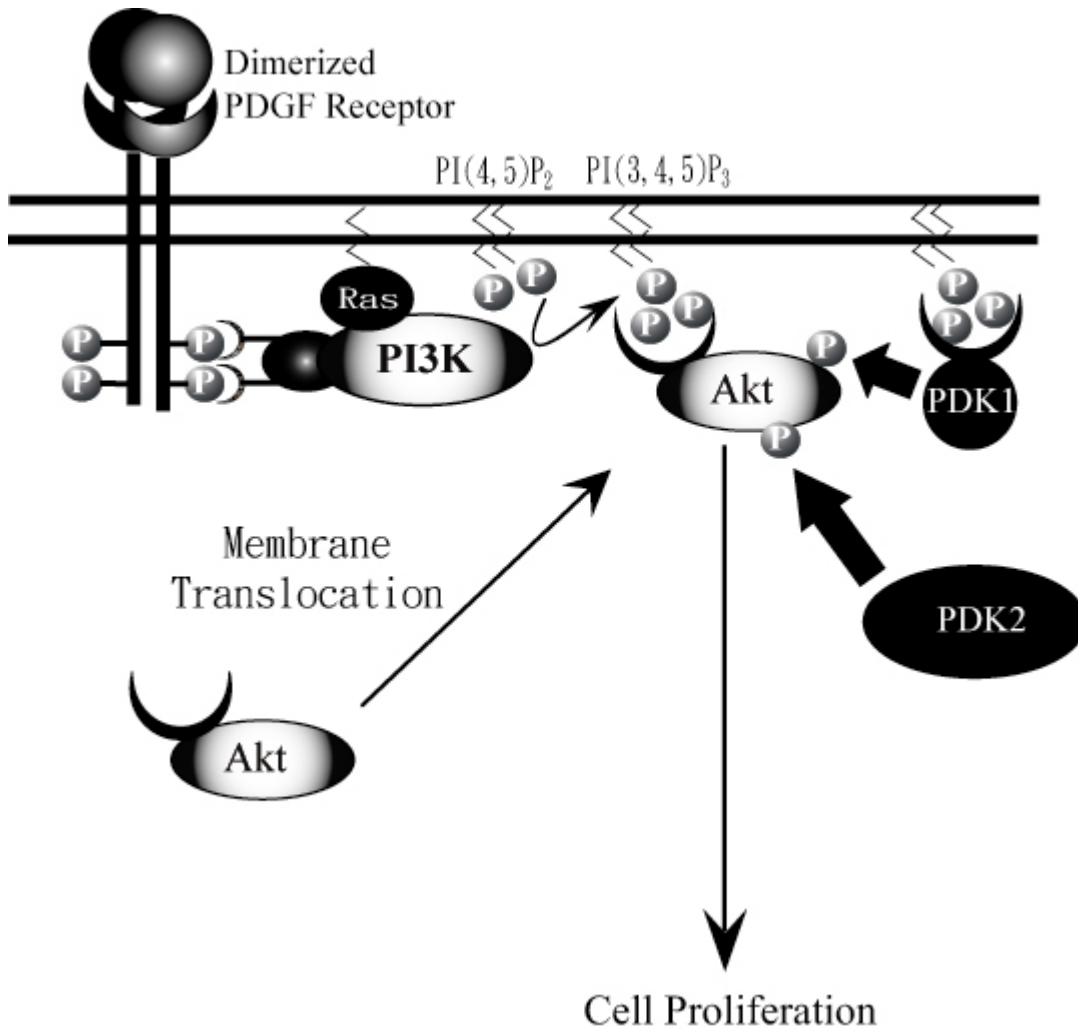
protein serine/threonine kinases Akt (also known as protein kinase B (PKB)) and phosphoinositide-dependent kinase 1 (PDK-1).

Akt belongs to the AGC superfamily (protein kinase A (PKA)/protein kinase G/protein kinase C-like). As with other AGC members, Akt is regulated by upstream second messengers and secondary, activating enzymes. In mammals, there are three isoforms of Akt (Akt-1, 2, and 3). All three isoforms contain a PH domain and PH domain binding to PI(3,4,5)P<sub>3</sub> or PI(3,4)P<sub>2</sub> results in Akt recruitment from the cytosol to the plasma membrane, where Akt is thought to undergo a conformational change and become activated through phosphorylation of two residues (Schael and Woodgett, 2003).

In unstimulated cells, Thr308 in the flexible loop (T-loop) of Akt-1 is largely unphosphorylated, acting to inhibit kinase function. When phosphorylated, the T-loop is modified, allowing access to ATP and the kinase substrate (Schael and Woodgett, 2001). It is now clear that Thr308 in Akt-1 is phosphorylated by PDK-1 (Stokoe et al., 1997; Stephens et al., 1997; Alessi et al., 1997a,b), and this is the major input required for the Akt activation. PDK-1 also contains a PH domain, which also interacts with PI(3,4,5)P<sub>3</sub> and PI(3,4)P<sub>2</sub>. The rate of Akt phosphorylation by PDK-1 is greatly enhanced by the inclusion of PI(3,4,5)P<sub>3</sub> and PI(3,4)P<sub>2</sub>, which concentrate both Akt and PDK-1 (Currie et al., 1999).

The other residue associated with Akt activation is at Ser473 (in Akt-1). Phosphorylation of Ser473 is required for Akt activity (Schubert et al., 2000), but the mechanism is not completely understood. There is evidence implicating both Akt autophosphorylation (Toker and Newton, 2000) and phosphorylation by other serine kinases, like the integrin-linked kinase (ILK) (Persad et al., 2001), although contrary results suggest

that pSer473 kinase activity is distinct from ILK and PDK-1 (Hill et al., 2002). A recent study has provided persuasive evidence that a rapamycin-insensitive form of the mammalian target of rapamycin (mTOR) protein kinase, in complex with Rictor:GβL mediates the phosphorylation of Akt at Ser473 (Sarbasov et al., 2005).



**Figure 1.2. A model for PI3K/Akt pathway.** PI3K binds to phosphorylated receptors through its p85 regulatory subunit, activating the p110 catalytic subunit. The active PI3K generates PI(3,4,5)P<sub>3</sub> that recruit cytosolic Akt and PDK-1 through their PH domains. Akt is phosphorylated and activated at the plasma membranes by PDK-1/2.

#### **1.4. Ras/Erk Pathway and Biological Functions**

The mitogen-activated protein kinase (MAPK) pathway is another primordial pathway controlling fundamental cellular processes. The arrangement of MAPK cascades includes a small GTPase working upstream of three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates MAPK. Here we focus on the regulation of Erk pathway, which features Ras as the GTPase, Raf as MAPKKK, MEK as MAPKK, and Erk as MAPK.

The small GTP-binding protein Ras is a crucial downstream effector of the PDGFR. The Ras/Raf/MEK/Erk pathway is at the heart of signaling networks that govern proliferation, differentiation and cell survival. Curiously, Erk activation has been associated with both stimulation and inhibition of cell proliferation (Kolch, 2000). Further study is required to fully elucidate the complexity of this pathway and its connections to cell behaviors.

##### **1.4.1. Ras and its regulatory proteins**

Grb2 is an adaptor molecule consisting of two SH3 domain and one SH2 domains. The SH2 domain of Grb2 can bind directly to two sites in autophosphorylated PDGF  $\beta$ -receptors (Tyr716 and Tyr775) (Fig. 1.1) (Arvidsson et al., 1994; Ruusala et al., 1998). Grb2 can also bind indirectly to PDGF  $\beta$ -receptors, i.e. to the adaptor Shc (Yokote et al., 1994) or to the tyrosine phosphatase SHP-2 (Li et al., 1994) after these molecules have bound to the PDGF  $\beta$ -receptors and become phosphorylated. The SH3 domains of Grb2 mediate binding of Sos1 (son of sevenless 1), a guanine nucleotide exchange factor (GEF) for Ras, which converts inactive Ras-GDP to active Ras-GTP (Schlessinger, 1993; Chardin et

al., 1993). This binding promotes the translocation of Sos1 to the plasma membrane and positions it in direct proximity to membrane-bound Ras. RasGAP is a protein with two SH2 domains that binds to Tyr771 of the activated PDGF  $\beta$ -receptor (Fig. 1.1.) (Fantl et al., 1992; Kazlauskas et al., 1992). RasGAP is important for negative control of Ras activation because it converts Ras-GTP to Ras GDP, by accelerating the intrinsic GTP hydrolysis activity of Ras.

Three isoforms of Ras (as known as p21<sup>ras</sup>) protein, H-Ras, N-Ras, and K-Ras, are expressed in mammalian cells. Ras proteins function as regulated GDP/GTP molecular switches that control diverse signaling networks. The GTP-bound conformation of Ras is active since it can interact with effector proteins, whereas the GDP-bound conformation can not. The dissociation of GDP from Ras is rate-limiting, with nucleotide-free Ras rapidly binding to GTP, which is 10 - 20 times more abundant than GDP in the cytosol. GEFs, such as Sos1, are important in Ras functions because GEFs increase the dissociation rate of GDP bound to Ras (Mitin et al., 2005). Ras-bound GTP is hydrolyzed to GDP by the intrinsic GTPase of Ras, which is very slow and must be catalyzed by GAPs (Bourne et al., 1991).

In most cases, the oncogenic mutants of RAS introduce amino-acid substitutions at three residues: G12, G13 and Q61 (Barbacid, 1987). The major role of oncogenic RAS mutant (such as G12V H-Ras) is to impair the intrinsic and GAP-accelerated GTPase activity, thereby generating active GTP-bound RAS molecules (Trahey and McCormick, 1987); on the other hand, dominant-negative mutant (such as S17N H-Ras) has a preferential affinity for GDP versus GTP binding (Feig et al., 1988) and are thought to sequester GEFs and,

thus, block activation of endogenous Ras. More recently, Ras mutants have been used to correlate effector interaction with biological function, providing further evidence of branch points in Ras signaling (Rodriguez-Viciano et al., 1994; Rodriguez-Viciano et al., 1996; Klinghoffer et al., 1996). In this study, we use the constitutively activated mutant G12V H-Ras and dominant negative mutant S17N H-Ras.

#### **1.4.2. Raf/MEK/Erk cascade**

The best-characterized Ras-dependent signaling pathway is the Raf/MEK/Erk cascade. Mammals possess three Raf serine/threonine kinases: Raf-1, A-Raf, and B-Raf. The ubiquitously expressed Raf-1 is certainly the best studied (Qi and Elion, 2005).

The activation of Raf-1 involves membrane recruitment and forming a high affinity complex with Ras-GTP (Van Aelst et al., 1993; Moodie et al., 1993; Zhang et al., 1993; Vojtek et al., 1993), dephosphorylation of an inhibitory site (Ser259) (Abraham et al., 2000; Dhillon et al., 2002), followed by phosphorylation of Ser338 (Diaz et al., 1997), as well as phosphorylation of the activation loop (Chong et al., 2001). These sites are conserved in A-Raf; however, in B-Raf the equivalent of Raf-1 Ser338 is constitutively phosphorylated and not regulated by Ras (Ser445 in B-Raf) (Mason et al., 1999).

Ras actually binds to two sites on Raf-1. Ras interaction with Ras-binding domain (RBD) of Raf-1 alone is sufficient for the recruitment of Raf-1 from the cytosol to the plasma membrane, but Ras interaction with the cysteine-rich domain (CRD) of Raf-1 is essential for efficient activation of Raf-1 (Brtva et al., 1995; Roy et al., 1997). Ras binding promotes conformational changes that relieve Raf-1 autoinhibition, facilitate the phosphorylation of

activating sites (Terai and Matsuda, 2005), and induce finally heterodimerization with B-Raf (Garnett et al., 2005; Rushworth et al., 2006).

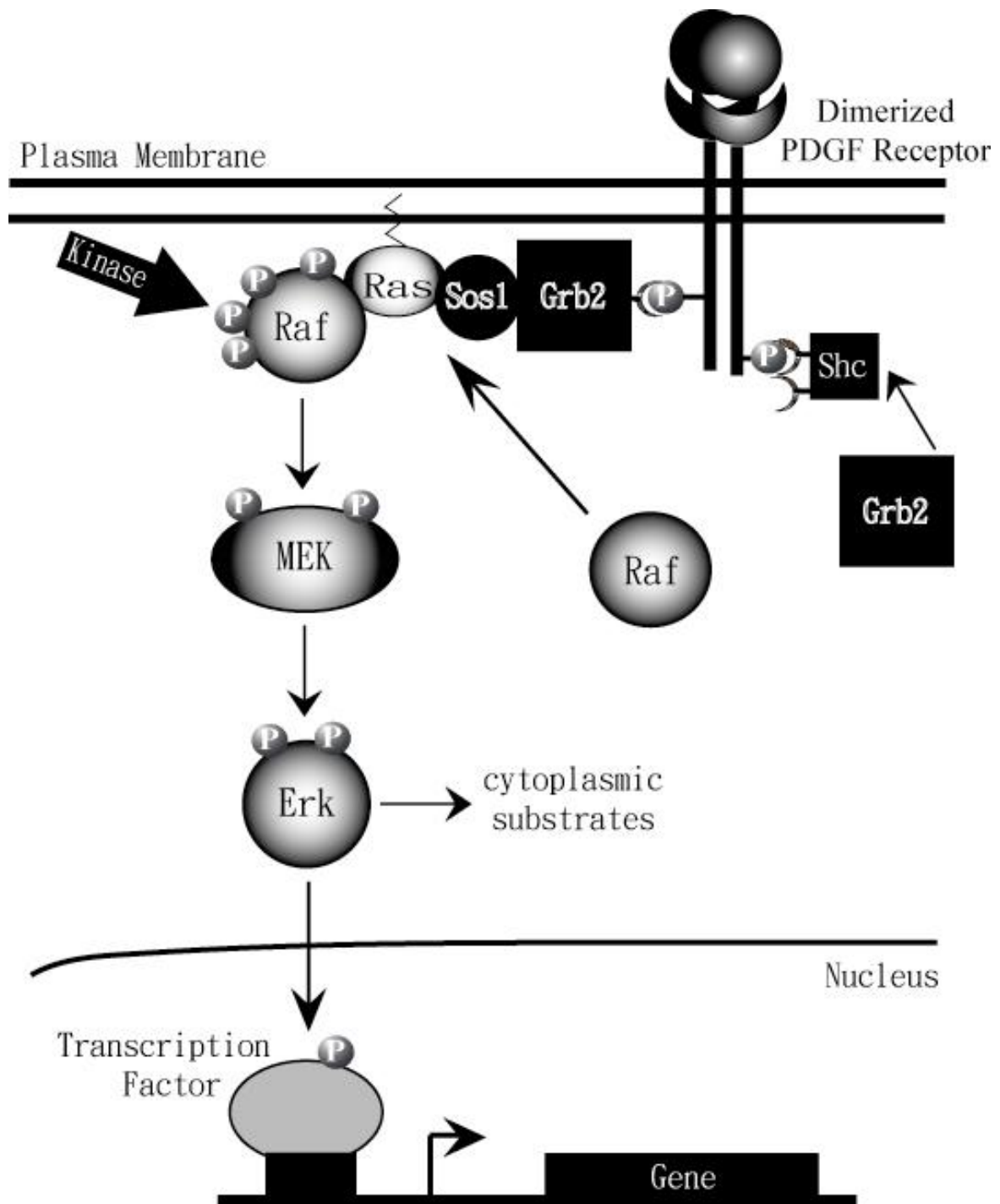
All three Raf isoforms share MEKs as the only commonly accepted downstream substrates. Raf kinases have shown to selectively phosphorylate and activate MEK-1 and MEK-2 (Schaeffer and Weber, 1999). PD098059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] is a synthetic inhibitor that selectively blocks the activation of MEK-1 and, to a lesser extent, the activation of MEK-2 (Alessi et al., 1995; Dudley et al., 1995). MEKs belong to the rare breed of dual-specificity kinases that can phosphorylate both threonine and tyrosine residues. Both MEK isoforms can activate the downstream Erk-1 and Erk-2 (also called p44 and p42 MAPK respectively based on the molecular weight: 43/44 and 41/42 kDa) via phosphorylation of Thr202 and Tyr204 in Erk-1 and Thr183 and Tyr185 in Erk-2 (Kolch, 2000).

#### **1.4.3. ERK and biological functions**

Erk is a highly conserved serine/threonine kinase that phosphorylates various substrates, including many enzymes, transcription factors and cytoskeletal proteins. Erk regulates growth factor-stimulated cell cycle progression, cell differentiation, migration, and survival. It remains uncertain how the activation of this one protein transduces multiple signals from extracellular stimuli to specific cell responses; however accumulating evidence suggests that differences in duration, magnitude and subcellular compartmentalization of Erk activity generate variations in signaling output (Ebisuya et al., 2005).

In PC12 cells, NGF (nerve growth factor)-mediated sustained activation of Erks can

lead to a differentiation response, whereas transient activation in response to epidermal growth factor (EGF) stimulation leads to proliferation (Qiu et al., 1992; Traverse et al., 1992); however, PDGF-induced proliferation requires sustained Erk activity in fibroblasts (Weber et al., 1997). Sustained Erk activation is associated with translocation of Erks to the nucleus. Therefore, transient activation will have different consequences for gene expression compared with sustained activation, because nuclear accumulation of active Erk will result in phosphorylation of transcription factors (Marshall, 1995; Wellbrock et al., 2004).



**Figure 1.3. A model for Ras/Erk pathway.** PDGF dimer binds to PDGF receptor that initiates this signaling pathway. The Grb2-Sos1 complex, which contains guanine nucleotide exchange factor activity, binds to the receptor and elevates Ras-GTP level. Ras-GTP recruits Raf, that phosphorylates and activates MEK, which in turn activates Erk and cell proliferation.

## 1.5. Signal Transduction Network

A complex network of signaling pathways is activated in cells in response to PDGF. Individual enzymes can receive input from several pathways, in which case an important signaling pathway is influenced by the activity of another. This type of integration process is known as crosstalk. As well as crosstalks between pathways, feedback loops can occur, in which a downstream component in a pathway either positively or negatively influences the activity of an upstream component. Signal integration also occurs at the level of gene expression. Different signals affect the expression of different genes, which may influence the network (Haugh and Weiger; 2006) (Fig. 1.4). These interactions result in network that is very complex and nonintuitive. A systematic analysis of these interactions could be useful in understanding the properties of signaling network.

### 1.5.1. Crosstalk from Ras to PI3K

Ras-GTP can bind to and activate the p110 $\alpha$  catalytic subunit of PI3K *in vitro*. Thus, Ras might act as an upstream positive effector of both the Raf/MEK/Erk pathway and the PI3K/Akt pathway (Katz and McCormick 1997). However, the weakness of this hypothesis is the inability to detect a stable G12V Ras/p110 $\alpha$  (or p110 $\gamma$ ) complex *in vivo*, even when both are transiently overexpressed (Rubio et al., 1997). G12V Ras significantly activates PI3K and increases PI(3,4,5)P<sub>3</sub>/PI(3,4)P<sub>2</sub> accumulation and Akt phosphorylation levels in cells (Rodriguez-Viciano et al., 1994; Rodriguez-Viciano et al., 1996; Klippel et al., 1996; Chan et al., 2002), suggesting that the interaction is sufficient for the activation and membrane localization of PI3K; however, S17N Ras blocks receptor-mediated modulation of

endogenous Ras-GTP, and the resulting effects on growth factor-mediated PI3K activation range from partial inhibition to no inhibition (Rodriguez-Viciano et al., 1994; Franke et al., 1995; Rodriguez-Viciano et al., 1996; Klinghoffer et al., 1996; Klippel et al., 1996), implying that Ras-PI3K crosstalk appears to be dependent on the cellular context.

### **1.5.2. Crosstalk from PI3K to Ras/Erk**

While Ras might contribute to the activation of PI3K through direct binding, crosstalk signals also present between PI3K and proteins in the Ras/Erk pathway. However, the overall effect of PI3K activation on Ras/Erk has been controversial because of reports of both positive and negative interactions.

Akt negatively regulates Raf activity, at least in certain cell contexts, apparently through direct phosphorylation of Raf-1 on Ser259 (Zimmermann and Moelling, 1999; Rommel et al., 1999; Guan et al., 2000; Reusch et al., 2001), which mediates binding of 14-3-3 protein and Raf-1 sequestration (Morrison and Cutler Jr, 1997). It has been proposed that if proliferation and survival are coregulated, the Akt-Raf interaction may ‘tip the scale’ in favor of cell survival (Scheid and Woodgett, 2000). Dephosphorylation of Ser259 by protein phosphatase 2A has been reported to be part of the Raf-1 activation mechanism (Dhillon et al., 2002).

On the other hand, different PI3K-dependent pathways apparently enhance Erk activation, via proteins in the Ras/Erk pathway both upstream and downstream of Ras.

Rac, which is a member of the Rho family of small GTPases (Hall, 1998; Ridley, 2001), is activated downstream of PI3K in response to PDGF and other stimuli (Ridley et al., 1992;

Wymann and Arcaro, 1994; Hawkins et al., 1995; Hooshmand-Rad et al., 1997); 3'PIs recruit certain GEFs to the plasma membrane, yielding higher GTP-bound fractions of Rac. It should be noted that Rac-GEFs could also be activated by PI3K-independent routes (Han et al., 1998; Pandey et al., 2000; Welch et al., 2003). GTP-bound Rac and Cdc42 (cell division cycle 42) specifically bind the serine/threonine kinases Pak (p21-associated kinase), causing autophosphorylation of Pak-1 on Thr423 that is required for full Pak1 activity (Yu et al., 1998; Gatti et al., 1999; Zenke et al., 1999); modification of Pak-1 on Thr423 by PDK-1 in a PI3K-independent manner is also suggested (King et al., 2000). Pak then induced phosphorylation of Raf-1 on Ser338 (King et al., 1998; Sun et al., 2000; Chaudhary et al., 2000; Li et al., 2001; Zang et al., 2002) and/or phosphorylation of MEK-1 on Ser298 (Slack-Davis et al., 2003; Beeser et al., 2005; Park et al., 2007) to activate Erk.

A second possible pathway linking PI3K to Raf is via activation of PLC and PKC (protein kinase C). PLC- $\gamma$ 1 contains two PH domains and catalyzes the hydrolysis of PI(4,5)P<sub>2</sub>, creating the second messengers inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). These second messengers are known to stimulate the release of Ca<sup>2+</sup> from internal stores and to activate PKC, respectively (Wahl and Carpenter, 1991). PKC isoforms are divided into three groups: conventional PKC (cPKC), novel PKC (nPKC) and atypical PKC (aPKC). The activation of cPKCs is regulated by both DAG and Ca<sup>2+</sup>, whereas the activation of nPKCs is Ca<sup>2+</sup>-independent. The activation of aPKCs is both DAG- and Ca<sup>2+</sup>-independent (Newton, 1997; Nishizuka, 1992). However, as there is not a significant overlap in the PKC isoforms known to respond to PI3K signaling (Toker et al., 1994; Derman et al., 1997; Le Good et al., 1998) and those previously reported to activate Raf

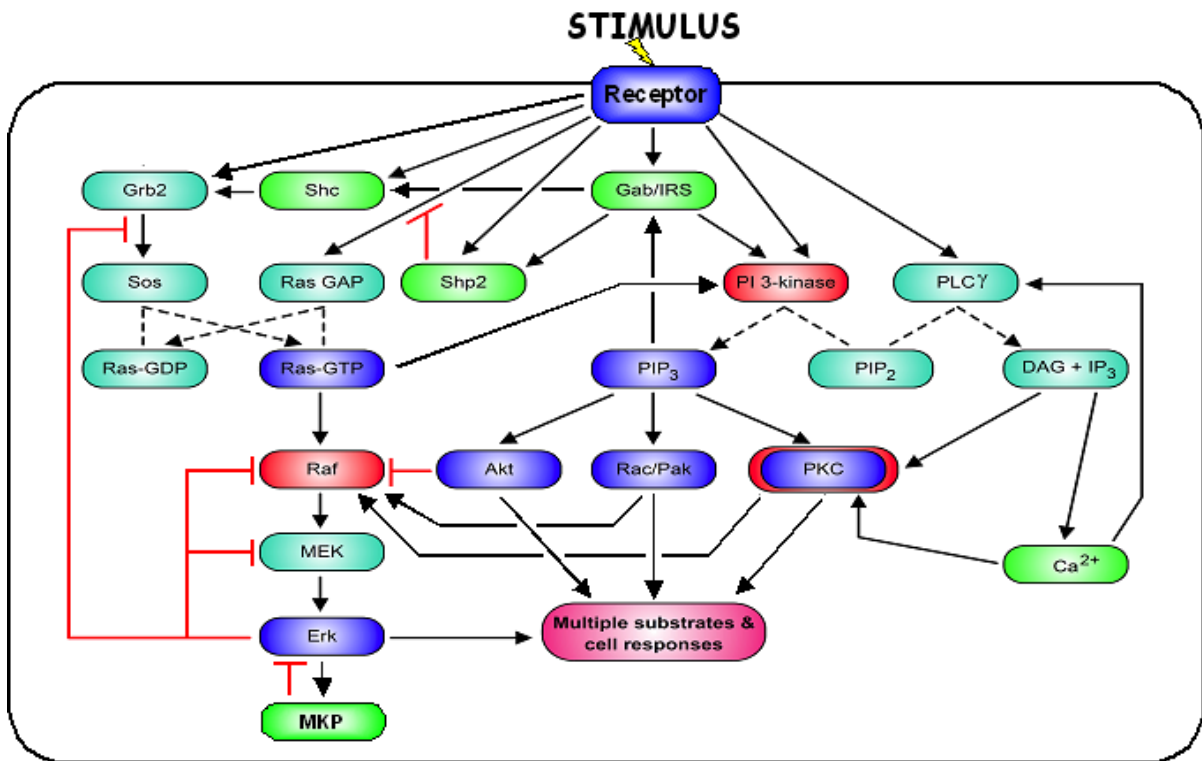
(Kolch et al., 1993; Carroll and May, 1994; Marquardt et al., 1994; VanRenterghem et al., 1994; Ueda et al., 1996; Corbit et al., 2003). Some PKC isoforms (e.g., PKC $\zeta$ ) constitute downstream targets of PI3K and are capable of stimulating Raf-1 independently of or in parallel to Ras (Chou et al., 1998; Schönwasser et al., 1998). Nevertheless, this mechanism may be tenuous in our cells.

Both of the pathways described above are consistent with previous reports that PI3K inhibitors abrogate Raf/MEK/Erk activation but not Ras-GTP loading (Cross et al., 1994; Karnitz et al., 1995; King et al., 1997). Other reports, however, indicate a role for PI3K upstream of Ras (Hu et al., 1995; Hawes et al., 1996; DePaolo et al., 1996; Wennström and Downward, 1999), although the mechanisms by which 3'PIs might promote Ras-GTP loading are unclear. Two possible mediators are Grb2-associated binder-1 (Gab1) (Gu and Neel, 2003) and centaurin- $\alpha$ 1 (Hayashi et al., 2006). Gab1 contains a PH domain and contributes to the activation of Ras by recruiting ubiquitous SH2 domain-containing protein tyrosine phosphatase (Shp2) and Grb2-Sos. Recent evidence showed that this ability of promoting Ras activation is only in response to a low, but not high, level of epidermal growth factor receptor (EGFR) stimulation (Sampaio et al., 2008). Under strong stimulation, Gab1/Shp2 was massively recruited by autophosphorylated EGFR via Grb2 binding. In contrast, under low stimulation, attracting Gab1 through PI(3,4,5)P<sub>3</sub> is a more efficient process (Sampaio et al., 2008). The second possible mediator, centaurin- $\alpha$ 1, contains two PH domains, which have been reported to bind PI(3,4,5)P<sub>3</sub> (Tanaka et al., 1997). The centaurin- $\alpha$ 1 can activate Ras/ERK pathway, and this activation requires the PI3K-dependent recruitment of centaurin- $\alpha$ 1 to the plasma membrane (Hayashi et al., 2006).

Taken together, what is clear is that the potential effects of modulating PI3K activation on the Ras/Erk pathway are many, and the net outcome depends on the stimulus and cell type.

### **1.5.3. Negative feedback interactions in Ras/Erk pathway**

Another complex feature of signal transduction networks is the influence of positive and negative feedback interactions, which can be self-contained within a pathway or involve pathway crosstalk. Within the Ras/Erk pathway, at least two distinct negative feedback loops have been established. First, Erk and/or MEK can desensitize Ras through hyper-phosphorylation of the Ras-GEF Sos (Cherniak et al., 1994; Waters et al., 1995a/b; de Vries-Smits et al., 1995; Langlois et al., 1995; Rozakis-Adcock et al., 1995; Buday et al., 1995; Porfiri and McCormick, et al., 1996; Chen et al., 1996; Holt et al., 1996; Klarlund et al., 1996; Corbalan-Garcia et al., 1996); in accord with this mechanism, MEK inhibition yields a more sustained Ras-GTP response in EGF-stimulated fibroblasts (Haugh et al., 1999). Second, MAPK phosphatase (MKP) is a dual specificity phosphatase (DSP) that dephosphorylates Ser/Thr and Tyr residues on MAPKs. MKP-1 dephosphorylates ERK1/2 *in vitro*, and expression of MKP-1 in cells blocks activation of ERK1/2 (Alessi et al., 1993; Sun et al., 1993; Zheng and Guan, 1993); ERK can induce *mkp-1* gene expression at the transcriptional level (Brondello et al., 1997), increase half-life of MKP-1 by phosphorylation of Ser359 and Ser364 on MKP-1 (Brondello et al., 1999), and enhance MKP-1 phosphatase activity (Slack et al., 2001), suggesting a negative feedback loop to inhibit the Erk activity (Bhalla et al., 2002).



**Figure 1.4. Network diagram depicting complex interactions between the PI3K/Akt and Ras/Erk pathways (adapted from Haugh and Weiger, 2006)** Arrow diagram illustrating interactions among signaling proteins mediated by receptor tyrosine kinases (RTKs) and other cell surface receptors. Many of these interactions represent crosstalk between major signaling pathways, such as the Ras and phosphoinositide 3-kinase (PI3K) pathways, which converge on the mitogen-activated protein kinases, Erk-1/2.

## **1.6. Mathematical modeling of signal transduction pathways and networks**

Since the 1990s, quantitative modeling has emerged as a powerful tool to explain the overwhelmingly complex interactions and to interpret the meaning of experimental data (Bhalla and Iyengar, 1999; Haugh et al., 2000; Tyson et al., 2003; Ma'ayan et al., 2005; Kholodenko, 2006; Haugh and Weiger, 2006; Janes and Yaffe, 2006). These mechanistic models, i.e. models based on established principles of physical chemistry and mechanics, were created with the initial purpose to understand the temporal dynamics of signaling responses. Kinetic models of specific pathways, in conjunction with experimental data, were used to extract important clues regarding the underlying biochemical mechanisms involved and their integrated behavior. Of particular relevance here are those models that have lent insight into the dynamics of signaling pathway crosstalk (Bhalla and Iyengar, 1999; Bhalla et al., 2002; Kholodenko et al., 2002; Hatakeyama et al., 2003; Kiyatkin et al., 2006).

In previous work, we acquired quantitative biochemical measurements of PDGF receptor phosphorylation and activation of the PI3K/Akt pathway in NIH 3T3 fibroblasts, and we formulated kinetic models of these processes. Hence, we have a good handle on the underlying mechanisms and their dose response behavior. The pathway exhibits saturation at the level of PI3K, such that all of the PI3K is recruited at submaximal receptor phosphorylation (Park et al., 2003).

In this study, a mathematical model was constructed based on known or plausible signaling mechanisms in the PDGF receptor signaling network, with appropriate simplifications, as described in detail in the Appendix. This mathematical model was mainly built by Professor Jason Haugh and Murat Cirit, and could be a useful tool to explain

our result. Through a kinetic analysis of PI3K and Ras/Erk signaling in the PDGF receptor system, complemented by the quantitative modeling, we find that PI3K signaling affects Erk activation both upstream and downstream of Ras, and that Ras and PI3K account for most if not all of the pathways from PDGF receptors to Erk. The magnitudes of the Ras- and PI3K-dependent effects are shown to depend strongly on the stimulation conditions, in ways that are readily explained based on the saturability of the respective pathways and negative feedback mechanisms. Interestingly, crosstalk from the Ras/Erk pathway to PI3K is shown to be far less significant, an observation that we speculate is tied to the ability of PDGF receptors to directly and potently activate PI3K signaling. Through modeling and computation, the magnitudes of PI3K-dependent crosstalk mechanisms are quantified, and predictions are generated to inform further refinement of those mechanisms.

## **CHAPTER 2. MATERIALS AND METHODS**

### **2.1. Reagents**

All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). Human recombinant PDGF-BB was from Peprotech (Rocky Hill, NJ). Antibodies against Ras (Y13-259, agaroseconjugated), Akt-1/2 N terminus, Erk-1 C terminus, and MKP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Akt pSer473, Erk pThr202/pTyr204 and anti-rabbit IgG HRP conjugated antibody were from Cell Signaling Technology (Beverly, MA). All pharmacological inhibitors were from Calbiochem (San Diego, CA); where applicable, cells were pre-incubated with the inhibitor for 30-60 minutes prior to PDGF stimulation. Unless otherwise noted, all other reagents were from Sigma-Aldrich (St. Louis, MO).

### **2.2. Plasmid and retroviral infection**

S17N and G12V H-Ras plasmids were used as PCR templates for cloning into the Not I/BamHI sites of the retroviral vector pBM-IRES-Puro, a kind gift from Drs. Steven Wiley and Lee Opresko (Pacific Northwest National Labs). All constructs were confirmed by DNA sequencing. The ecotropic  $\phi$ NX packaging cells were transfected by calcium phosphate precipitation for 4 hours with 15  $\mu$ g pBM-IRES-Puro construct per 100 mm plate. After replacing the medium, viral supernatants were collected at 24, 28 and 32 hours after transfection, filtered, supplemented with 5  $\mu$ g/ml polybrene, and used for serial infection of NIH 3T3 fibroblasts. Where applicable, NIH 3T3 cells were serially infected with

retrovirus bearing empty vector, S17N H-Ras, or G12V H-Ras and selected using puromycin (2 µg/ml) prior to each experiment.

### **2.3. Cell culture and lysate preparation**

NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, VA) and variant cell lines were cultured at 37°C, 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (BSA), 2mM L-glutamine, and the antibiotics penicillin and streptomycin. Where applicable, Ras expression was maintained under puromycin selection. Cells were serum-starved in DMEM containing 0.1% FBS, 2mM L-glutamine, and the antibiotics penicillin and streptomycin, and 1 mg/ml fatty acid-free BSA, for 3 hours prior to PDGF-BB stimulation. PDGF-BB was added to each plate at the final concentration indicated. The cells were washed once with ice-cold Dulbecco's phosphate-buffered saline (PBS) and then lysed in ice-cold buffer containing 50mM HEPES, pH 7.4, 100mM NaCl, 10% v/v Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50mM β-glycerophosphate, pH 7.3, 5 mM sodium fluoride, 1mM EGTA, and 10 µg/ml each aprotinin, leupeptin, pepstatin A, and chymostatin. After scraping insoluble debris and transferring to an Eppendorf tube, the lysates were vortexed briefly, incubated on ice for 10 minutes, and clarified by centrifugation. The supernatants were collected and stored frozen at -70°C.

### **2.4. Quantitative immunoblotting**

Pooled cell lysates were subjected to SDS-PAGE gels using standard techniques. After

gel electrophoresis, proteins were transferred to PVDF membrane (Immobilon, Millipore, Bedford, MA) and probed with the indicated antibodies. The blots were incubated with chemiluminescence substrates (Pierce, Rockford, IL) and imaged using a high sensitivity CCD camera (BioRad Fluor S-Max, Hercules, CA).

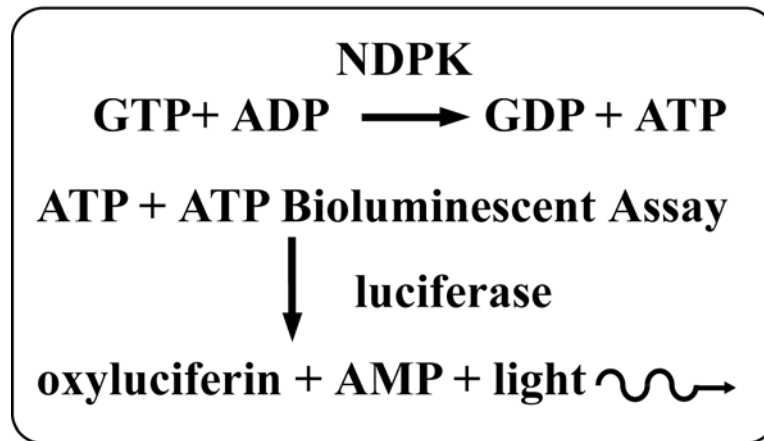
## **2.5. Ras-GTP biochemical assays**

To determine the amounts of Ras-bound GTP, eluted from anti-Ras immunoprecipitates (Y13-259, agarose-conjugated), the coupled NDPK (nucleoside 5'-diphosphate kinase; Sigma N2635)/luciferase assay of Boss and colleagues (Scheele et al., 1995; Sharma et al., 1998) was used (Fig. 2.1a). A significant reduction in background was gained by preparing an exhaustively dialysed NDPK stock. This allowed us to pre-incubate 50 µl of GTP/GDP sample with 50 µl of FL-AAM (firefly luciferase/ATP assay mixture; Sigma), supplemented with 100 nM purified ADP, in order to deplete any remaining ATP contamination in the sample or ADP stock. To this, 50 m-units of the NDPK was added to initiate the reaction, and light production was integrated over 10 min in a PerkinElmer 96-well-plate-reading luminometer. Ras-bound GTP+GDP was quantified by first phosphorylating GDP to GTP in the presence of pyruvate kinase and phospho(enol)pyruvate (Sigma P9136 and P7127 respectively) (Sharma et al. 1998). GDP and GTP standards, pre-treated in this manner, yielded near identical standard curves (Fig. 2.1b&c). These assays were performed as described previously (Kaur et al., 2006).

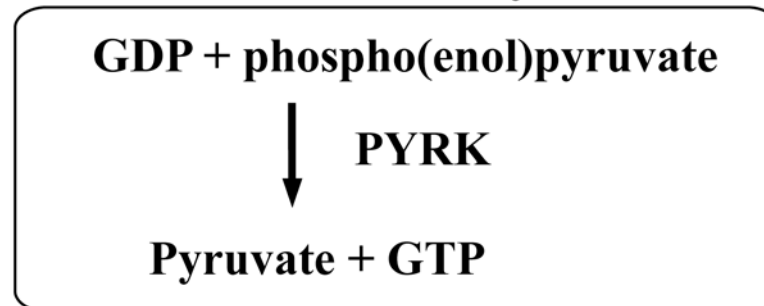
**Figure 2.1. Ras-GTP biochemical assays.** *a.* The reactions involved in Ras-GTP assays. *b* & *c.* Standard curve showing linearity of the assay and low fmol sensitivity (*b.* raw data of light output vs. time for various GTP amounts).

a.

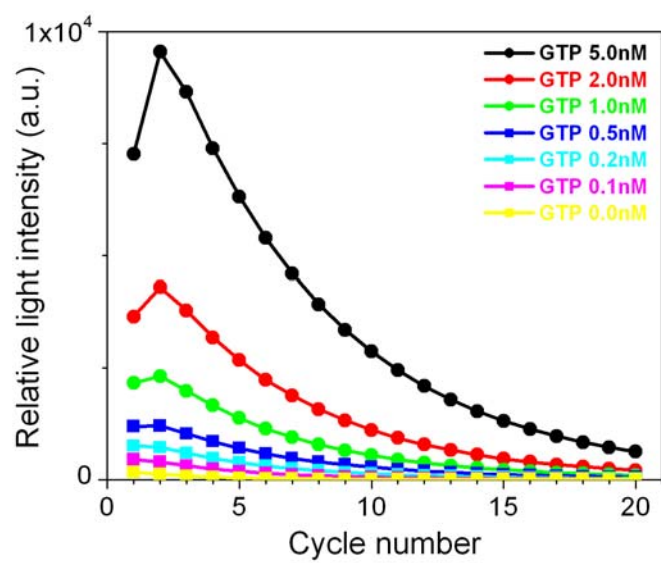
### **GTP assay**



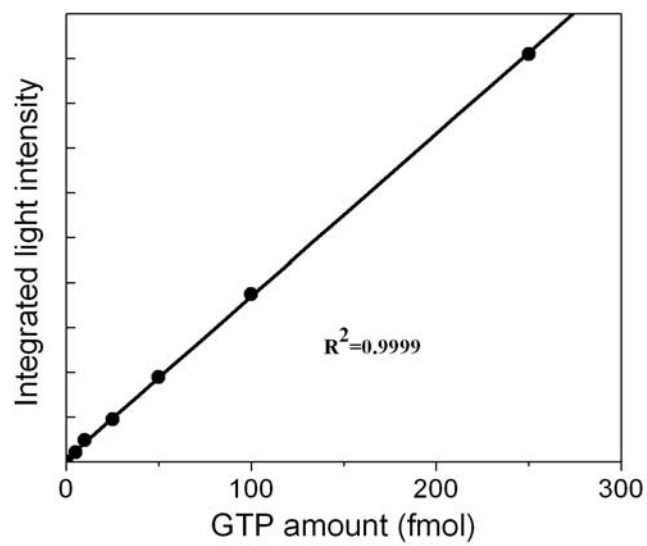
### **GDP assay**



b.



c.



## **2.6. Data normalization and statistical analysis**

All biochemical data were first normalized by an appropriate loading control as stated in the corresponding figure legend (e.g., phosphorylated Erk-1/2 was normalized by total Erk-1). To evaluate the consistency of relative trends across independent experiments, the data were further normalized as follows. Sets of data collected on different days were multiplied by normalization factors so as to minimize the average coefficient of variation (standard deviation/mean) for the time course of cells stimulated with 1 nM PDGF concentration under control conditions (DMSO only or pBM-IRES-Puro vector only); each normalization factor was then applied to the rest of the data collected on the same day (control conditions with lower PDGF concentrations and either inhibitor or Ras variant expression conditions). Where applicable, normalized means for control and perturbed conditions were compared by student's t-test, using the most conservative model (two-tails, unequal variance).

## **2.7. Kinetic modeling and analysis** (work mainly done by Professor Jason Haugh and Murat Cirit)

A mathematical model was constructed based on known or plausible signaling mechanisms, with appropriate simplifications, as described in detail in the Appendix. The model has 18 time-dependent variables and a total of 44 rate constants or other adjustable parameters, of which 10 were assigned fixed values based on previous work. Remaining parameters were specified using a computational scheme based on the Metropolis algorithm, which is well-suited for kinetic parameter estimation constrained by biochemical data (Violin et al., 2008). Our variation of the method, implemented in MATLAB (The MathWorks),

works as follows (see Appendix for details). Starting with initial parameter values, the model output is computed and aligned with the data, and the quality of fit is evaluated. Next, new values are chosen according to normal distributions centered on the previous values; the width of the distribution is a parameter of the algorithm. If specified error criteria are met, the new parameter set is accepted and used as the nexus for choosing the next parameter values; otherwise, the procedure is repeated with the previous parameter set. If the starting parameter set (determined based on a separate error minimization subroutine) is nearly optimal, then the parameter sets generated in this manner constitute an ensemble that fit the data almost equally well. After compiling the ensemble, the model output is recalculated for each parameter set, and at each time point, an ensemble mean and standard deviation are calculated.

## **CHAPTER 3. RESULTS**

Adapted from Wang et al., PI3K-dependent crosstalk interactions converge with Ras as quantifiable inputs integrated by Erk, submitted for publication.

### **3.1. PDGF-stimulated Akt and Erk phosphorylation as a function of time and PDGF dose**

Intracellular signals are dynamic (Hunter, 2000; Pawson, 2004), and time courses are perhaps the most fundamental type of signaling data set. We measured the time-course data; using time-course plots to visualize changes in signals over time while keeping the quantitative information of the original data. Initially we examined the kinetic phosphorylation of Akt and Erk under control conditions (DMSO only or pBM-IRES-Puro vector only) in NIH 3T3 fibroblasts, in response to different PDGF-BB concentrations (0.03, 0.1, 0.3 or 1 nM), by quantitative immunoblotting. The levels of phosphorylated protein were normalized by an appropriate loading control (e.g. phosphorylated Erk-1/2 was normalized by total Erk-1), measured in parallel, which was not significantly affected by PDGF stimulation. PDGF-stimulated Akt and Erk phosphorylation depend on both time and PDGF dose (Fig. 3.1&3.2). These data sets provide a clear picture of our system.

### **3.2. PI3K is effectively insulated from crosstalk**

To ascertain the degree of cooperation between PDGF receptors and Ras in the PI3K/Akt pathway, we modulated the levels of Ras-GTP through the expression of G12V H-Ras or S17N H-Ras, quantified dynamic Akt phosphorylation by quantitative

immunoblotting for different combinations of PDGF concentrations and time points.

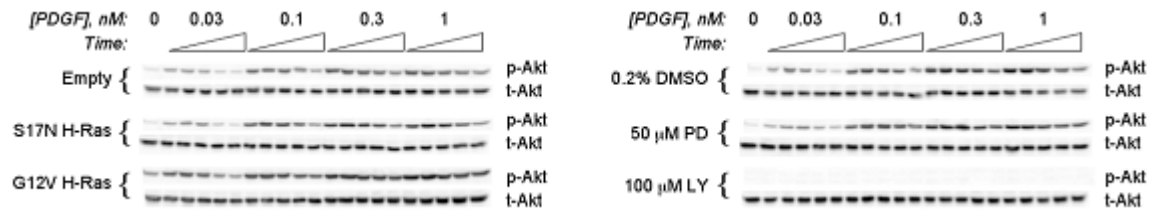
Previous experiments confirmed that the expression of S17N H-Ras in our cells, by retroviral infection and puromycin selection, prevents Ras-GTP loading in response to PDGF stimulation. Ras-GTP levels followed the prospective trend (S17N<control<<G12V) (Kaur et al., 2006). In this study, we observed PI3K signaling, manifested as Akt phosphorylation, is not significantly affected by either blocking (by expression of S17N Ras) nor elevating (by expression of G12V Ras) activation of Ras, except for a higher basal Akt phosphorylation level in unstimulated G12V Ras cells (Fig. 3.1). This result suggests PDGFR-mediated PI3K signaling is not affected by Ras-GTP levels in our system.

The PDGF-stimulated increase in Ras-GTP is transient, due to a negative feedback loop mediated by downstream components, Erk. Inhibition of Erk signaling could help to increase the duration of Ras activation. In this study, we showed blocking MEK and Erk activation (using the MEK inhibitor PD098059) has not a significant effect on PDGF-stimulated activation of Akt (Fig. 3.1). We also confirmed that the PD098059 completely blocked PDGF-stimulated Erk-1/2 phosphorylation (Fig. 3.2) and, as we will show, leads to higher, more sustained Ras-GTP levels in our cells (Fig. 3.5), presumably by relieving feedback desensitization of mSos (Waters et al., 1995a; Klarlund et al., 1995; Langlois et al., 1995).

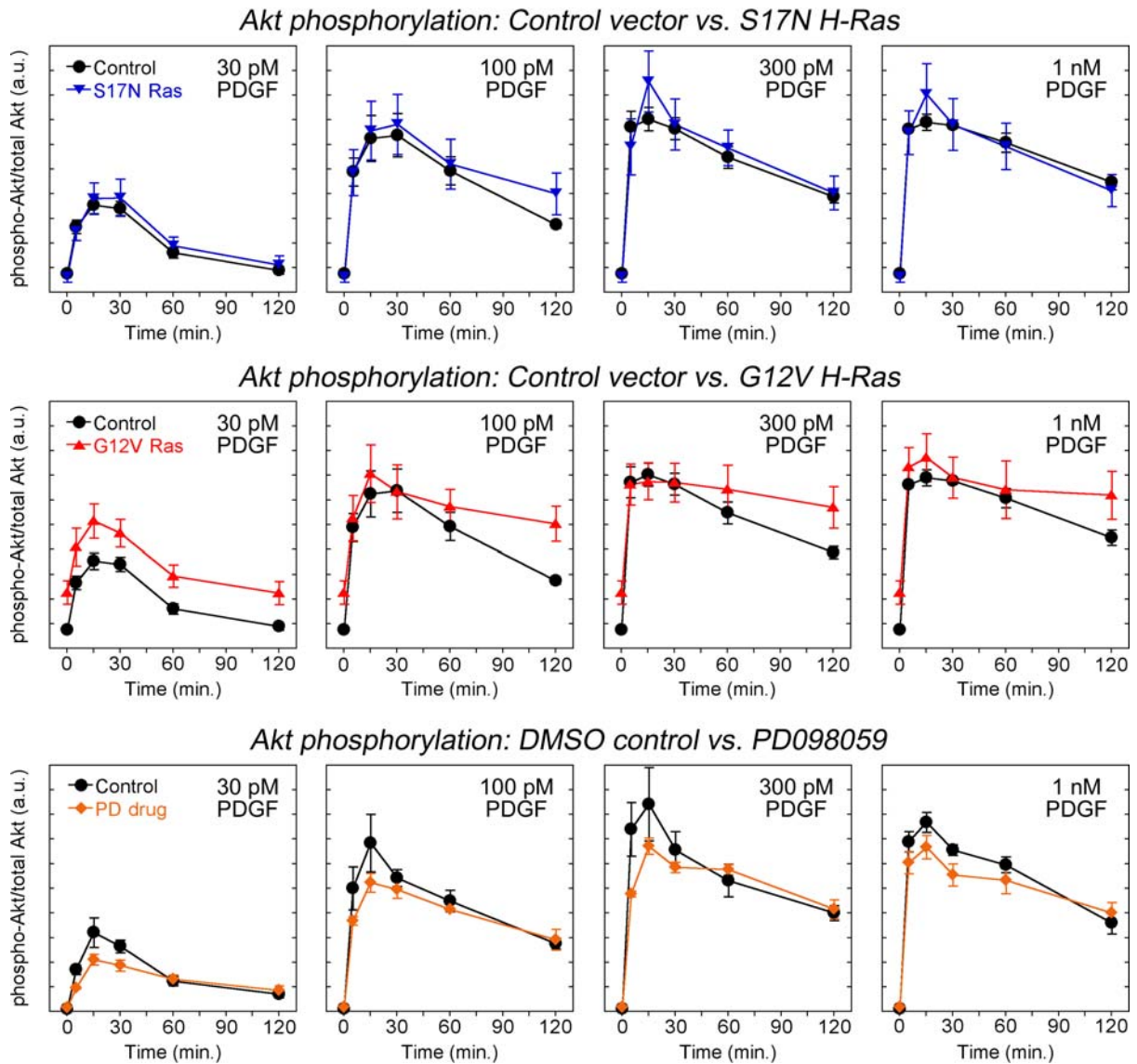
Taken together, neither expression of dominant-negative Ras nor the MEK inhibitor PD098059 has a significant effect on Akt phosphorylation, suggesting the PI3K pathway is affected very little by crosstalk from Ras.

**Figure 3.1. Systematic analysis of PDGF-stimulated Akt phosphorylation kinetics.** *a.* Immunoblots, representative of 5 or 6 independent experiments, used to quantify relative amounts of phosphorylated Akt (p-Akt; Ser473) and total Akt (t-Akt) are shown. NIH3T3 fibroblasts were modulated by retroviral induction of dominant-negative (S17N) or constitutively active (G12V) H-Ras expression or incubation with inhibitors of PI3K (100  $\mu$ M LY294002) or MEK (50  $\mu$ M PD098059). The respective controls are empty vector or vehicle only (0.2% DMSO). Lysates were prepared from cells that were unstimulated or stimulated with the indicated PDGF-BB concentration for various times (5, 15, 30, 60, or 120 min.). *b.* & *c.* Quantification of Akt phosphorylation, normalized as described under methods, comparing either S17N or G12V Ras expression (*a*,  $n = 6$ ) or MEK inhibition (*b*,  $n = 5$ ) with their respective controls. Values are reported as mean  $\pm$  s.e.m., and comparisons to control are by student's t-test: \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

a.



b. & c.



### **3.3. PI3K and Ras make independent contributions to Erk signaling**

In previous work, we showed that the expression of S17N Ras prevents PDGF-stimulated Ras-GTP loading in our cells (Kaur et al., 2006). In this study, to examine the signal strength from Ras-GTP, which exerts a positive influence on downstream Raf/MEK/Erk cascade, we modulated the levels of Ras-GTP through the expression of G12V H-Ras or S17N H-Ras and quantified dynamic Erk-1/2 phosphorylation by quantitative immunoblotting. The results reveal that when Ras is inhibited (by expression of S17N H-Ras), PDGF-stimulated Erk phosphorylation is partially inhibited (Fig. 3.2). Expression of constitutively active (G12V) Ras yields an elevated level of Erk phosphorylation prior to stimulation but also a compressed PDGF dose response (Fig. 3.2), consistent with high constitutive expression of mitogen-activated protein kinase phosphatase (MKP) as reported previously (Kaur et al., 2006).

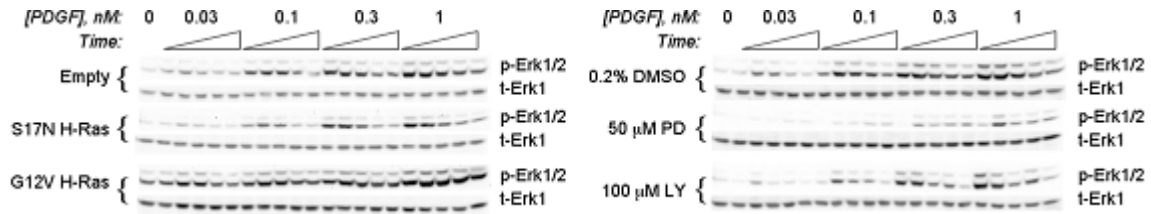
To examine the overall effect of PI3K, which might exert both positive and negative effects on Erk phosphorylation, we used the PI3K inhibitor LY294002. The results reveal that blocking PI3K partially inhibits PDGF-stimulated activation of Erk (Fig. 3.2). We have confirmed that the LY294002 completely blocked PDGF-stimulated activation of Akt (Fig. 3.1) and control compound LY303511, which is pharmacologically similar to LY294002 except that it does not inhibit PI3K (Knight et al., 2006; Gharbi et al., 2007), does not affect Erk phosphorylation significantly (Fig. 3.3).

Taken together, blocking either activation of Ras or of PI3K partially inhibits PDGF-stimulated activation of Erk. The degree of Erk inhibition depends strongly on both stimulation dose and time; no single combination of these conditions is indicative of the

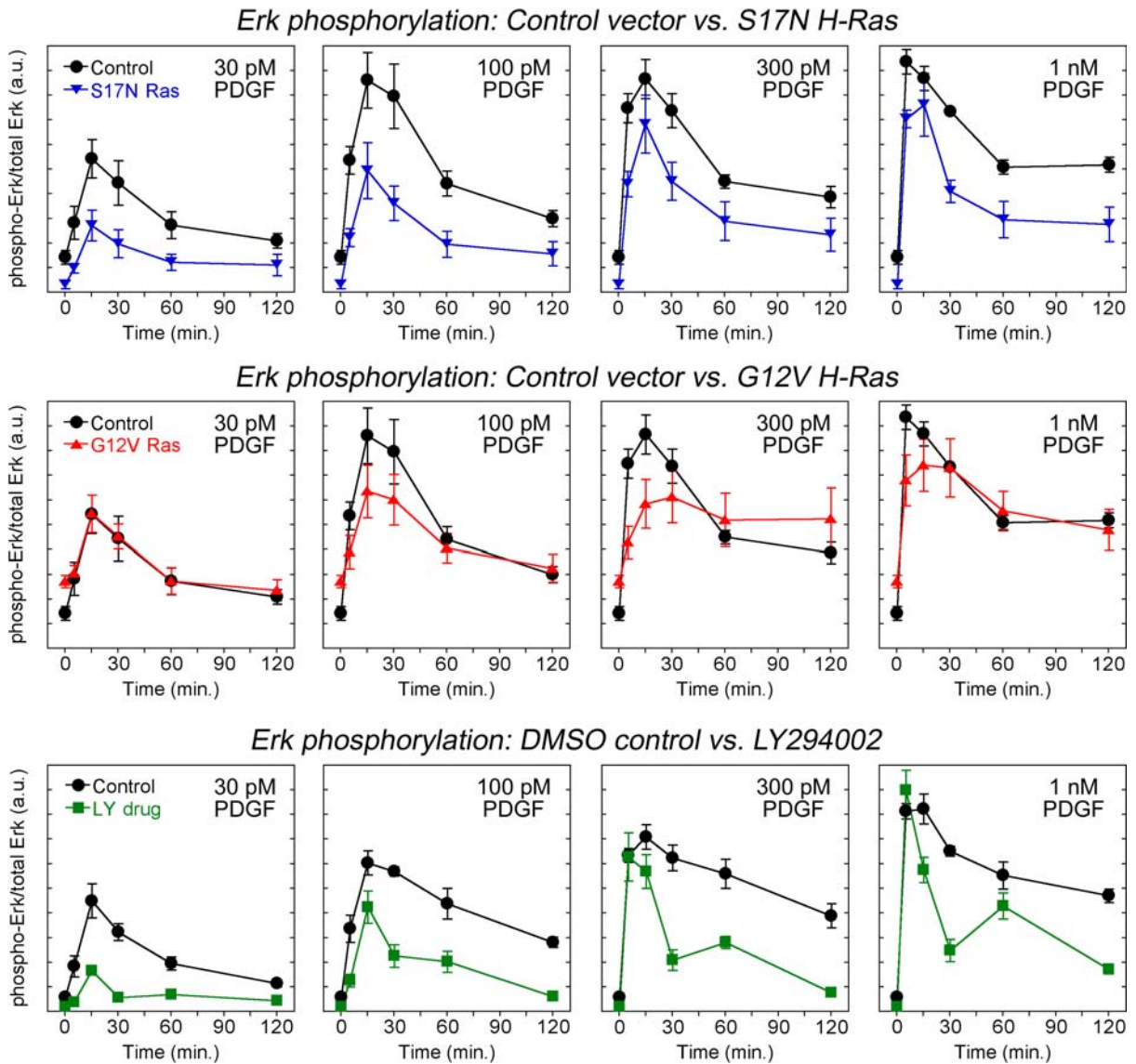
relative roles of Ras and PI3K in activating Erk. At short stimulation times, PI3K inhibition significantly affects Erk activation at low but not high PDGF concentrations, in accord with a previous study (Duckworth and Cantley, 1997); however, even at saturating PDGF concentrations, the kinetics of Erk phosphorylation in PI3K-inhibited cells are less sustained. The effects of Ras inhibition are similar, except that Erk phosphorylation is less sensitive to PDGF concentration and shows a lower initial rate at higher PDGF doses.

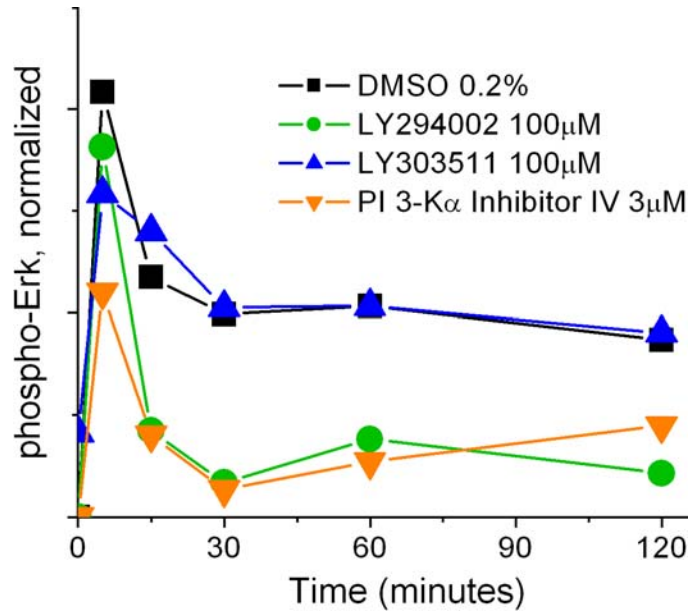
**Figure 3.2. Systematic analysis of PDGF-stimulated Erk phosphorylation kinetics.** *a.* Same as in Figure 3.1a, except that representative immunoblots for phosphorylated Erk (p-Erk1/2; pThr202/pTyr204) and total Erk (t-Erk1) are shown. *b.* Same as in Figure 3.1b, except quantification is for Erk phosphorylation. *c.* Same as in Figure 3.1c, except quantification is for Erk phosphorylation, and the comparison is between PI3K inhibition and the DMSO control.

a.



b. & c.





**Figure 3.3. Specificity of PI3K inhibition.** The PI3K inhibitor LY294002 is known to have off-target effects. LY303511 has the same or similar pharmacological profile with respect to those targets but does not inhibit PI3K. The results shown, representative of two independent experiments, confirm that LY303511 does not significantly affect Erk phosphorylation, stimulated by PDGF (1 nM) and quantified by immunoblotting (phospho-Erk/total Erk), and that another PI3K inhibitor yields similar effects as LY294002.

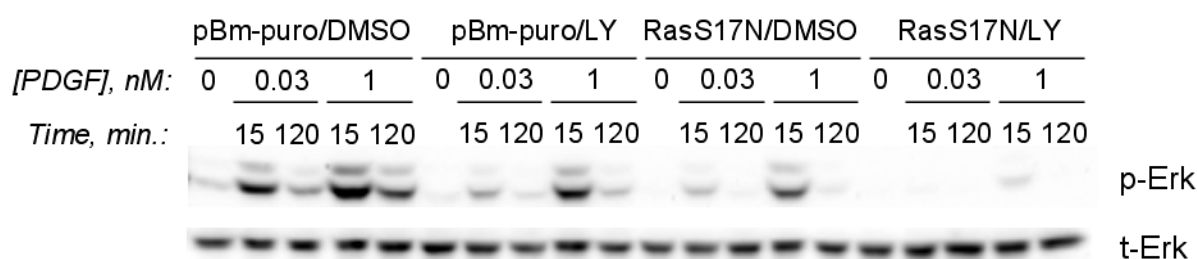
### **3.4. PI3K and Ras account for Erk activation**

Blocking either Ras or PI3K only partially inhibits Erk. These results suggested two possible Erk activation mechanisms, or combinations thereof. One possibility is that Ras and PI3K make independent contributions that operate in parallel to activate Erk; alternatively, these two pathways are linked by a common mechanism (e.g., PI3K is required for Ras activation), in which case at least one other pathway would need to be identified to account for the residual Erk phosphorylation seen when either Ras or PI3K is inhibited. In each of three independent experiments, simultaneous inhibition of both Ras and PI3K completely blocked PDGF-stimulated Erk phosphorylation (Fig. 3.4). It confirmed that Ras and PI3K make independent contributions to Erk activation and excluded the possibility that Ras- and PI3K-independent pathways are involved. We conclude that Ras and PI3K are responsible for all of the major pathways from PDGF receptors to Erk, and at least one mode of PI3K-dependent crosstalk to Erk operates in parallel with Ras. Incidentally, this result also provides further confirmation that Ras function is effectively blocked in our S17N H-Ras-expressing cells.

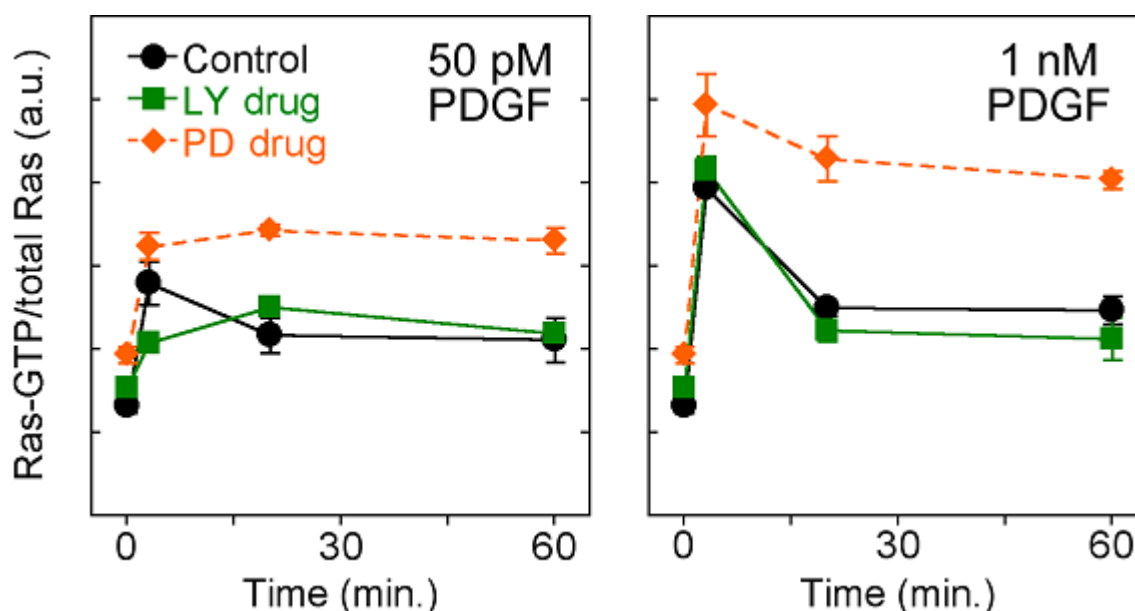
### **3.5. PI3K enhances Erk activation at points both upstream and downstream of Ras**

Having established a role for PI3K-dependent signaling to Erk in parallel with Ras, we sought to determine whether or not PI3K also partially contributes to the PDGF receptor-mediated activation of Ras. Using a coupled enzymatic assay that quantifies the amounts of GTP and the sum of GDP + GTP eluted from anti-Ras immunoprecipitates (Scheele et al., 1995; Kaur et al., 2006), we measured the kinetics of PDGF-stimulated Ras-GTP loading for selected conditions (Fig. 3.5). In each of three independent experiments, incubation with the PI3K inhibitor LY294002 reduced the initial rate of Ras-GTP loading at a low PDGF concentration (50 pM), whereas Ras-GTP loading kinetics were minimally affected at 1 nM PDGF. Interestingly, the plateau level of Ras-GTP in PI3K-inhibited cells was similar to that of control cells, even at the lower dose of PDGF. Combined with the results described above establish a role for PI3K-dependent signaling to Erk in parallel with Ras, we concluded that PI3K affects Erk activation at points both upstream and downstream of Ras.

As alluded to earlier, Ras-GTP loading is also affected by a known negative feedback loop that destabilizes Ras-GEF recruitment. Consistent with such a mechanism, incubation with the MEK inhibitor PD098059 yielded a higher Ras-GTP level (Fig. 3.5). The feedback loop might also explain the effect of PI3K inhibition seen at lower PDGF concentrations. Erk phosphorylation is dramatically reduced under those conditions (Fig. 3.2b), and thus the feedback regulation might be relieved. The net effect of reduced but stable Ras-GEF recruitment would be a lower initial rate and more sustained level of Ras-GTP loading.



**Figure 3.4. Ras and PI3K account for all of the major PDGF-stimulated pathways that converge on Erk.** NIH 3T3 fibroblasts were infected with retrovirus produced from empty pBM-puro vector or vector with dominant-negative (S17N) H-Ras, pretreated with either DMSO vehicle control or LY294002, then stimulated with PDGF-BB (dose and time as indicated). Whereas inhibition of Ras or PI3K only partially blocks Erk phosphorylation, consistent with Fig. 3.2, blocking both Ras and PI3K abolishes PDGF-stimulated Erk phosphorylation. The immunoblot shown is representative of three independent experiments.

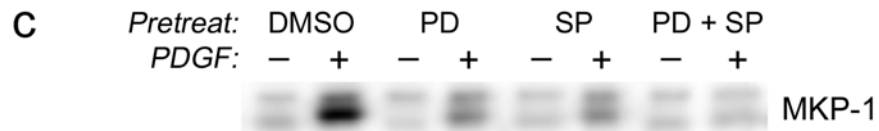
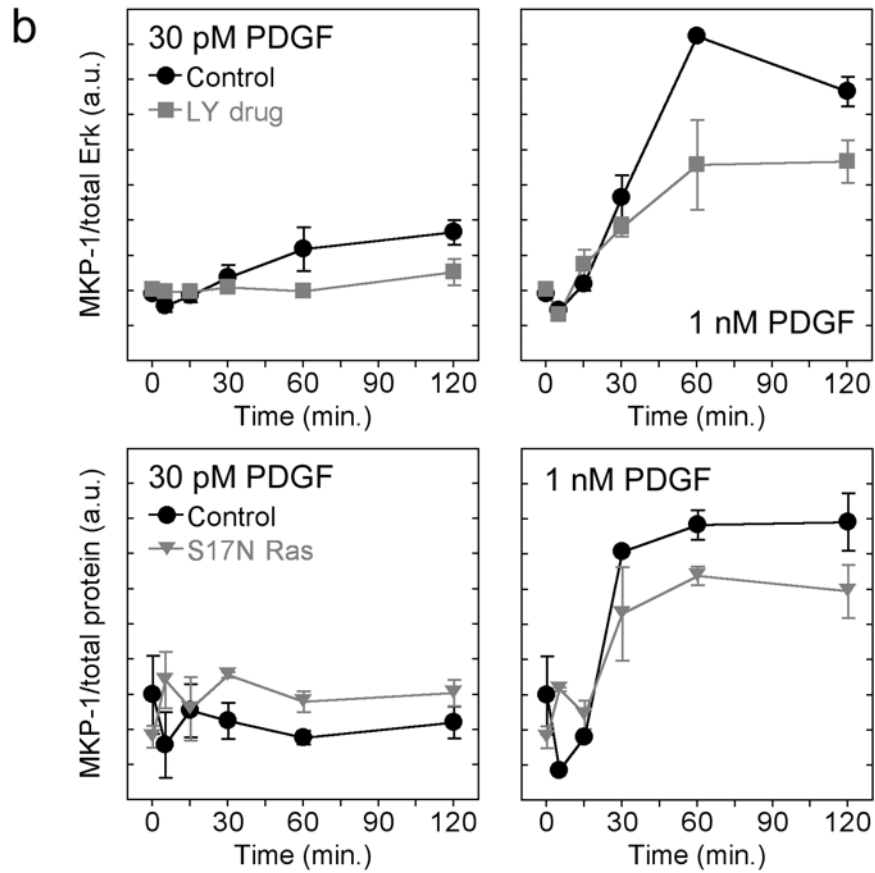
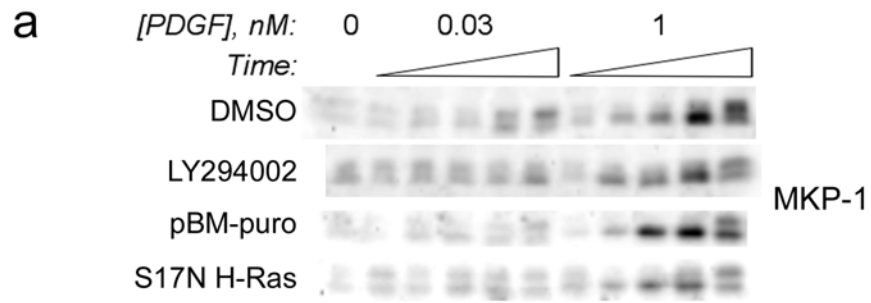


**Figure 3.5. Quantitative Ras-GTP loading measurements: characterization of Erk-dependent feedback regulation and PI3K-dependent crosstalk.** NIH 3T3 fibroblasts were either unstimulated or stimulated with 50 pM or 1 nM PDGF-BB for 3, 20, or 60 min. Pretreatments were control (0.2% DMSO), LY294002 (100  $\mu$ M), or PD098059 (50  $\mu$ M). Ras-GTP levels were measured using a quantitative enzymatic assay and normalized as described under Methods, and the values are reported as mean  $\pm$  s.e.m. ( $n = 3$ ).

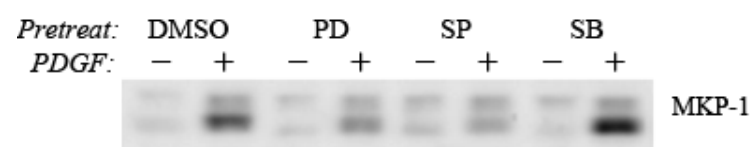
### **3.6. Multiple negative feedback loops shape the integrated Erk phosphorylation response**

In addition to feedback regulation of Ras-GEF, we also characterized the upregulation of MKP expression induced by Erk and other mitogen-activated protein kinases (Brondello et al., 1997; Bhalla et al., 2002; Plows et al., 2002) (Fig. 3.6a&b). MKP-1 expression is upregulated ~ 3-fold in response to 1 nM PDGF, after a lag time of approximately 15 minutes. At 30 pM PDGF, which elicits roughly half-maximal Erk phosphorylation (Fig. 3.2b&c), MKP-1 expression increases only slightly, suggestive of a signaling threshold for triggering the feedback loop. A similar MKP-1 response is observed when either Ras or PI3K is inhibited, with a partial reduction in the fold-induction (Fig. 3.6b). As expected, inhibition of MEK/Erk or c-Jun N-terminal kinase (JNK) (but not of p38; Fig. 3.6d) significantly reduces the induction of MKP-1 expression, and simultaneous inhibition of MEK and JNK blocks MKP-1 upregulation completely (Fig. 3.6c).

**Figure 3.6. Delayed onset and threshold for MKP-1 upregulation, a known negative feedback loop in the Erk pathway.** *a.* Immunoblots of MKP-1 levels in control, PI3K-inhibited, or S17N H-Ras-expressing cells that were unstimulated or stimulated with PDGF-BB for 5, 15, 30, 60, or 120 min.; each blot is representative of 2 or 3 independent experiments. *b.* Quantification of MKP-1 expression, with either total Erk1 or total protein as the loading control and normalized as described under Methods. Values are reported as mean  $\pm$  s.e.m. ( $n = 3$  for DMSO and LY294002;  $n = 2$  for control vector and S17N Ras). *c.* MEK/Erk and JNK pathways contribute to feedback upregulation of MKP-1. PDGF stimulation was 1 nM for 60 min. MEK or/and JNK were inhibited by incubation with 50  $\mu$ M PD098059 (PD) and 25  $\mu$ M SP600125 (SP), respectively. The DMSO concentration was 0.25% in all cases. The blot is representative of two independent experiments. *d.* p38 pathway does not contribute to feedback upregulation of MKP-1. Same as in *c*, except that p38 was inhibited by incubation with 25  $\mu$ M SB203580 (SB).



d



### **3.7. Parsing the magnitudes of PI3K-dependent crosstalk interactions in the PDGF receptor signaling network** (work mainly done by Professor Jason Haugh and Murat Cirit)

Synthesizing the data assembled, a conceptual model of the PDGF receptor signaling network is now clear (Fig. 3.7a). Yet, the unique dose response and kinetic information in the data set allows for a more quantitative description through mathematical modeling and analysis. The relatively coarse granularity of our model, described in detail in the Appendix, reflects a careful balance between the level of molecular detail included and both the uncertainty of those details and the ability to specify model parameters. The model builds upon previous work characterizing PDGF receptor and PI3K activation dynamics in these cells (Park et al., 2003).

A total of 34 unspecified parameter values were estimated using a Monte Carlo-based algorithm that directly and globally compares the model output to the experimental data (Appendix); thus, an ensemble of 10,000 parameter sets that fit the data almost equally well was assembled. This approach embraces the inherent ‘sloppiness’ of kinetic models, which prohibits the reliable identification of precise parameter values yet allows for predictions based on collective fits to data (Gutenkunst et al., 2007). The model output is expressed as an ensemble average (Violin et al., 2008) and compared to the corresponding data (Fig. 3.7b). Additional data used to constrain the algorithm indicates the degree of saturation in the MEK/Erk pathway (Fig. 3.8). Taken together, the data force the model to reconcile time- and PDGF dose-dependent features of the network observed under control and perturbed conditions. In the context of the model, the strength of the inputs converging on MEK/Erk determines the saturability of Erk phosphorylation with respect to PDGF dose and also the

degree to which the response adapts.

Parameter statistics (Appendix) show that many parameters tend to be pegged at arbitrarily high values, suggesting ways to simplify the model without a significant change in output. For example, the algorithm consistently picked parameter sets in which the enzymes operate far from saturation. This observation facilitates our analysis of Ras- and PI3K-dependent MEK phosphorylation, in which we examine the catalytic efficiency of each phosphorylation step relative to that of the opposing dephosphorylation reaction (Fig. 3.7c). The results reveal a consistent ratio of PI3K- and Ras-dependent contributions, as indicated by points lying on a diagonal line, with the Ras-dependent pathway tending to be more potent under maximal PDGF stimulation (Fig. 3.7c). To further quantify these pathways, we formulated a single number, the MEK activation comparator (MAC), which compares their capacities to generate dually phosphorylated MEK (ratio of PI3K-dependent/Ras-dependent; see Appendix). Over the parameter set ensemble, the median MAC value is 0.22, with quartile values of 0.17 and 0.30; that is, the PI3K-dependent MEK activation pathway is roughly one-fifth as potent as the Ras-dependent pathway under maximal PDGF stimulation in our model. However, the MAC does not account for the feedback desensitization of Ras-GTP loading, which attenuates the magnitude of the Ras-dependent input.

Upstream of Ras, a similar analysis was performed for PI3K-dependent and PI3K-independent modes of Ras-GEF recruitment, which are characterized by specific model parameters (Fig. 3.7d). Together, these modes determine the saturability of Ras-GTP loading with respect to PDGF dose. While most of the parameter sets in the ensemble included a significant PI3K-dependent contribution, a small fraction of them ignored the

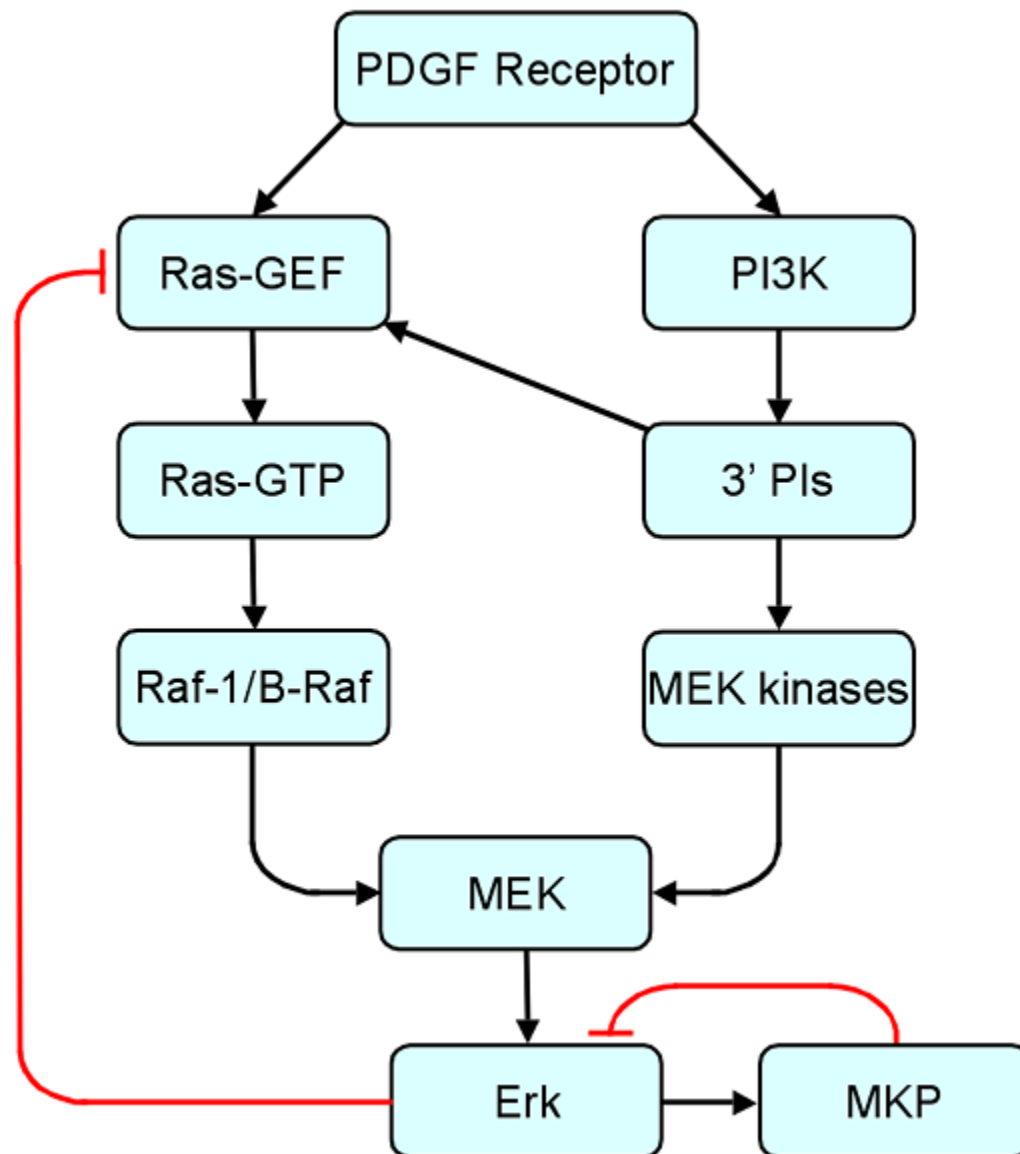
PI3K-dependent mode (Fig. 3.7d). Defining the GEF recruitment comparator (GRC) as the ratio of PI3K-dependent/PI3K-independent modes at low PDGF concentrations (see Appendix), the median GRC value for the ensemble is 1.4, with quartile values of 0.7 and 2.0.

### **3.8. Model predictions and potential for model refinement** (work mainly done by Professor Jason Haugh and Murat Cirit)

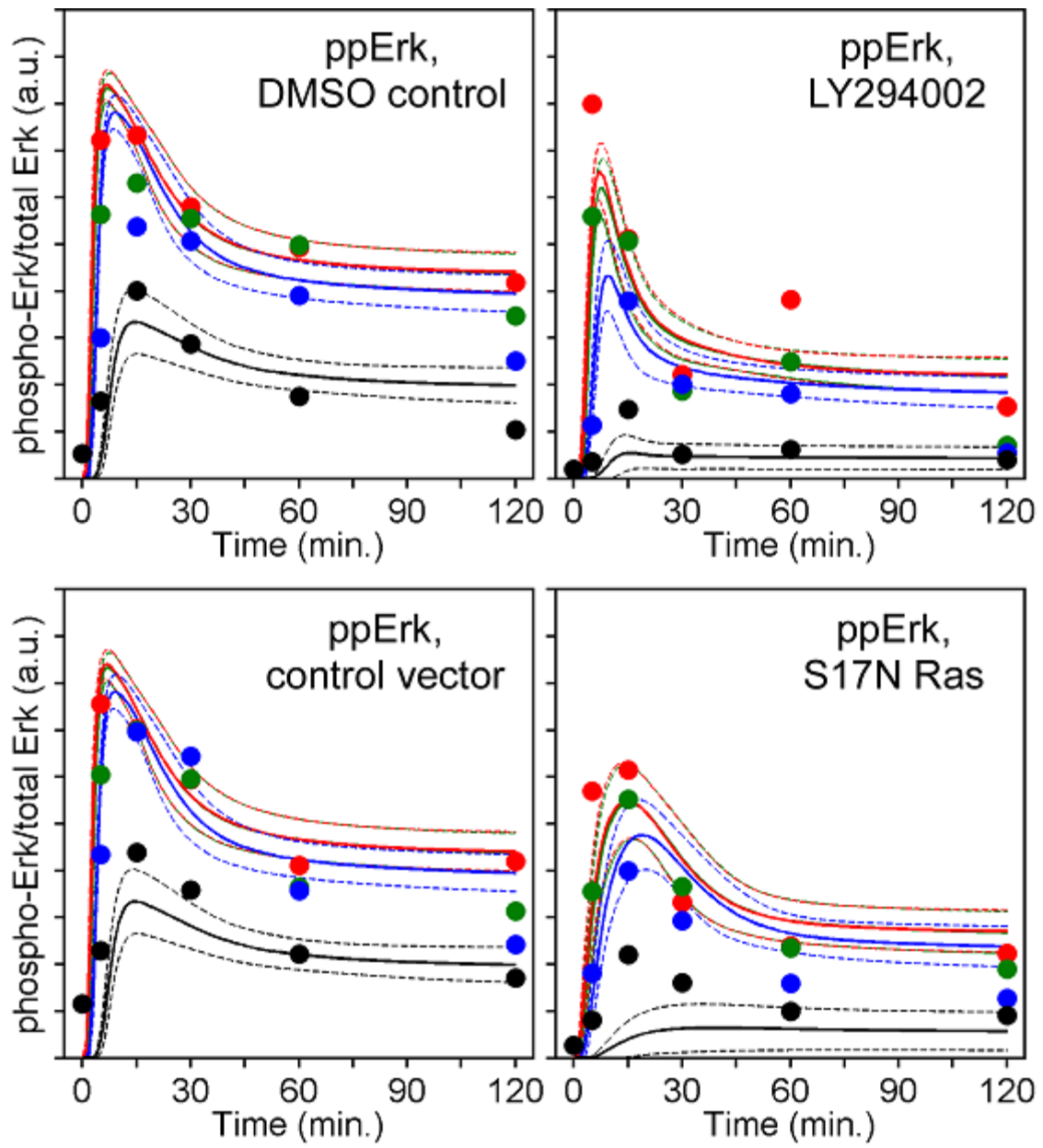
We have shown how data-driven analysis of a kinetic model can be used to quantify inputs to signaling pathways. Another use of this approach is to generate hypothetical predictions with an eye towards future experiments. Here, we focus on the PI3K-dependent crosstalk impinging upstream and downstream of Ras (Fig. 3.7e). Whereas both mechanisms are blocked by PI3K inhibition, the model ensemble predicts unique kinetic signatures that might be expected if either mechanism were silenced selectively. At lower PDGF concentrations, it is predicted that blocking either mechanism would yield Erk phosphorylation levels that are intermediate between control and PI3K-inhibited conditions, but the key discriminator is the Erk phosphorylation kinetics observed with a high PDGF dose; there, if the crosstalk mechanism upstream of Ras is blocked, the kinetics are predicted to be similar to control conditions, whereas if the mechanism downstream of Ras is blocked, the kinetics are predicted to be similar to the PI3K-inhibited case (Fig. 3.7e). The basis for this result stems from the saturability of Ras-GEF recruitment, wherein high PDGF concentrations stimulate maximal Ras-GTP loading even in the absence of PI3K signaling.

**Figure 3.7. Computational modeling and analysis of the PDGF receptor signaling network.** *a.* Minimal conceptual model of the network with crosstalk interactions from PI3K lipid products (3' PIs) affecting Ras-specific GEF (e.g., Grb2-Sos complex) recruitment and activation of MEK kinases. It is recognized that those crosstalk effects might entail multiple steps, and that a more refined model would account for the effect of JNK activation on the MKP negative feedback loop. *b.1 - b.3.* A quantitative kinetic model of the network was formulated, and a Monte Carlo scheme was used to collect an ensemble of parameter sets that fit our data set well (Appendix). The solid curves represent ensemble means, and the dashed curves are mean  $\pm$  s.d. ( $n = 10,000$ ). The data values used to constrain the model (symbols) are also shown (see also Fig. 3.8). PDGF concentrations are: red, 1 nM; green, 300 pM; blue, 100 pM; gray, 50 pM; black, 30 pM. *c.* Quantification of Ras- and PI3K-dependent MEK phosphorylation pathways. For each parameter set described in *b*, the quantity  $C_{xij}$  is defined as the maximum catalytic efficiency of pathway  $i$  ( $i = 1$ , Ras-dependent;  $i = 2$ , PI3K-dependent) towards site  $j$  on MEK divided by the catalytic efficiency of the corresponding phosphatase reaction (Appendix). On the dashed line, the two pathways are equally potent by this measure. *d.* Quantification of the PI3K-independent and PI3K-dependent modes of Ras-GEF recruitment. These are characterized by the model parameters  $K_{GR}$  and  $K_{GP}$ , respectively, plotted here for each parameter set in the ensemble. The factor of 123 is a scaling factor (Appendix); when  $K_{GP} = K_{GR}/123$  (dashed line), the two modes contribute equally to Ras-GEF recruitment in the limit of low PDGF concentration. *e.* Model predictions based on ensemble averaging. Predictions are based on hypothetical interventions by which the PI3K-dependent crosstalk upstream or downstream of Ras are selectively blocked (Appendix), which can be compared with control and PI3K-inhibited (all crosstalk blocked) conditions. Solid curves represent ensemble means, and dashed curves are mean  $\pm$  s.d. ( $n = 10,000$ ). PDGF concentrations are: red, 1 nM; black, 30 pM.

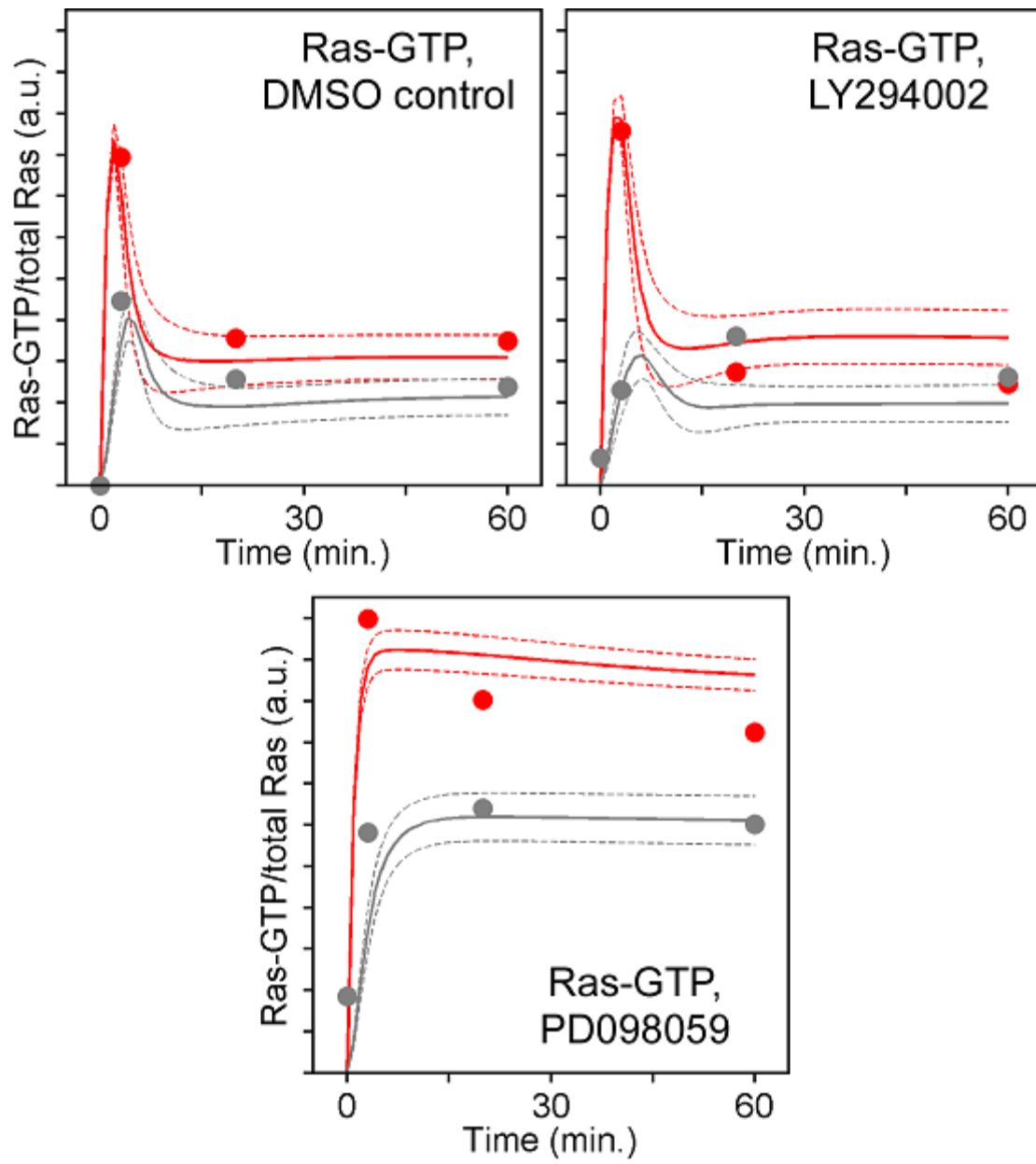
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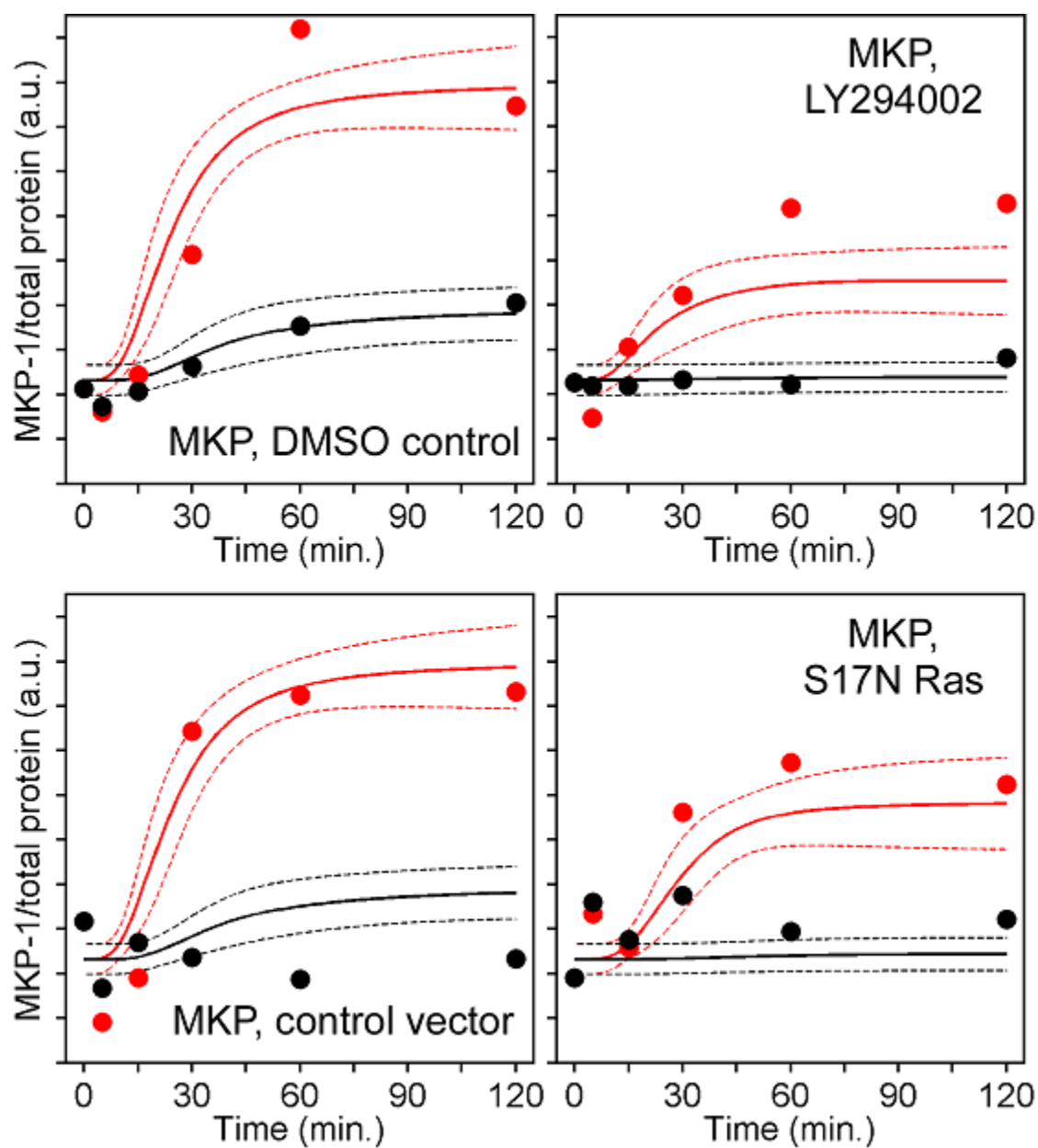
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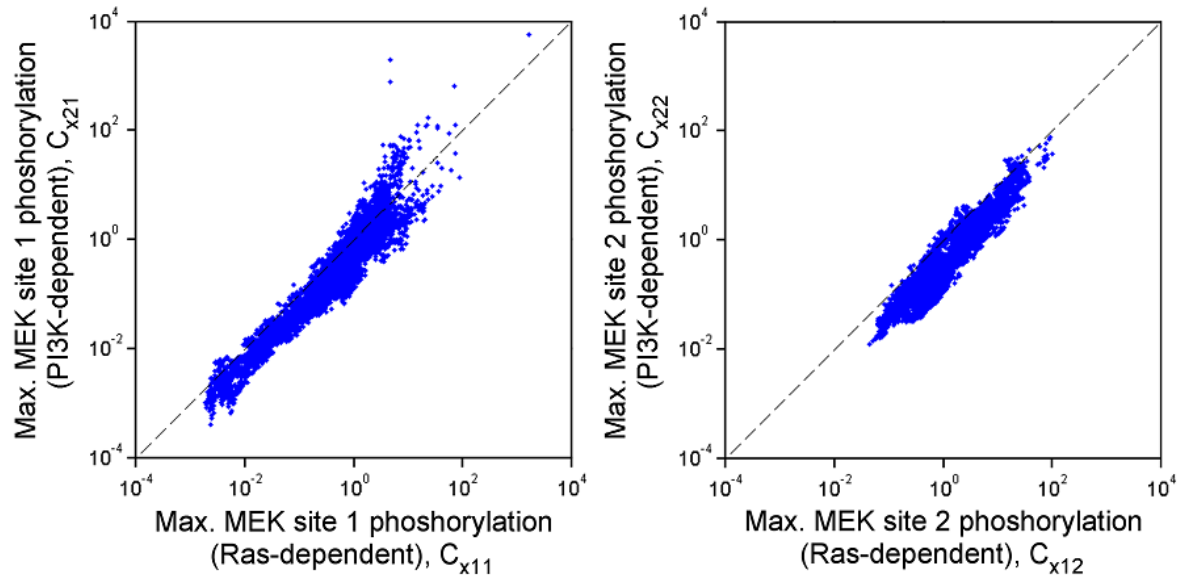
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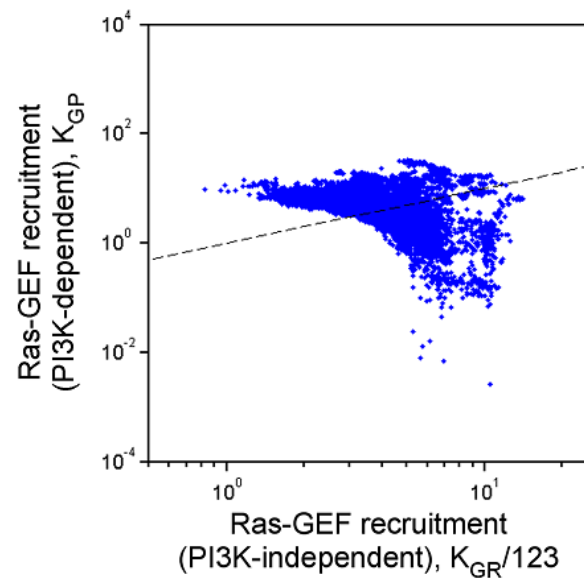
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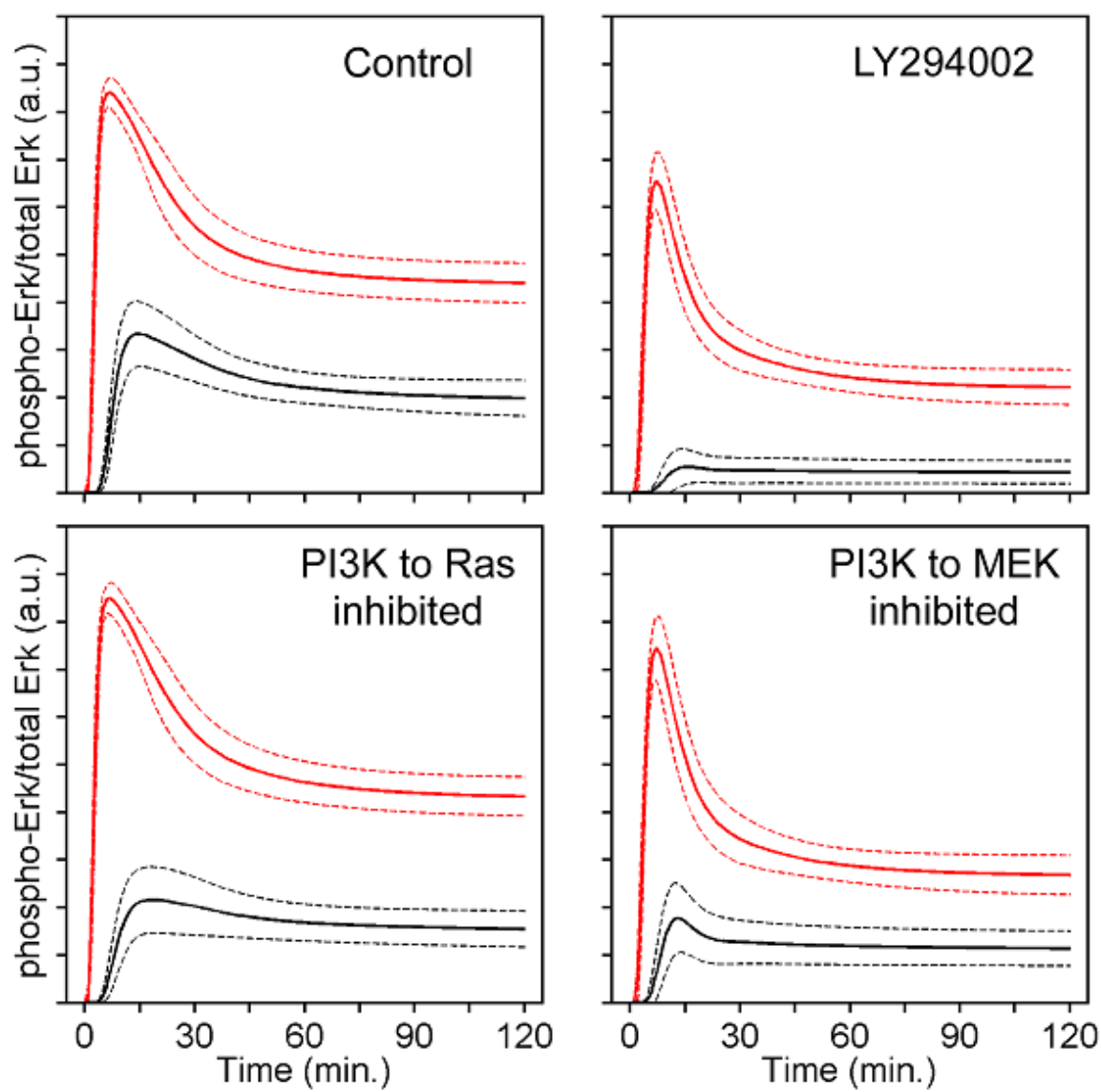
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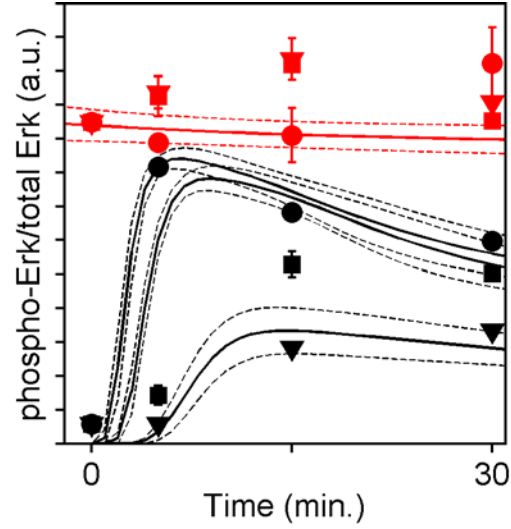


d.



e.





**Figure 3.8. Saturation of the Erk cascade using phorbol ester.** Cells were pretreated for 15 minutes with DMSO only (black symbols) or 200 nM PMA (red symbols), prior to stimulation with PDGF-BB: 30 pM (triangles), 100 pM (squares), or 1 nM (circles). Erk phosphorylation was determined in duplicate by quantitative immunoblotting (phospho-Erk/total Erk), and the values are expressed as mean  $\pm$  s.e.m. ( $n = 2$ ). The error bars are shown for illustrative purposes only; they are equivalent to the range divided by 1.41). The results indicate that PDGF does not stimulate additional Erk phosphorylation in PMA treated cells, suggesting that the pathway is fully activated. In the cells not treated with PMA, the PDGF-stimulated response is 80-90% of the saturation level. The solid curves represent the corresponding ensemble means for the kinetic model, and the dashed curves are mean  $\pm$  s.d. ( $n = 10,000$ ).

### 3.9. Conclusions

The unique data set obtained for the PDGF receptor system has enabled the systematic characterization of multiple crosstalk interactions and regulatory feedback loops in a signaling network.

Crosstalk from the Ras/Erk pathway to PI3K is far less significant, which might be considered surprising in light of the interaction between Ras-GTP and PI3K catalytic subunit. Apparently, Ras does not appreciably complement the already tight binding between PDGF receptors and PI3K (Kazlauskas and Cooper, 1990), whereas Ras might play a more significant role in conjunction with receptors/adaptors that bind PI3K regulatory subunit with lower affinity (Kaur et al., 2006).

PI3K is strongly activated and plays a prominent role in this system, and Ras- and PI3K-dependent pathways converge as distinct inputs to Erk activation that we have quantified experimentally and through computational modeling. At lower PDGF concentrations, Erk activation qualitatively follows AND logic, with both inputs required for a robust response, whereas it follows OR logic at higher PDGF concentrations, wherein either pathway is sufficient.

In addition to its contribution(s) in parallel with Ras, PI3K signaling also enhances Ras-GTP loading. Two distinct negative feedback loops were also identified and characterized: the desensitization of Ras-GTP loading and the upregulation of MKP-1.

Through modeling and computation, the magnitudes of PI3K-dependent crosstalk mechanisms are quantified, and predictions are generated to inform further refinement of those mechanisms.

## **CHAPTER 4. PRELIMINARY EXPERIMENTS FOR FUTURE RESEARCH DIRECTIONS**

The overall effect of PI3K activation on Ras/Erk was confirmed as positive in our system. To advance this line of inquiry, the PI3K-dependent intermediates that execute the crosstalk to Erk will need to be quantitatively characterized.

A strong candidate in that regard is the Rac/Pak pathway, which might contribute to activation of Erk via Raf-1 and/or MEK-1. pBM-puro retroviral vectors encoding dominant-negative (S17N) and constitutively active (Q61L) Rac1 mutants have been constructed and successfully integrated in our cells, and preliminary results show that dominant-negative Rac partially affects Erk signaling (Fig. 4.1). Phosphorylation of Raf-1 on Ser338, a crucial step in the activation of Raf-1 by oncogenic Ras and growth factors, can be mediated by Pak-1. We have confirmed that this phosphorylation site is sensitive to PI3K inhibition, however it is also sensitive to Ras inhibition in our system (Fig. 4.2); Pak also phosphorylates MEK-1 on Ser298 to activate Erk. Beeser et al. (2005) reported inhibition of Pak kinase activity (by expression of a specific inhibitor, the Pak1 inhibitor domain (PID), or by small interference RNA (siRNA)) dramatically decreased phosphorylation of MEK-1 on Ser298 and activation of Erk in response to PDGF in NIH 3T3 cells (Beeser et al., 2005). To analyze this crosstalk, we can assess the phosphorylation of MEK-1 as the output by using immunoblotting. A good anti-phospho MEK-1 Ser298 antibody was commercially available (Biosource International).

Another candidate is PDK-1, which might contribute to activation of Pak-1 and/or

MEK-1/2. Pak-1 is specifically phosphorylated at Thr423 in response to PDGF in NIH 3T3 cells in a reaction that requires catalytically active PDK-1; however, PDK-1 phosphorylation of Pak-1 was not blocked by pretreatment with wortmannin or when PDK-1 was mutated to prevent PI(3,4,5)P<sub>3</sub> binding (King et al., 2000), indicating this process is PI3K-independent; PDK-1 can also phosphorylates MEK-1 and MEK-2 on Ser222 and Ser226, respectively, to activate Erk. These phosphorylations were known to be essential for maximum MEK and Erk activities (Sato et al., 2004). To further study these two crosstalks, we can access the outputs by using immunoblotting, and antibodies against phospho-Pak1(Thr423/Pak2(Thr402) and phospho-MEK-1/2(Ser217/221) (Cell Signaling) are commercially available.

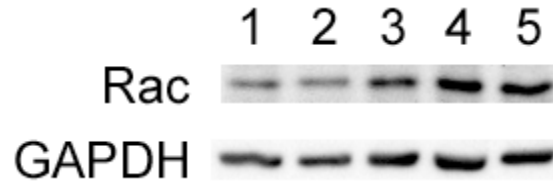
PLC/PKC pathway was also suggested as a possible pathway linking PI3K to Raf; however, inhibition of PDGF-stimulated PKC activation by 1  $\mu$ M calphostin C only modestly inhibits Erk (Fig. 4.3; similar results were obtained with another PKC inhibitor, BIM1, at 1  $\mu$ M), indicating this mechanism may be tenuous in our cells.

Such PI3K-dependent modes of Erk activation could mask a more subtle negative regulation of the Erk pathway, through Akt for example; however, we found that overexpression of HA-Akt-1 does not significantly affect PDGF-stimulated Erk phosphorylation in our cells, despite a 2-fold increase in phosphorylated Akt (Fig. 4.4).

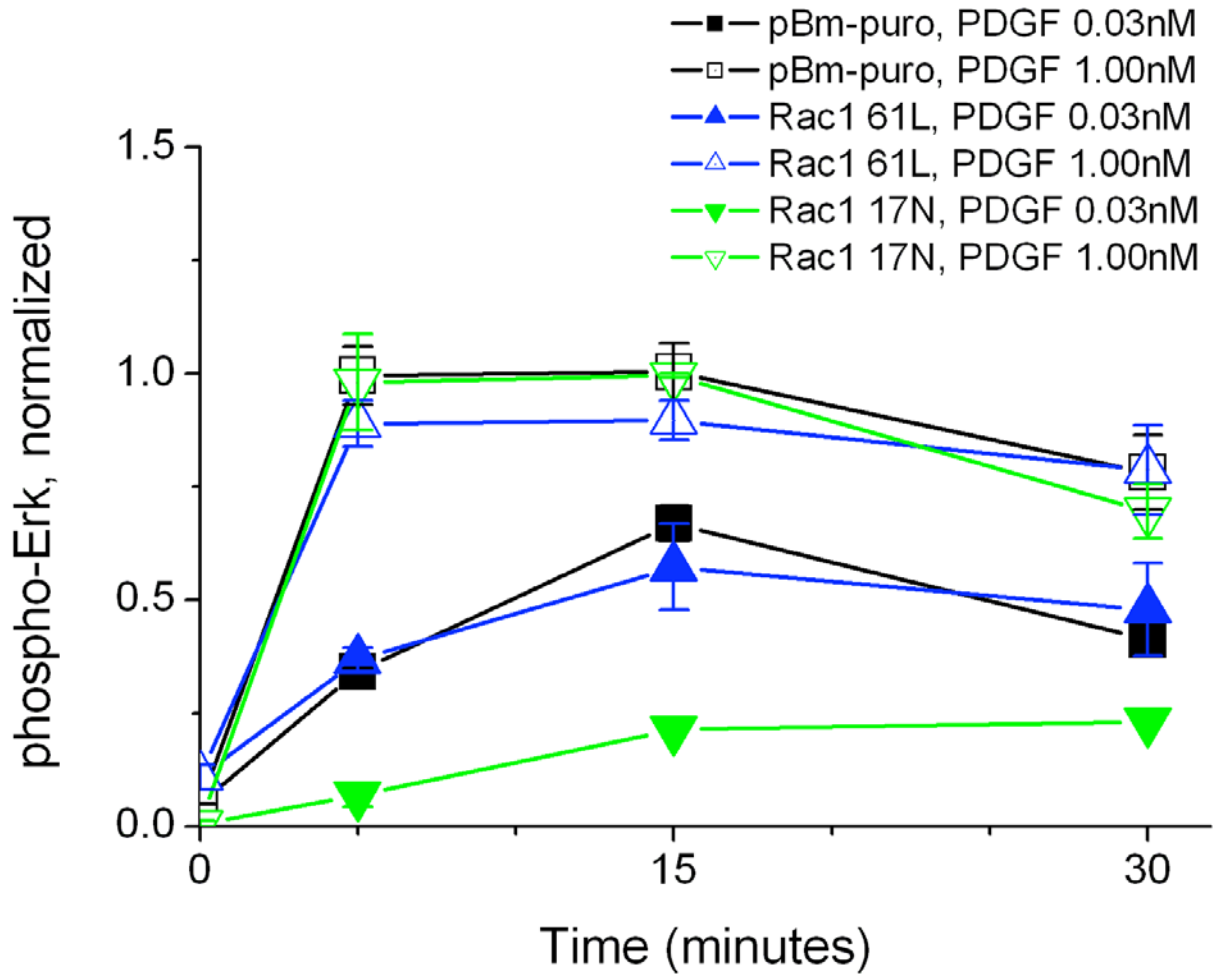
PI3K lipid products apparently contribute upstream of Ras as well, perhaps through direct recruitment of Ras-specific GEF activity via Gab1/Shp2 or through mediators such as centaurin- $\alpha$ 1. If these hypothetical pathways could be tested by selectively blocking (by expressing mutant form that is unable to bind PI(3,4,5)P<sub>3</sub> or by using siRNA), the mechanisms and signal strengths of these crosstalk interactions could be characterized.

The precise role of MKPs *in vivo* is not yet fully understood. It is probable that there is some degree of redundancy between MKPs. For example, the phosphatase MKP-3 is specific to the Erks and *in vivo* study has highlighted the role of MKP-3 as a negative feedback regulator of fibroblast growth factor (FGF) signaling in mouse embryos (Li et al., 2007). More work is required to access the functional role of individual MKPs in our system.

a

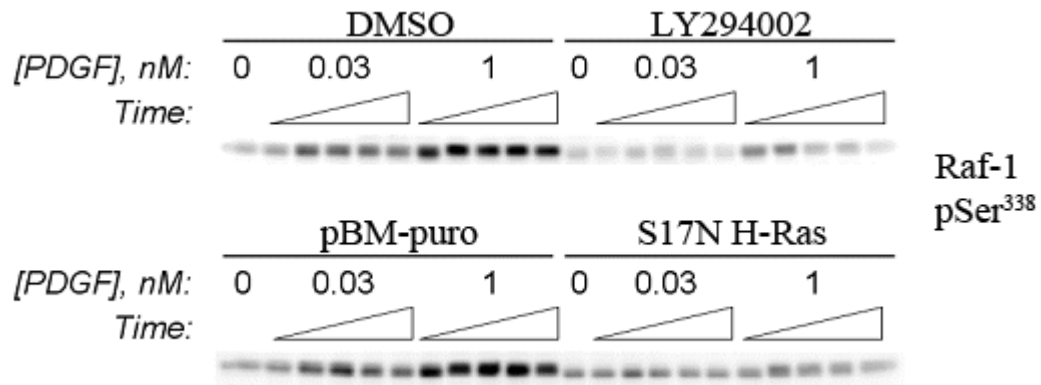


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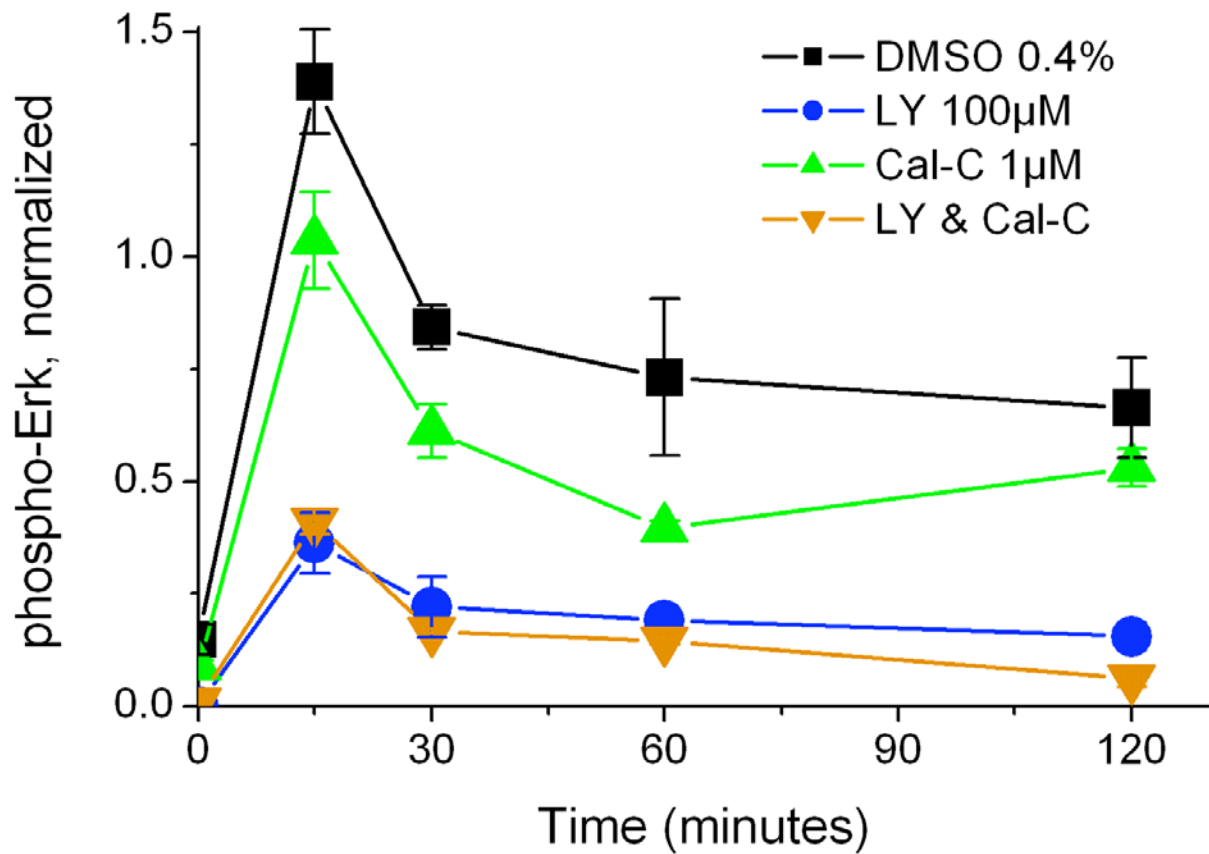


**Figure 4.1. Preliminary results suggest that Rac inhibition alters the Erk dose response.**

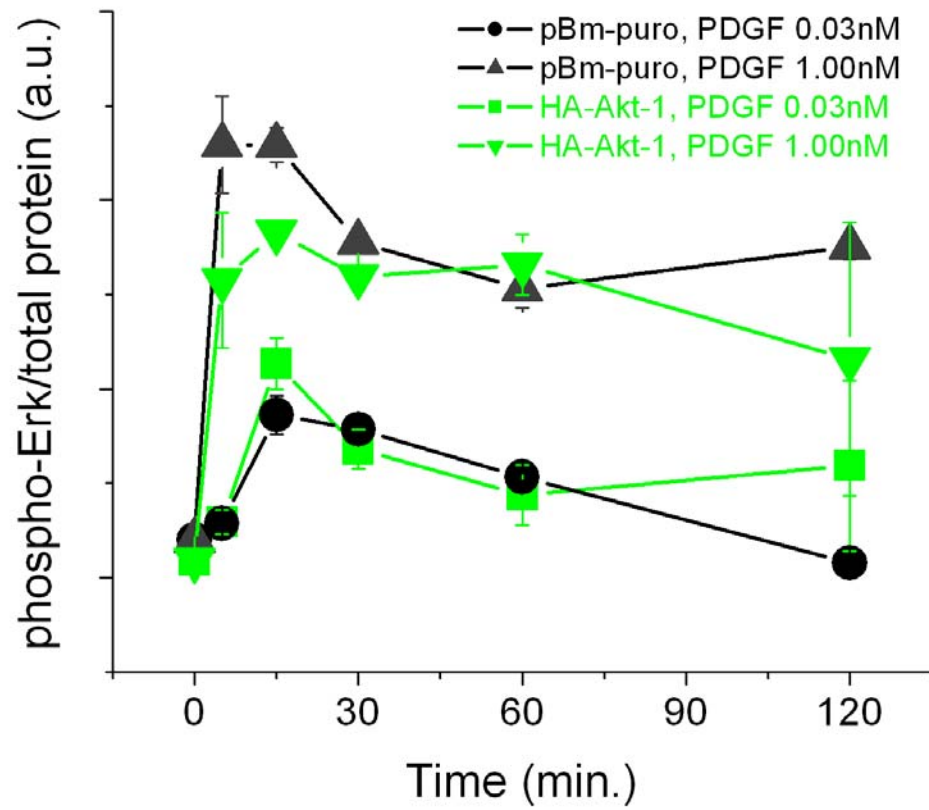
a. Rac expression retrovirally induced with 1) nothing; 2) empty vector; 3) wild-type Rac1; 4) Q61L Rac1; or 5) S17N Rac1. Overexpression is ~2-fold. b. S17N Rac 1 significantly inhibits Erk activation at low PDGF concentration ( $n = 2$ ; mean  $\pm$  s.e.m.).



**Figure 4.2. Preliminary data linking Raf-1 phosphorylation to PI3K and Ras signaling.** Immunoblots used to quantify relative amounts of phosphorylated Raf-1 (p-Akt; Ser338) are shown. NIH fibroblasts were modulated by retroviral induction of dominant-negative (S17N) expression or incubation with inhibitors of PI3K (100  $\mu$ M LY294002). The respective controls are empty vector or vehicle only (0.2% DMSO). Lysates were prepared from cells that were unstimulated or stimulated with the indicated PDGF-BB concentration for various times (5, 15, 30, 60, or 120 min.). Each blot is representative of 3 independent experiments. The mode of Raf-1 regulation does not map to either of the independent pathways from PDGF receptors to MEK/Erk.



**Figure 4.3. Minor role for PKC in PDGF-stimulated Erk activation.** The PKC inhibitor calphostin C (Cal-C, 1  $\mu$  M) only modestly alters Erk activation in our system ( $n = 2$ ; mean  $\pm$  s.e.m.). Similar results were obtained with another PKC inhibitor, BIM1, at 1  $\mu$  M.



**Figure 4.4. HA-Akt-1 overexpression does not significantly affect PDGF-stimulated Erk phosphorylation in NIH3T3 fibroblasts.** NIH3T3 fibroblasts were modulated by retroviral induction of HA-Akt-1 expression. The control is empty vector. ( $n = 2$ ; mean  $\pm$  s.e.m.).

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## Appendix

## APPENDIX : Kinetic modeling and analysis

(Work mainly done by Professor Jason Haugh and Murat Cirit)

### I. Kinetic Model

#### I.A. Receptor and PI3K Activation

##### I.A.1 PDGF Receptor Binding/Dimerization/Trafficking

The starting point for the kinetic model of the PDGF receptor signaling network was our previous model of receptor and PI3K activation, which was validated by quantitative experiments in the same cells as used here (1,2). Definitions and base values of the relevant rate constants are listed in Table S1. Defining  $R$ ,  $C_1$ , and  $C_2$  as the density of free receptors, 1:1 receptor-ligand complexes, and functional receptor dimers in the membrane, respectively, and  $[L]$  as the concentration of ligand (PDGF) added at time  $t = 0$ ,

$$C_1 = [L]R/K_{D,L} ;$$

$$\frac{d(R + C_1)}{dt} = V_s - k_t(R + C_1) + 2(k_{-x}C_2 - k_xC_1^2);$$

$$\frac{dC_2}{dt} = k_xC_1^2 - (k_{-x} + k_e)C_2;$$

$$R(0) = R_0 = V_s/k_t; \quad C_1(0) = C_2(0) = 0 .$$

Here,  $K_{D,L}$  is the dissociation constant characterizing 1:1 complex formation (fast on-off kinetics),  $V_s$  is the receptor insertion rate, and the rate constants  $k_x$ ,  $k_{-x}$ ,  $k_t$ , and  $k_e$  characterize receptor dimerization, dimer uncoupling, constitutive membrane turnover, and induced

endocytosis of receptor dimers, respectively. Receptor densities are nondimensionalized by scaling  $R$ ,  $C_1$ ,  $C_2$ , and  $V_s$  by the initial surface receptor expression level,  $R_0$ ; substituting its definition from above and simplifying,

$$\frac{dr}{dt} = \left( 1 + \frac{[L]}{K_{D,L}} \right)^{-1} \left[ k_t(1-r-c_1) + 2(k_{-x}c_2 - k_x R_0 c_1^2) \right]; r(0) = 1; \text{ (Eq. S1)}$$

$$c_1 = [L]r/K_{D,L}; \quad \text{(Eq. S2)}$$

$$\frac{dc_2}{dt} = k_x R_0 c_1^2 - (k_{-x} + k_e)c_2; c_2(0) = 0. \quad \text{(Eq. S3)}$$

The output of this model is the fraction of receptors in dimers as a function of time, given by  $2c_2(t)$ . The parameter values were taken from the papers cited above, except for the basal receptor turnover rate constant  $k_t$ .

### I.A.2 PI3K Recruitment and 3' PI Accumulation

We next present a model of PI3K recruitment and 3' PI production. In keeping with the notation used previously (2,3), we refer to the fraction of the PI3K enzyme recruited as  $e_{PI3K}$ , and the dimensionless 3' PI messenger density is given by  $m_{3PI}$ . The rate of 3' PI accumulation in response to PDGF in fibroblasts is limited by 3' PI turnover, not the recruitment of PI3K (1,4). Hence, it is justified to assume pseudo-equilibrium for PI3K binding:

$$e_{PI3K}(t) = \frac{[2\alpha_{PI3K}c_2(t) - e_{PI3K}(t)][1 - e_{PI3K}(t)]}{\kappa_{PI3K}},$$

$$\alpha_{PI3K} = A_{mem}R_0/E_{PI3K,Tot}; \quad \kappa_{PI3K} = V_{cyt}K_{D,PI3K}/E_{PI3K,Tot}.$$

The dimensionless parameters  $\alpha_{PI3K}$  and  $\kappa_{PI3K}$  are cast in terms of  $A_{mem}$ , the surface area of the plasma membrane,  $E_{PI3K,Tot}$ , the total number of PI3K molecules per cell,  $V_{cyt}$ , the volume of the cytosol, and  $K_{D,PI3K}$ , the equilibrium dissociation constant for the receptor/PI3K interaction. The equation above is rearranged to obtain

$$e_{PI3K}(t) = \frac{1 + \kappa_{PI3K} + 2\alpha_{PI3K}c_2(t) - \sqrt{(1 + \kappa_{PI3K} + 2\alpha_{PI3K}c_2(t))^2 - 8\alpha_{PI3K}c_2(t)}}{2}; \quad (\text{Eq. S4})$$

The stimulated accumulation of 3' PI lipids, with the normal basal level subtracted, is modeled in dimensionless form as follows (1-3):

$$\frac{dm_{3PI}}{dt} = k_{3PI}(e_{PI3K} - m_{3PI}); m_{3PI}(0) = 0. \quad (\text{Eq. S5})$$

The base values of the parameters for this portion of the model ( $\alpha_{PI3K}$ ,  $\kappa_{PI3K}$ , and  $k_{3PI}$ ), listed in Table S1, were assigned values that are quantitatively consistent with the data in our previous papers on PI3K signaling and hence were not subject to parameter fitting based on the new data presented in this paper.

**Table S1: Kinetic model parameter definitions and values, PDGF receptor/PI3K module.**

| <i>Parameter</i> | <i>Description</i>                                | <i>Value</i>             |
|------------------|---------------------------------------------------|--------------------------|
| $K_{D,L}$        | PDGF single-site dissociation constant            | 1.5 nM                   |
| $k_x R_0$        | Dimerization rate constant                        | $0.3 \text{ min}^{-1}$   |
| $k_{-x}$         | Dimer uncoupling rate constant                    | $0.07 \text{ min}^{-1}$  |
| $k_e$            | Dimer endocytosis rate constant                   | $0.2 \text{ min}^{-1}$   |
| $k_t$            | Basal receptor turnover rate constant             | $0.005 \text{ min}^{-1}$ |
| $\alpha_{PI3K}$  | Receptor/PI3K expression ratio                    | 80                       |
| $\kappa_{PI3K}$  | Dimensionless receptor-PI3K dissociation constant | 0.3                      |
| $k_{3PI}$        | 3' PI turnover rate constant                      | $1.0 \text{ min}^{-1}$   |

## I.B. Ras/Erk Pathway

Based on our Ras-GTP and phospho-Erk data, the model at least needs to include the following processes: (i) recruitment of Ras-guanine nucleotide exchange factor (GEF) activity from the cytoplasm, controlled by the densities of activated receptors and 3' PI, mediating an increase in Ras-GTP level; (ii) activation of Raf and other MEK kinases, controlled by the densities of Ras-GTP and 3' PI; (iii) dual phosphorylation/dephosphorylation of MEK and of Erk; and (iv) negative feedback loops mediated by Erk affecting desensitization of GEF recruitment and up-regulation of MKP-1 expression. These aspects of the model are discussed below. Most of the Ras/Erk pathway parameters were estimated using a Monte-Carlo algorithm, described in detail in Part II of this Appendix. This strategy does not identify a “best-fit” value for each parameter but rather an ensemble of parameter sets that fit the data almost equally well. Definitions of the kinetic parameters and statistics concerning their estimation are summarized in Table S2.

### I.B.1 GEF Recruitment and Ras-GTP Accumulation

For simplicity we do not model explicitly the various adaptor proteins involved in PDGF receptor-mediated Ras-GEF recruitment, such as Grb2, Shc, and Gab-1. Fractional GEF recruitment,  $e_{GEF}(t)$ , is assumed to respond rapidly to changes in the density of receptor dimers,  $c_2(t)$ , and 3' PI lipids,  $m_{3PI}(t)$ , according to the following approximate equilibrium-binding relation.

$$e_{GEF}(t) = [K_{GR}c_2(t) + K_{GP}m_{3PI}(t)]f_{GEF}(t) - e_{GEF}(t).$$

Other functions of  $c_2$  and  $m_{3PI}$  on the right-hand side of this equation were evaluated (e.g., adding a dependence on the product of  $c_2$  and  $m_{3PI}$ ); not surprisingly, the nature of the experimental data does not adequately constrain the model to the extent that significant deviations from the assumed linear model are favored. Rearranging,

$$e_{GEF}(t) = \left[ \frac{K_{GR}c_2(t) + K_{GP}m_{3PI}(t)}{1 + K_{GR}c_2(t) + K_{GP}m_{3PI}(t)} \right] f_{GEF}(t). \quad (\text{Eq. S6})$$

The dimensionless affinity parameter  $K_{GR}$  (GEF/Receptor) is analogous to the parameter grouping  $2\alpha_{PI3K}/(1+\kappa_{PI3K})$  for receptor-mediated PI3K recruitment, described under section A.2. The model considers that 3' PI lipids might present or recruit independent binding sites for GEF, characterized by the dimensionless affinity constant  $K_{GP}$  (GEF/Phosphoinositide). The final component of the GEF recruitment model is the function  $f_{GEF}(t)$ , representing the fraction of the intracellular GEF available for recruitment, which is subject to feedback from MEK/Erk (section I.B.3).

The modeling of Ras-GTP accumulation is treated as in past models (5,6). Defining  $M_{Ras-GTP}$  and  $M_{Ras,Tot}$  as the area densities of membrane-associated Ras-GTP and total Ras (GTP- and GDP-bound), respectively,

$$\frac{dM_{Ras-GTP}}{dt} = (k_0 + k_{GEF}e_{GEF})(M_{Ras,Tot} - M_{Ras-GTP}) - k_{GAP}M_{Ras-GTP}.$$

The rate constants  $k_0$ ,  $k_{GEF}$ , and  $k_{GAP}$  characterize basal GDP/GTP exchange, maximal receptor-mediated activation of GEF activity, and GTP hydrolysis catalyzed by GTPase-accelerating proteins (GAPs), respectively. We define the dimensionless  $m_{Ras}(t)$  by

analogy to  $m_{3PI}(t)$  in that  $m_{Ras} = 0$  when  $e_{GEF} = 0$  (representing the basal state) and  $m_{Ras} = 1$  when  $e_{GEF} = 1$ . Manipulation of the equation above gives

$$\begin{aligned} \frac{dm_{Ras}}{dt} &= k_{Ras} [(1 + \Gamma)e_{GEF} - (1 + \Gamma e_{GEF})m_{Ras}], m_{Ras}(0) = 0; \\ k_{Ras} &= k_0 + k_{GAP}; \Gamma = k_{GEF} / k_{Ras}. \end{aligned} \quad (\text{Eq. S7})$$

In this particular model, we do not consider receptor-mediated activation of GTPase-accelerating protein (GAP) activity, but at least in rough terms one could in any case consider the gain parameter  $\Gamma$  to represent the ratio of GEF/GAP activities under maximal stimulation conditions.

This portion of the model was parameterized as follows. Based on previous experiments (7), it is known that only a small fraction of Ras in our cells is converted to the GTP-bound form, consistent with  $\Gamma \ll 1$  and  $m_{Ras} \approx e_{GEF}$  at steady state; hence,  $\Gamma = 0.1$  was chosen as an arbitrary, order-of-magnitude estimate. Ras-GTP levels peak at  $t \sim 3$  minutes or earlier, and the temporal resolution of our kinetic data does not allow for accurate estimation of the effective rate constant  $k_{Ras}$ . A sufficiently high value of  $1 \text{ min}^{-1}$  was therefore assigned. The values of the 2 parameters characterizing GEF recruitment ( $K_{GR}$ ,  $K_{GP}$ ) were subject to our parameter estimation algorithm.

### *I.B.2 Ras- and PI3K-dependent Activation of the Erk cascade*

Ras and PI3K are responsible for activating serine-threonine kinases that activate MEK, which in turn activates Erk. There are multiple isoforms of Raf (notably, Raf-1 and B-Raf) and also other MEK kinases (e.g., Pak, PDK-1). In the minimal mathematical description, we identify and model two modes of activation at this level:

1. Mode 1 ( $x_1$ ): Ras-dependent, PI3K-independent. This is the only mode leading to MEK/Erk activation in PI3K-inhibited cells.
2. Mode 2 ( $x_2$ ): Ras-independent, PI3K-dependent. This accounts for MEK/Erk activation in S17N Ras-expressing cells.

The dimensionless variables  $x_1$  and  $x_2$  are assumed to be independent; that is, they represent either distinct enzymes or activation of the same enzyme with most of it remaining in the inactive state.

MEK and Erk are successively activated via dual phosphorylation mechanisms that are thought to be distributive (nonprocessive); i.e., MEK must be engaged by a MEK kinase in separate encounters to be phosphorylated on its two activation sites, and likewise for Erk phosphorylation by MEK. The dual phosphorylation mechanism has interesting theoretical properties that have been characterized by other groups over the years (8-12), and so we wish to maintain that character without sacrificing model simplicity (in terms of the number of adjustable parameters). We define  $y$ ,  $y_p$ , and  $y_{pp}$  as the fractions of MEK that are unphosphorylated, mono-phosphorylated, and dually phosphorylated, respectively, and  $z$ ,  $z_p$ , and  $z_{pp}$  as the corresponding fractions of Erk. There are also phosphatases,  $yph$  and  $zph$ , which dephosphorylate MEK and Erk, respectively.

Our model assumes quasi-steady state for the enzyme-substrate complexes (Michaelis-Menten kinetics). A notable assumption here is that the substrates are in excess relative to the enzymes; it is fully recognized that this assumption might not be strictly satisfied inside the cell, and this aspect of the model can be refined as additional data come to light. We do allow competition for common enzymes; unphosphorylated and

mono-phosphorylated forms of MEK and Erk compete with each other for the upstream kinase, and the phosphorylated forms compete with each other for binding to the corresponding phosphatase. Further, active MEK kinase can be saturated by inactive MEK so as to reduce the rate of MEK kinase dephosphorylation, and saturation of active MEK by inactive Erk reduces the rate of MEK dephosphorylation. The quasi-steady state expressions for the enzyme-substrate complexes, denoted by (enzyme • substrate), are as follows.

$$\begin{aligned}
(x_1 \ y) &\approx \frac{(x_1)_{\text{free}} (y)_{\text{free}}}{\tilde{K}_{M,x11}} \approx \frac{(x_1)_{\text{free}} y}{\tilde{K}_{M,x11}}; & (x_1 \ y_p) &\approx \frac{(x_1)_{\text{free}} (y_p)_{\text{free}}}{\tilde{K}_{M,x12}} \approx \frac{(x_1)_{\text{free}} y_p}{\tilde{K}_{M,x12}}; \\
(x_1)_{\text{free}} &\approx \frac{x_1}{1 + y/\tilde{K}_{M,x11} + y_p/\tilde{K}_{M,x12}}; \\
(x_2 \ y) &\approx \frac{(x_2)_{\text{free}} (y)_{\text{free}}}{\tilde{K}_{M,x21}} \approx \frac{(x_2)_{\text{free}} y}{\tilde{K}_{M,x21}}; & (x_2 \ y_p) &\approx \frac{(x_2)_{\text{free}} (y_p)_{\text{free}}}{\tilde{K}_{M,x12}} \approx \frac{(x_2)_{\text{free}} y_p}{\tilde{K}_{M,x12}}; \\
(x_2)_{\text{free}} &\approx \frac{x_2}{1 + y/\tilde{K}_{M,x21} + y_p/\tilde{K}_{M,x22}}; \\
(y_{pp} \ z) &\approx \frac{(y_{pp})_{\text{free}} (z)_{\text{free}}}{\tilde{K}_{M,y1}} \approx \frac{(y_{pp})_{\text{free}} z}{\tilde{K}_{M,y1}}; & (y_{pp} \ z_p) &\approx \frac{(y_{pp})_{\text{free}} (z_p)_{\text{free}}}{\tilde{K}_{M,y2}} \approx \frac{(y_{pp})_{\text{free}} z_p}{\tilde{K}_{M,y2}}; \\
(y_{pp})_{\text{free}} &\approx \frac{y_{pp}}{1 + z/\tilde{K}_{M,y1} + z_p/\tilde{K}_{M,y2}}; \\
(yph \ y_p) &\approx \frac{(yph)_{\text{free}} (y_p)_{\text{free}}}{\tilde{K}_{M,yph1}} \approx \frac{(yph)_{\text{free}} y_p}{\tilde{K}_{M,yph1}}; \\
(yph \ y_{pp}) &\approx \frac{(yph)_{\text{free}} (y_{pp})_{\text{free}}}{\tilde{K}_{M,yph2}} \approx \frac{(yph)_{\text{free}} y_{pp}}{1 + z/\tilde{K}_{M,y1} + z_p/\tilde{K}_{M,y2}}; \\
(yph)_{\text{free}} &\approx \frac{yph(1 + z/\tilde{K}_{M,y1} + z_p/\tilde{K}_{M,y2})}{(1 + z/\tilde{K}_{M,y1} + z_p/\tilde{K}_{M,y2})(1 + y_p/\tilde{K}_{M,yph1}) + y_{pp}/\tilde{K}_{M,yph2}}; \\
(zph \ z_p) &\approx \frac{(zph)_{\text{free}} (z_p)_{\text{free}}}{\tilde{K}_{M,zph1}} \approx \frac{(zph)_{\text{free}} z_p}{\tilde{K}_{M,zph1}}; & (zph \ z_{pp}) &\approx \frac{(zph)_{\text{free}} (z_{pp})_{\text{free}}}{\tilde{K}_{M,yph2}} \approx \frac{(zph)_{\text{free}} z_{pp}}{\tilde{K}_{M,yph2}}; \\
(zph)_{\text{free}} &\approx \frac{zph}{1 + z_p/\tilde{K}_{M,zph1} + z_{pp}/\tilde{K}_{M,zph2}}.
\end{aligned}$$

The dimensionless parameters  $\tilde{K}_{M,xi1}$  and  $\tilde{K}_{M,xi2}$  are Michaelis constants, scaled by the total MEK concentration, characterizing the first and second phosphorylations of MEK by enzyme  $x_i$ ;  $\tilde{K}_{M,y1}$  and  $\tilde{K}_{M,y2}$  are the corresponding Michaelis constants for Erk phosphorylation by active MEK,  $\tilde{K}_{M,yph1}$  and  $\tilde{K}_{M,yph2}$  are the corresponding Michaelis constants for MEK dephosphorylation, and  $\tilde{K}_{M,zph1}$  and  $\tilde{K}_{M,zph2}$  are the corresponding

Michaelis constants for Erk dephosphorylation.

The conservation equations for the MEK kinases assume that these enzymes are mostly maintained in their inactive states, and that their deactivation is far from saturation (pseudo-first order). As noted above, however, we account for the potential saturation of each active MEK kinase by inactive MEK. It is noted that the PI3K-dependent mechanism could rightly be modeled as a sequence of two or more steps, because unlike Ras-GTP, 3' PIs are probably not capable of directly interacting with the MEK kinase(s) (with the exception of PDK-1). To reduce the number of parameters, we lump these processes into a single, rate-limiting step for  $x_2$ ; however, we allow for partial saturation of this mechanism with respect to the 3' PI level, by including a saturation parameter,  $K_{x2}$ . Cast in terms of dimensionless concentrations, the rate equations for the MEK kinases are

$$\frac{dx_1}{dt} = k_{d,x1} \left( m_{Ras} - \frac{x_1}{1 + y/\tilde{K}_{M,x11} + y_p/\tilde{K}_{M,x12}} \right); x_1(0) = 0; \quad (\text{Eq. S8})$$

$$\frac{dx_2}{dt} = k_{d,x2} \left[ \frac{(1 + K_{x2})m_{3PI}}{1 + K_{x2}m_{3PI}} - \frac{x_2}{1 + y/\tilde{K}_{M,x21} + y_p/\tilde{K}_{M,x22}} \right]; x_2(0) = 0; \quad (\text{Eq. S9})$$

For MEK, the conservation equations are

$$\frac{dy}{dt} = -\sum_{i=1}^2 \frac{\tilde{V}_{\max,xi1} x_i y / \tilde{K}_{M,xi1}}{1 + y/\tilde{K}_{M,xi1} + y_p/\tilde{K}_{M,xi2}} + \frac{\tilde{V}_{\max,yph1} y_p / \tilde{K}_{M,yph1}}{1 + y_p/\tilde{K}_{M,yph1} + y_{pp}/\tilde{K}_{M,yph2}}; y(0) = 1; \quad (\text{Eq. S10})$$

$$\frac{dy_{pp}}{dt} = \sum_{i=1}^2 \frac{\tilde{V}_{\max,xi2} x_i y_p / \tilde{K}_{M,xi2}}{1 + y / \tilde{K}_{M,xi1} + y_p / \tilde{K}_{M,xi2}} - \frac{\tilde{V}_{\max,yph2} y_{pp} / \tilde{K}_{M,yph2}}{(1 + z / \tilde{K}_{M,y1} + z_p / \tilde{K}_{M,y2}) (1 + y_p / \tilde{K}_{M,yph1}) + y_{pp} / \tilde{K}_{M,yph2}}; y_{pp}(0) = 0; \quad (\text{Eq. S11})$$

$$y_p = 1 - y - y_{pp}. \quad (\text{Eq. S12})$$

As shown in Eqs. S10 & S11, the model allows  $x_1$  and  $x_2$  to possess distinct catalytic properties with respect to MEK phosphorylation. The parameters  $\tilde{V}_{\max,xi1}$  and  $\tilde{V}_{\max,xi2}$  account for the abundance of the  $i$ th MEK kinase mode at maximal stimulation as well as its  $k_{cat}$  values for the first and second phosphorylations of MEK, respectively. They are scaled by the total concentration of MEK and therefore have units of inverse time. Corresponding parameters are specified for the single MEK phosphatase ( $yph$ ), which is assumed to have constant abundance, and for Erk phosphorylation/dephosphorylation. The rate expressions for Erk phosphorylation are

$$\frac{dz}{dt} = -\frac{\tilde{V}_{\max,y1} y_{pp} z / \tilde{K}_{M,y1}}{1 + z / \tilde{K}_{M,y1} + z_p / \tilde{K}_{M,y2}} + \frac{\tilde{V}_{\max,zph1} e_{ph} z_p / \tilde{K}_{M,zph1}}{1 + z_p / \tilde{K}_{M,zph1} + z_{pp} / \tilde{K}_{M,zph2}}; z(0) = 1; \quad (\text{Eq. S13})$$

$$\frac{dz_{pp}}{dt} = \frac{\tilde{V}_{\max,y2} y_{pp} z_p / \tilde{K}_{M,y2}}{1 + z / \tilde{K}_{M,y1} + z_p / \tilde{K}_{M,y2}} - \frac{\tilde{V}_{\max,zph2} e_{ph} z_{pp} / \tilde{K}_{M,zph2}}{1 + z_p / \tilde{K}_{M,zph1} + z_{pp} / \tilde{K}_{M,zph2}}; z_{pp}(0) = 0 \quad (\text{Eq. S14})$$

$$z_p = 1 - z - z_{pp}. \quad (\text{Eq. S15})$$

The function  $e_{ph}(t)$  represents the dimensionless abundance of the dual-specificity phosphatase that dephosphorylates Erk and thus counteracts active MEK; this phosphatase is

subject to up-regulation through a MAPK-dependent negative feedback loop (section I.B.3 below).

Even with a fair number of parameter-reducing simplifications, modeling the Erk cascade introduces a relatively large number of adjustable rate constants (23). This number may be reduced if one or more of the enzymes can be assumed to operate far from saturation ( $\tilde{K}_M$  values  $\gg 1$ ).

### *I.B.3 Negative Feedback Loops Eliciting GEF Desensitization and Up-regulation of MKP-1*

These negative feedback loops are embodied by the functions  $f_{GEF}(t)$  and  $e_{ph}(t)$ , as introduced in sections I.B.1 and I.B.2, respectively. GEF desensitization, which involves Erk-dependent hyperphosphorylation, affects the fraction of GEF available,  $f_{GEF}(t)$ .

$$\frac{df_{GEF}}{dt} = -k_{FB,f} \left[ \frac{z_{pp}^n}{z_f^n + z_{pp}^n} f_{GEF} - \frac{1}{K_f} (1 - f_{GEF}) \right]; f_{GEF}(0) = 1; \quad (\text{Eq. S16})$$

This expression allows for potentially switch-like or a more graded transition (Hill coefficient  $n \geq 1$ ) as well as fast or slow “reset” kinetics. As shown in Table 2, one could set  $n = 1$  without affecting the model output significantly. The rate constant  $k_{FB,f}$  defines the time scale associated with the feedback loop, and  $K_f$  defines its gain (maximum ratio of desensitization and reset frequencies).

For  $e_{ph}$ , the model needs to account for the observation that MKP-1 appears with a delay of  $\sim 15$  minutes. Thus, we impose a variable  $w$ , possibly representing a transcription factor activity, which builds up slowly and switches on MKP-1 expression.

$$\frac{dw}{dt} = k_{d,w} (z_{pp} - w); w(0) = 0; \quad (\text{Eq. S17})$$

$$\frac{de_{ph}}{dt} = k_{FB,ph} \left[ \frac{w^p}{W_{ph}^p + w^p} - \frac{1}{K_{ph}} (e_{ph} - 1) \right]; e_{ph}(0) = 1. \quad (\text{Eq. S18})$$

These two Erk-dependent feedback loops introduce 9 additional parameters.

### **I.C. Modeling Molecular Perturbations Affecting Erk Phosphorylation**

PI3K inhibition is modeled by setting  $m_{3PI} = 0$ , which affects the Ras/Erk pathway both upstream and downstream of Ras. MEK inhibition, which affects Ras-GEF desensitization, is modeled by setting  $f_{GEF} = 1$ . S17N Ras is modeled by setting  $m_{Ras} = 0$ . Chronic activation by phorbol ester is modeled by assuming that MEK activation is saturated ( $y_{pp}$  set to 1).

**Table S2: Kinetic model parameters, Ras/Erk pathway module.** A Monte-Carlo algorithm was used to estimate all but two of these parameter values, producing an ensemble of parameter sets that fit the data set almost equally well. See Part II of this Supplement for more details. \* These two parameters were fixed. † The Hill coefficients  $n$  and  $p$  were constrained to be no lower than 1. Highlighted values are deemed arbitrarily high (yellow) or low (cyan).

| <i>Parameter</i>                         | <i>Description</i>                                        | <i>Minimum</i> | <i>Lower<br/>Quartile</i> | <i>Median</i> | <i>Upper<br/>Quartile</i> | <i>Maximum</i> |
|------------------------------------------|-----------------------------------------------------------|----------------|---------------------------|---------------|---------------------------|----------------|
| $K_{GR}$                                 | Affinity constant, GEF/receptor binding                   | 102            | 394                       | 495           | 640                       | 1730           |
| $K_{GP}$                                 | Affinity constant, 3' PI-dependent GEF binding            | 0.00266        | 3.47                      | 5.09          | 6.51                      | 32.7           |
| $k_{Ras}$                                | Characteristic rate constant, Ras-GTP loading             |                |                           | 1/min *       |                           |                |
| $I$                                      | Maximally stimulated GEF/GAP activity ratio               |                |                           | 0.1 *         |                           |                |
| $k_{d,x1}$                               | MEK kinase deactivation rate constant (Ras-activated)     | 0.203/min      | 0.561/min                 | 0.745/min     | 1.21/min                  | 12.9/min       |
| $k_{d,x2}$                               | MEK kinase deactivation rate constant (PI3K-activated)    | 0.305/min      | 1.77/min                  | 2.85/min      | 11.2/min                  | 282/min        |
| $K_{x2}$                                 | Saturation constant, PI3K-dependent MEK kinase activation | 0.251          | 5.25                      | 6.77          | 10.2                      | 31.0           |
| $\tilde{V}_{\max,x11}/\tilde{K}_{M,x11}$ | Cat. efficiency, MEK --> pMEK (Ras-activated)             | 0.0579/min     | 0.516/min                 | 1.18/min      | 1.62/min                  | 71.3/min       |
| $\tilde{K}_{M,x11}$                      | Mich. constant, MEK --> pMEK (Ras-activated)              | 0.343          | 20.1                      | 30.3          | 250                       | 2570           |

Table S2 (continued)

|                                            |                                                  |                           |           |           |           |          |
|--------------------------------------------|--------------------------------------------------|---------------------------|-----------|-----------|-----------|----------|
| $\tilde{V}_{\max,x21}/\tilde{K}_{M,x21}$   | Cat. efficiency, MEK --> pMEK (PI3K-activated)   | 0.0302/min                | 0.236/min | 0.405/min | 0.907/min | 353/min  |
| $\tilde{K}_{M,x21}$                        | Mich. constant, MEK --> pMEK (PI3K-activated)    | 0.0568                    | 13.7      | 21.6      | 203       | 3710     |
| $\tilde{V}_{\max,yph1}/\tilde{K}_{M,yph1}$ | Catalytic efficiency, pMEK --> MEK               | $5.6 \times 10^{-4}$ /min | 1.65/min  | 4.40/min  | 14.7/min  | 483/min  |
| $\tilde{K}_{M,yph1}$                       | Michaelis constant, pMEK --> MEK                 | 0.573                     | 23.0      | 44.0      | 97.8      | 445      |
| $\tilde{V}_{\max,x12}/\tilde{K}_{M,x12}$   | Cat. efficiency, pMEK --> ppMEK (Ras-activated)  | 0.115/min                 | 3.54/min  | 4.71/min  | 15.5/min  | 146/min  |
| $\tilde{K}_{M,x12}$                        | Mich. constant, pMEK --> ppMEK (Ras-activated)   | 2.81                      | 18.6      | 45.5      | 114       | 758      |
| $\tilde{V}_{\max,x22}/\tilde{K}_{M,x22}$   | Cat. efficiency, pMEK --> ppMEK (PI3K-activated) | 0.0318/min                | 1.09/min  | 2.41/min  | 9.45/min  | 77.6/min |
| $\tilde{K}_{M,x22}$                        | Mich. constant, pMEK --> ppMEK (PI3K-activated)  | 0.876                     | 9.59      | 15.7      | 31.6      | 878      |
| $\tilde{V}_{\max,yph2}/\tilde{K}_{M,yph2}$ | Catalytic efficiency, ppMEK --> pMEK             | 0.233/min                 | 4.20/min  | 6.77/min  | 9.07/min  | 52.2/min |
| $\tilde{K}_{M,yph2}$                       | Michaelis constant, ppMEK --> pMEK               | 1.05                      | 7.99      | 12.7      | 42.5      | 254      |

Table S2 (continued)

|                                            |                                              |                           |           |           |           |                        |
|--------------------------------------------|----------------------------------------------|---------------------------|-----------|-----------|-----------|------------------------|
| $\tilde{V}_{\max,y1}/\tilde{K}_{M,y1}$     | Catalytic efficiency, Erk --> pErk           | 0.862/min                 | 6.57/min  | 11.8/min  | 52.8/min  | > 10 <sup>4</sup> /min |
| $\tilde{K}_{M,y1}$                         | Michaelis constant, Erk --> pErk             | 0.0146                    | 9.91      | 31.9      | 45.5      | 890                    |
| $\tilde{V}_{\max,zph1}/\tilde{K}_{M,zph1}$ | Catalytic efficiency, pErk --> Erk           | 1.1x10 <sup>-4</sup> /min | 0.167/min | 0.451/min | 1.11/min  | 63.2/min               |
| $\tilde{K}_{M,zph1}$                       | Michaelis constant, pErk --> Erk             | 0.275                     | 8.27      | 14.0      | 37.0      | 167                    |
| $\tilde{V}_{\max,y2}/\tilde{K}_{M,y2}$     | Catalytic efficiency, pErk --> ppErk         | 0.669/min                 | 8.16/min  | 31.9/min  | 66.9/min  | 7730/min               |
| $\tilde{K}_{M,y2}$                         | Michaelis constant, pErk --> ppErk           | 0.0481                    | 4.21      | 8.81      | 80.7      | 944                    |
| $\tilde{V}_{\max,zph2}/\tilde{K}_{M,zph2}$ | Catalytic efficiency, ppErk --> pErk         | 0.0263/min                | 0.122/min | 0.228/min | 0.493/min | 13.7/min               |
| $\tilde{K}_{M,zph2}$                       | Michaelis constant, ppErk --> pErk           | 1.10                      | 9.98      | 31.5      | 195       | 797                    |
| $k_{FB,f}$                                 | Feedback rate constant, GEF desensitization  | 0.104/min                 | 0.763/min | 0.976/min | 1.56/min  | 23.7/min               |
| $Z_f$                                      | Dimensionless threshold, GEF desensitization | 0.00829                   | 0.146     | 0.272     | 0.507     | 2.50                   |
| $n$                                        | Hill coefficient, GEF desensitization        | 1.00 <sup>†</sup>         | 1.02      | 1.03      | 1.05      | 1.48                   |

Table S2 (continued)

|             |                                            |                   |            |            |            |           |
|-------------|--------------------------------------------|-------------------|------------|------------|------------|-----------|
| $K_f$       | Gain coefficient, GEF desensitization      | 1.04              | 3.16       | 3.76       | 5.17       | 16.6      |
| $k_w$       | Delay rate constant, MKP up-regulation     | 0.00646/min       | 0.0187/min | 0.0333/min | 0.0914/min | 0.478/min |
| $k_{FB,ph}$ | Feedback rate constant, MKP up-regulation  | 0.0375/min        | 0.998/min  | 2.34/min   | 3.69/min   | 60.6/min  |
| $W_{ph}$    | Dimensionless threshold, MKP up-regulation | 0.107             | 0.248      | 0.385      | 1.11       | 12.3      |
| $p$         | Hill coefficient, MKP up-regulation        | 1.10 <sup>†</sup> | 1.76       | 1.98       | 2.56       | 24.7      |
| $K_{ph}$    | Gain coefficient, MKP up-regulation        | 1.35              | 3.08       | 4.64       | 25.1       | 8055      |

## II. Parameter Estimation

### II.A. Metropolis Algorithm

#### II.A.1 Description of the Algorithm

The values of all but 2 of the 36 parameters listed in Table S2 were subject to a Monte Carlo estimation routine based on the Metropolis algorithm (13). The algorithm was implemented in MATLAB (MathWorks, Natick, MA), adapted from code provided by Tim Elston (Department of Pharmacology, UNC-Chapel Hill) (14). The following data sets were used to constrain the model: dually phosphorylated Erk under DMSO control, Ras-inhibited, and PI3K-inhibited conditions, Ras-GTP under control, PI3K-inhibited, and MEK-inhibited conditions, and MKP expression under control and Ras-inhibited conditions. In order to set all of the data on a similar scale, the mean of the normalized data values under control conditions, 1 nM PDGF stimulation, were set to 1. Later, the data presented in Fig. 3.8. were incorporated to constrain the saturation level of ppErk (stimulated by phorbol ester); those data were aligned with the other ppErk data by minimizing the sum of the squared deviations between the corresponding time points in control cells stimulated with 1 nM PDGF.

The algorithm works as follows.

- 1) An initial set of parameters is chosen. For the exponents  $n$  and  $p$  (Table S2), which were constrained to be no less than 1, the corresponding parameter value in the algorithm was added to its lower limit (e.g.,  $n = 1 + x$ ).

- 2) The dimensionless model output is computed using the stiff solver ode15s.
- 3) The model outputs based on the current parameter set are modified by alignment factors to directly compare with data, one each for ppErk, Ras-GTP, and MKP expression. The values of these three factors,  $a_j$ , are chosen such that the sum of squared deviations (SSD) for each of the three data types  $j$  (ppErk, Ras-GTP, MKP-1), comparing measured and calculated values at each data point  $i$ ,

$$SSD_j = \sum_i (y_{measured,ij} - a_j y_{model,ij})^2,$$

is minimized. This step is done by systematically subdividing the range of possible values until each  $SSD_j$  can no longer be reduced by more than 0.1%. For example, for a dimensionless variable between 0 and 1 and corresponding data with a peak value greater than 1 in arbitrary units, we know that  $a_j > 1$ , in which case we know that  $0 < 1/a_j < 1$ . The minimum SSD values thus obtained are saved and used to evaluate the closeness of fit, as described in the following section.

- 4) A new set of parameters is determined from the old set as follows.

$$k_{i,new} = k_{i,old} (1 + \alpha \text{randn}),$$

where  $k_i$  is one of the model parameters, and randn is a random number drawn for each parameter from a normal distribution centered on zero with  $\sigma = 1$ . Thus,  $\alpha$  is a parameter of the algorithm that governs how much the parameter values tend to change between iterations. Its value affects the efficiency of the algorithm, and after extensive experimentation we concluded that a value of  $\alpha = 0.05$  is close to optimal

for this application. That value was used throughout the analysis reported here. If any of the new parameters is below  $10^{-4}$  or greater than  $10^4$ , the new value is thrown out, and another value is drawn based on the old value.

5) Steps 2-4 are repeated using the new parameter set, and its  $SSD_j$  are evaluated.

If defined criteria are satisfied (see section II.A.2 below), the new parameter set is accepted; otherwise, it is thrown out, and the previous set is used again.

6) The procedure is repeated until the desired number of accepted parameter sets is achieved. All of the accepted parameter sets are saved in a matrix for further analysis.

#### *II.A.2 Generation of a Parameter Ensemble*

The strategy for using the algorithm was as follows. First, we established a suitable initial parameter set. This was done by randomly varying the parameters as described above until a weighted sum of SSD values converged to a near-minimal value; in this exercise, it was confirmed that different starting guesses resulted in approximately the same value of the weighted SSD. Once a reference parameter set was established, it was used as the starting point for an extensive search of the parameter space, with the goal of collecting parameter sets that fit the data nearly as well as or better than the initial parameter set. A parameter set was selected if it produced a SSD value less than 1.5 for each of the following data subsets: ppErk with DMSO, ppErk in S17N Ras cells, ppErk in LY294002-treated cells, Ras-GTP measurements, MKP-1 measurements, and ppErk with DMSO or PMA. After some experimentation, it was found that an additional criterion was needed to ensure that the

ppErk, S17N Ras data for the lowest PDGF concentration (30 pM) was fit adequately, and hence the parameter sets also had to have a SSD < 0.35 for those particular data points. Finally, to improve the quality of fit of the PI3K-inhibited ppErk data, the parameter sets obtained were sorted according to their SSD value for those data, and sets with SSD < 1.0 for that subset of data were selected. Statistics for this ensemble, representing 10,000 of the “best” parameter sets (out of > 60,000 initially chosen), are summarized in Table S2.

## II.B. Ensemble Averaging and Analysis

With the ensemble of parameter sets saved as a matrix, MATLAB was used to recalculate the model output for each parameter set and store those values in a larger matrix. For each experimental condition and time point, an ensemble mean and standard deviation ( $n = 10,000$ ) were computed, and these values were used to compare the model with the experimental data in Fig. 3.7b. To predict the outcomes of certain perturbations, namely the inhibition of PI3K-dependent crosstalk to Ras-GEF or to MEK (Fig. 3.7e), the corresponding changes in the parameter values ( $K_{GP} = 0$  or  $\tilde{V}_{\max,x21} = \tilde{V}_{\max,x22} = 0$ , respectively) were made in each of the 10,000 parameter sets, and the mean and standard deviation of the model output was recomputed.

The analysis of MEK phosphorylation presented in Fig. 3.7c was carried out as follows. The maximum phosphorylation of MEK by pathway  $i$  ( $i = 1$  for Ras-dependent,  $i = 2$  for PI3K-dependent) on site  $j$  ( $j = 1$  or  $2$ ) is characterized by the ratio of catalytic efficiencies,

$$C_{xij} = \frac{\tilde{V}_{\max,xij} / \tilde{K}_{M,xij}}{\tilde{V}_{\max,yphj} / \tilde{K}_{M,yphj}}$$

Fig. 3.7c shows scatter plots of maximum PI3K-dependent phosphorylation of site 1 ( $C_{x21}$ ) versus maximum Ras-dependent phosphorylation of site 1 ( $C_{x11}$ ) and of maximum PI3K-dependent phosphorylation of site 2 ( $C_{x22}$ ) versus maximum Ras-dependent phosphorylation of site 2 ( $C_{x12}$ ), with each parameter set in the ensemble represented as a dot. The MEK activation comparator (*MAC*), referred to in the main text, incorporates the phosphorylation of both MEK sites and was calculated as follows. Suppose that the MEK kinases and phosphatase are far from saturation and that only one of the two MEK activation pathways is present and maximally activated ( $x_i = 1$ ). If such a system were allowed to reach steady state, Eqs. S10-S12 reduce to

$$\begin{aligned} y_p &= C_{xi1} y; y_{pp} = C_{xi2} y_p; \\ y + y_p + y_{pp} &= 1; \\ y_{pp} &= \frac{\Psi_i}{1 + \Psi_i}; \Psi_i = \frac{C_{xi1} C_{xi2}}{1 + C_{xi1}}. \end{aligned}$$

The *MAC* ratio compares the capacity for activation of  $y_{pp}$  thus obtained for the PI3K-dependent pathway to that of the Ras-dependent pathway, according to

$$MAC = \frac{\Psi_2}{\Psi_1} = \left( \frac{C_{x21} C_{x22}}{1 + C_{x21}} \right) / \left( \frac{C_{x11} C_{x12}}{1 + C_{x11}} \right)$$

The analysis of Ras-GEF recruitment presented in Fig. 3.7d was carried out as follows. It is apparent from Eq. S6 that GEF recruitment, as a fraction of the amount

available, is determined by the magnitude of

$$K_{GR}c_2(t) + K_{GP}m_{3PI}(t)$$

A balanced comparison of the two terms in this sum, representing the PI3K-independent and PI3K-dependent GEF recruitment modes, is complicated by the fact that the dimensionless variables  $c_2$  and  $m_{3PI}$  are normalized differently. However, in the limit of low PDGF concentrations (with  $e_{PI3K} \ll 1$ ),  $m_{3PI}$  is proportional to  $c_2$  at quasi-steady state (Eq. S4 & S5), with

$$m_{3PI} = e_{PI3K} \approx \frac{2\alpha_{PI3K}}{1 + \kappa_{PI3K}} c_2 \quad (e_{PI3K} \ll 1, \text{ quasi-steady state}).$$

For the parameters used here, the proportionality constant is equal to 123. Fig. 3.7d shows a scatter plot of PI3K-dependent GEF recruitment, expressed as the value of  $K_{GP}$ , versus the corresponding PI3K-independent GEF recruitment, expressed on a comparable scale as the value of  $K_{GR}$  divided by 123; each parameter set in the ensemble is represented as a dot. The GEF recruitment comparator (*GRC*) compares these contributions in terms of a ratio,

$$GRC = \left( \frac{2\alpha_{PI3K}}{1 + \kappa_{PI3K}} \right) \frac{K_{GP}}{K_{GR}} = 123 \frac{K_{GP}}{K_{GR}}.$$

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