CIRIT, MURAT. Predictive Computational Models of Mitogen-activated Protein Kinase Signaling Networks Driven by Quantitative Measurements of System Dynamics. (Under the direction of Jason M. Haugh.)

Signal transduction networks in mammalian cells, comprised of a limited set of interacting biochemical pathways, are accessed by various growth factor and cytokine receptors to elicit distinct cell responses. This raises the question as to how specificity of the stimulus-response relationship is encoded at the molecular level. In mammalian cells, the Ras/ERK pathway controls cell proliferation and other responses stimulated by growth factors, and several crosstalk and feedback mechanisms affecting its activation have been identified. Here we present a systematic, comparative analysis of the signaling networks accessed by fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) receptors in mouse fibroblasts, in which the extracellular signal-regulated kinase (ERK) cascade is activated by Ras- and phosphoinositide 3-kinase (PI3K)-dependent pathways and is subjected to three layers of ERK-dependent feedback: desensitization of Ras activation, negative regulation of MEK kinase (e.g. Raf) activities, and upregulation of dual-specificity ERK phosphatases. Through the formulation and analysis of data-driven mathematical models, we show that while the FGF stimulation of PI3K signaling is relatively weak, this deficiency is compensated for by a more potent, Ras-dependent activation of ERK. We take a systematic approach to parse the magnitudes of multiple regulatory mechanisms that attenuate ERK activation through canonical (Ras-dependent) and non-canonical (PI3K-dependent) pathways. Our results establish the second of these as the dominant mode of ERK self-regulation in mouse fibroblasts. We further demonstrate that models of signaling networks, trained on a sufficient diversity of quantitative data, can be reasonably comprehensive, accurate, and predictive in the dynamical sense.
Predictive Computational Models of Mitogen-activated Protein Kinase Signaling Networks Driven by Quantitative Measurements of System Dynamics

by
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DEDICATION

To my parents, Tanzer and Mustafa Cirit
BIOGRAPHY

Murat Cirit was born on June 6, 1981 in Istanbul, Turkey. He attended Istanbul (Erkek) Lisesi; one of the most prestigious high schools in Turkey. Here the boarding school environment conditioned him for the necessity of teamwork, discipline and high standards required in a competitive science field. After graduating Istanbul (Erkek) Lisesi, he enrolled at Middle East Technical University (METU) in Ankara, majoring in Chemical Engineering. His interest in biological sciences started during sophomore year in college. In the spring of 2005, he graduated with high honors in chemical engineering and minoring in biological sciences. To expand his knowledge and pursue a career in biomolecular engineering, he attended North Carolina State University in the fall of 2005 and started working with Dr. Jason M. Haugh on experimental cell biology research. Concurrently he was learning computational biology to master the interdisciplinary approach to cell biology research. During this time, he also received a minor degree in biotechnology and Grand Challenges in Biomanufacturing Graduate Fellowship from Golden LEAF Biomanufacturing Training and Education Center, which trained him in different areas within biotechnology. As an experimental and computational biologist, he wants to pursue his career in cancer research to implement a holistic and systematic approach for discovery.
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Life is in balance with its surroundings. Living cells regulate their functional responses to external signals, such as growth factors and hormones, which are typically recognized by cell surface receptors. Receptor tyrosine kinases (RTKs) and their ligands, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and their various isoforms, play important roles in cell proliferation, apoptosis, adhesion, migration, and differentiation in a broad spectrum of cell lines, and spurious signaling through these receptors has been implicated as a major contributing factor in cancer. Activated receptors mediate signal transduction by interacting with intracellular proteins that initiate intracellular pathways or cascades. Often, signal transduction results in the regulation of gene transcription. The canonical pathways do not operate in isolation, as they interact with and thus affect one another (crosstalk); the sequential pathway concept is further challenged by the regulation of signaling through negative feedback.

1.1 Protein Interactions

Central to the mechanisms of signal transduction are the interactions among intracellular proteins and with certain lipids and cofactor molecules. Several specific, modular interaction domains have been identified in signaling proteins, such as Src homology 2 (SH2), Src homology 3 (SH3), phosphotyrosine binding (PTB), and pleckstrin homology (PH) domains. The affinities of the interactions mediated by such domains, together with the catalytic properties of enzymes involved, determine the specificity and kinetic properties of signal transduction pathways.
**Src homology 2 (SH2) and Src homology 3 (SH3) domains**

Each SH2 domain consists of approximately 100 amino acids (Liu et al., 2006) and binds specific peptide sequences containing a phosphorylated tyrosine residue (Birge, Knudsen, Besser, & Hanafusa, 1996; Buday, 1999; Pawson, 2004). SH2 domains recognize sequences of four to seven residues (Liu et al., 2006; Pawson, Gish, & Nash, 2001), and the amino acids C-terminal to the phospho-tyrosine determine the specificity (Birge et al., 1996). More than 100 SH2 domains have been identified (Pawson, 1995). SH2 domain-containing proteins can be divided into two classes. The first comprises signaling proteins with enzymatic activity, such as Src family tyrosine kinases (from which SH2 domains were named), phospholipase C-\(\gamma\) (PLC-\(\gamma\)), p120 Ras-GAP, and SH2 domain containing protein tyrosine phosphatases 1 and 2 (Shp-1/2) (Buday, 1999; Schlessinger, 2000). Proteins in the second class do not have catalytic function but act as adaptor proteins linking various proteins in multi-molecular complexes; these include the p85 regulatory subunit of phosphoinositide 3-kinases (PI3Ks; see below), Grb2, Nck, Crk, and others (Buday, 1999; Pawson, 2002; Schlessinger, 2000; Schlessinger & Ullrich, 1992). The dissociation constant \(K_D\) of SH2 domain binding is typically in the range of 10 nM to 1 \(\mu\)M (Pawson, 1995). Many signaling proteins have tandem SH2 domains, which can yield high-avidity binding to dual phospho-tyrosine motifs in the low nM range.

SH3 domains also mediate protein-protein interactions. They bind proline-rich motifs of approximately 10 amino acids (Buday, 1999; Pawson, 1995). The specificity of different SH3 domains is determined by the amino acid residues adjacent to the prolines (Birge et al., 1996). Nearly 300 SH3 domains have been reported (Pawson, 2002), and the \(K_D\) values for SH3 domains have been found to lie in the range of 1-100 \(\mu\)M (Pawson, 1995).

**Phosphotyrosine binding (PTB) domains**

Like SH2 domains, PTB domains mediate association with tyrosine-phosphorylated proteins (Schlessinger, 2000); however, they are structurally distinct from SH2 domains (Kavanaugh, Turck, & Williams, 1995). PTB domains consist of approximately 160 amino acids, and unlike SH2 domains, PTB domains recognize amino acid residues N-terminal to
the phospho-tyrosine site. The $K_D$ values of PTB domains are similar in magnitude to SH2 domains, approximately 10-100 nM (Vandergeer & Pawson, 1995).

**Pleckstrin homology (PH) domains**

More than 200 PH domains have been identified by homology (Pawson, 2002; Schlessinger, 2000), and each is composed of approximately 120 amino acids (Musacchio, Gibson, Rice, Thompson, & Saraste, 1993). PH-containing proteins are generally cytoplasmic in unstimulated cells but associate with specific lipids at the plasma membrane (Pawson, 1995), mediating a membrane localization function (Falasca et al., 1998). They have been found in a wide variety of proteins, including serine/threonine and tyrosine-specific protein kinases, regulators of small GTPases, and cytoskeleton-modifying proteins.

### 1.2 Receptor Tyrosine Kinases

Receptor tyrosine kinases are one large family of cell surface receptors. These transmembrane proteins contain three major domains; an extracellular domain, a single transmembrane domain, and a cytoplasmic domain. The extracellular domain is responsible for binding to a specific ligand and transmission of the biological signal to the cytosolic domain. The cytosolic domain contains a conserved protein tyrosine kinase core and regulatory sequences that are subjected to autophosphorylation (Hubbard & Till, 2000; Pawson, 1995, 2002; Schlessinger, 2000; Schlessinger & Ullrich, 1992).

The receptor tyrosine kinases discussed most centrally in this chapter are the receptors for platelet-derived growth factor and fibroblast growth factor ligands.

#### 1.2.1 Platelet Derived Growth Factor Receptors and Their Ligands

Platelet-derived growth factor (PDGF) is a potent stimulator of cell growth, proliferation and migration (Deuel, Kawahara, Mustoe, & Pierce, 1991; Heldin, Ostman, & Ronnstrand, 1998; Seifert et al., 1989). Four single PDGF polypeptide chains, A, B, C, and D, have been identified. These polypeptide chains are linked by disulfide bonds to form five known dimeric isoforms: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD
(Fredriksson, Li, & Eriksson, 2004). Two structurally related PDGF receptors (PDGFRs), $\alpha$ and $\beta$, have been characterized. After ligand binding, these receptors can form either PDGFR-$\alpha \alpha$ and PDGFR-$\beta \beta$ homodimers or PDGFR-$\alpha \beta$ heterodimers (Emaduddin, Ekman, Ronnstrand, & Heldin, 1999; Kelly et al., 1991). PDGFR-$\alpha \alpha$ can bind to PDGF-AA, PDGF-BB, PDGF-AB, and PDGF-CC. The binding partners of PDGFR-$\alpha \beta$ are PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD. PDGFR-$\beta \beta$ homodimers can form in response to PDGF-BB or PDGF-DD binding (Li & Eriksson, 2003). PDGF-BB is the best characterized of the PDGFR ligands.

*Platelet Derived Growth Factor-BB (PDGF-BB)*

PDGF-BB stimulates cell proliferation, survival, motility and wound healing (Deuel et al., 1991; Heldin et al., 1998; Ross, Raines, & Bowenpope, 1986; Seifert et al., 1989). It is a 24 kDa disulfide-linked homodimer of two B chains totaling 218 amino acids. As outlined above, PDGF-BB promotes dimerization of both PDGFR isoforms.

*Structure of PDGFRs*

PDGFR-$\alpha$ is comprised of 1063 amino acid residues, and the PDGFR-$\beta$ contains 1067 residues. Mature forms of the $\alpha$- and $\beta$-receptors have molecular weights of 170 and 190 kDa, respectively (Heldin et al., 1998). The latter has been more extensively characterized from the standpoint of intracellular signaling. In contrast with EGFR family receptors, which contain only a single tyrosine kinase domain, each PDGFR has two tyrosine kinase domains.

*Receptor-Ligand Binding and Initiation of Signal Transduction*

After stimulation of PDGFRs with PDGF-BB, 2:1 receptor-ligand complexes are formed with effective $K_D = 0.1-1\text{nM}$ (Kelly et al., 1991; Park, Schneider, & Haugh, 2003). After dimerization, autophosphorylation of the intracellular domains occurs in *trans*, mediating intracellular signaling.
In PDGFR-α, several phosphorylated tyrosine residues have been identified. Phosphorylation of Tyr849 in the tyrosine kinase domain is important for activation of the kinase activity (Emaduddin et al., 1999; Heldin et al., 1998). There are 10 known PDGFR-α autophosphorylation sites: Tyr572, Tyr574, Tyr720, Tyr731, Tyr742, Tyr754, Tyr762, Tyr768, Tyr988, and Tyr1018 (Heldin et al., 1998). Binding partners include Src (Tyr572), PI3K (Tyr731 and Tyr742), the protein phosphatase Shp-2 (Tyr720 and Tyr754), the adaptor protein Crk (Tyr762), and PLC-γ1 (Tyr988 and Tyr1018) (Heldin et al., 1998).

There are 11 known autophosphorylation sites in PDGFR-β: Tyr579, Tyr581, Tyr716, Tyr740, Tyr751, Tyr763, Tyr771, Tyr775, Tyr857, Tyr1009, and Tyr1021 (Heldin et al., 1998; Kelly et al., 1991). Like PDGFR-α, phosphorylation of Tyr857 in the kinase domain is essential for kinase activity (Emaduddin et al., 1999; Heldin et al., 1998). PDGFR-β interactions with intracellular signaling proteins show a similar pattern as for PGDFR-α, with some notable differences. Src interacts with Tyr572 and Tyr581 via its SH2 domain (Heldin et al., 1998), and PI3K binds with Tyr740 and Tyr751 (Franke et al., 1995). Shp-2 binds to Tyr1009 with high affinity and to Tyr763 with lower affinity (Heldin et al., 1998). Phosphorylation of Tyr1021 allows high-affinity binding of PLC-γ (Heldin et al., 1998). In addition, the adaptor protein Grb2 associates with Tyr716, and Nckβ can interact with Tyr1009, via their SH2 domains (Heldin et al., 1998). The Ras-GTPase activating protein (p120 RasGAP) binds with high affinity to Tyr771 (Emaduddin et al., 1999; Heldin et al., 1998). Figure 1.1 summarizes the phosphorylation sites and corresponding binding partners of PDGFR-β homodimers.
1.2.2 Fibroblast Growth Factor Receptors and Their Ligands

The fibroblast growth factor receptor (FGFR) family consists of four members: FGFR-1 (known as flg), FGFR-2 (bek), FGFR-3 and FGFR-4. Fibroblast growth factors (FGFs) vary in size from 155 to 268 amino acids and share 33-65% amino acid sequence identity (Jaye, Schlessinger, & Dionne, 1992). FGFs consists of a family of heparin-binding polypeptides, recently including 18 members (De Marchis et al., 2002); these are acidic-FGF (aFGF or FGF-1), basic-FGF (bFGF or FGF-2), KGF (FGF-7), FGF-3, FGF-4 and so on. Of these, bFGF has been by far the best characterized.
Basic Fibroblast Growth Factor (bFGF)

bFGF plays a crucial role in cell proliferation, wound healing process and tumor angiogenesis (Pintucci et al., 2005). It is a single polypeptide (monomeric) with \( MW = 18 \) kDa (Gospodarowicz, Neufeld, & Schweigerer, 1986; Plotnikov, Schlessinger, Hubbard, & Mohammadi, 1999), and it is so named because of its basic pI of 9.6 (Gospodarowicz et al., 1986; Rifkin & Moscatelli, 1989). It contains two domains for heparin binding (Rifkin & Moscatelli, 1989).

Structure of FGFRs

FGFRs are approximately 125-145 kDa (Burgess & Maciag, 1989; Jaye et al., 1992). The extracellular domains of FGFRs contain three immunoglobulin-like (Ig-like) regions (two short domains and one long domain) (Ornitz et al., 1996). The binding specificity of the receptors has been mapped to specific sequence differences (Ornitz et al., 1996). FGFRs have a split kinase domain in their intracellular domains, which lies between residues 421 and 780 in FGFR-1 (Mohammadi et al., 1996; Mohammadi, Schlessinger, & Hubbard, 1996; Sorensen et al., 2006).

Receptor-Ligand Binding and Initiation of Signal Transduction

FGFR-1 shows the highest binding affinity to bFGF, with effective \( K_D = 25-50 \) pM (Ornitz & Leder, 1992). FGFR-2 (Dionne et al., 1990), FGFR-3 (Ornitz & Leder, 1992), and FGFR-4 (Ron et al., 1993) bind bFGF with lower, but still subnanomolar, affinity. bFGF competes with other FGFs for receptor binding (Rifkin & Moscatelli, 1989).

The binding of bFGF and FGFR occurs by a unique mechanism. In order to bind, the monomeric bFGF must be presented as a dimer, for which two different mechanisms have been proposed. The first mechanism is that bFGF binding to heparin in the extracellular matrix promotes bFGF dimerization, while also protecting bFGF from denaturation and enzymatic degradation. In the second mechanism, heparin sulfate proteoglycans (HSPGs) found on the cell surface serve as low affinity receptors (\( K_D = 2 \) nM) for bFGF. After bFGF-HSPG complexes are formed, high-affinity FGFRs are thought to interact with these
complexes, forming ternary complexes. bFGF can bind to FGFRs only when bound to either cell surface HSPGs or to free, soluble heparin or heparin-like molecules. Obviously, the recruitment of FGFR is essential for FGF-stimulated signal transduction (Baird, Schubert, Ling, & Guillemin, 1988; Bikfalvi, Klein, Pintucci, & Rifkin, 1997; Jaye et al., 1992; Klagsbrun & Baird, 1991; McKeehan & Kan, 1994; Yayon, Klagsbrun, Esko, Leder, & Ornitz, 1991).

Like the other receptor tyrosine kinases, ligand binding induces FGFR homo- and hetero-dimerization, and dimerized receptors are autophosphorylated (Mohammadi et al., 1991). Phosphorylated tyrosine residues in FGFR-1 are Tyr463, Tyr583, Tyr585, Tyr653, Tyr654, Tyr730 and Tyr766 (Mohammadi et al., 1996; Vainikka et al., 1994). The adaptor proteins Crk and FGF receptor substrate-2 (FRS2) bind to phosphorylated Tyr463 and Tyr585, respectively (Kouhara et al., 1997; Ong et al., 2001). The autophosphorylation sites Tyr653 and Tyr654 interact with Shc via its only SH2 and PTB domains (Klint, Kanda, & Claessonwelsch, 1995; Kouhara et al., 1997). Tyr-766 is a major site for SH2 domain binding of PLC-γ (Mohammadi et al., 1992; Mohammadi et al., 1991; Peters et al., 1992; Rhee & Bae, 1997; Vainikka et al., 1994). Grb2 does not bind directly to FGFR-1 (Klint et al., 1995). Figure 1.2 shows the phosphorylation sites and corresponding binding partners of FGFR1/FGFR1.
1.3 Signal Transduction Pathways

Upon ligand binding to a receptor and autophosphorylation of cytosolic domain of the receptor, SH2, SH3, PH and PTB-domain containing cytosolic proteins, such as kinases, adaptor proteins and phospholipases, initiate signal transduction cascades after membrane recruitment (Buday, 1999; Pawson, 1995, 2004; Schlessinger, 2000).

1.3.1 PI3-kinase Pathway

The phosphoinositide 3-kinase (PI3K) pathway plays important roles in cytoskeletal rearrangements, vesicle trafficking, migration, cell proliferation, and survival pathways and
has been investigated in the contexts of human diseases including diabetes and cancer (Cantley, 2002; Franke, Kaplan, & Cantley, 1997; Shaw & Cantley, 2006; Vivanco & Sawyers, 2002).

Class IA PI3Ks are heterodimeric complexes, comprised of a 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (Schlessinger, 2000). p85 proteins are adaptors that contain two SH2 domains and one SH3 domain (Aaronson, 1991). These SH2 domains are primarily responsible for binding of PI3K to activated growth factor receptors, most notably PDGFRs (Cooper & Kashishian, 1993; Escobedo, Kaplan, Kavanaugh, Turck, & Williams, 1991; Gu & Neel, 2003; McGlade et al., 1992). Other receptors, such as EGFR, lack binding sites for PI3K and therefore must utilize scaffold proteins such as Gab1, which can recruit PI3K activity after being phosphorylated by EGFR and other tyrosine kinases (Gu & Neel, 2003; Kiyatkin et al., 2006; Rodrigues, Falasca, Zhang, Ong, & Schlessinger, 2000). In FGFR signaling, Gab1 cannot associate with FGFRs; however, it has been shown that Gab1 can interact with FRS2:Grb2 complex (Gu & Neel, 2003; Ong et al., 2001). The binding of Gab1 to p85 is regulated as a consequence of Shp2-mediated dephosphorylation of Gab1 (Cunnick, Mei, Doupnik, & Wu, 2001; Gu & Neel, 2003; Shi, Yu, Park, Marshall, & Feng, 2000; Zhang et al., 2002). Membrane localization of PI3Ks generally promote their enzymatic activity, by virtue of enhanced access to their lipid substrates (Klippel et al., 1996). Class IA PI3Ks catalyze the conversion of PtdIns(4,5)P$_2$ to generate PtdIns(3,4,5)P$_3$ on the membrane (Di Paolo & De Camilli, 2006; Franke et al., 1995; Hawkins, Jackson, & Stephens, 1992; Jackson, Stephens, & Hawkins, 1992). Phosphatase and tensin homologue (PTEN) and other 3’-specific PI phosphatases are negative regulators of PI3K signaling, as they catalyze the reverse reaction (Rodrigues et al., 2000; Sun et al., 1999). In addition to the 3’-phosphatase action of PTEN, PtdIns(3,4,5)P$_3$ are also regulated by 4’- and 5’-phosphatases producing PtdIns3P and PtdIns(3,4)P$_2$, respectively. The best characterized of these enzymes are the Src homology 2 (SH2) domain-containing inositol 5-phosphatases 1 and 2 (SHIP1/2) (Leslie, Batty, Maccario, Davidson, & Downes, 2008; Rohrschneider, Fuller, Wolf, Liu, & Lucas, 2000; Weber et al., 2003). PtdIns(3,4,5)P$_3$ recruits a number of PH-domain-containing proteins to the plasma membrane, such as the serine-threonine kinases phosphoinositide-
dependent protein kinase 1 (PDK1) (Kandel & Hay, 1999; Millward, Zolniewicz, & Hemmings, 1999; Toker & Cantley, 1997) and Akt (Marte & Downward, 1997; Stokoe et al., 1997). PtdIns(3,4,5)P$_3$-bound PDK1 is thus able to phosphorylate Akt on Thr308 as an initial step in Akt activation (Hill, Feng, & Hemmings, 2002; Shaw & Cantley, 2006). PIP$_3$ is also important for Akt phosphorylation on a second site, Ser473 (Bayascas & Alessi, 2005). The phosphorylation of Akt on Thr308 and Ser473 is essential for its kinase activity (Kandel & Hay, 1999). Phosphorylated Akt mediates anti-apoptotic cell behavior and cell survival.

The kinase mammalian target of rapamycin (mTOR), which is found in two distinct complexes, mammalian TOR complex 1 (mTORC1) and mammalian TOR complex 2 (mTORC2), has recently been implicated in Akt regulation (Jacinto et al., 2006; Sarbassov, Ali, & Sabatini, 2005; Sarbassov, Guertin, Ali, & Sabatini, 2005; Shaw & Cantley, 2006; Wang, Harris, Roth, & Lawrence, 2007). The name mTOR derives from its association with the compound rapamycin, which has been used for many years as a drug that suppresses the immune system after organ transplantation. These two structurally and functionally distinct proteins share the same catalytic subunit, mTOR. Both complexes contain multiple tandem HEAT (for Huntington, EF3, A subunit of PP2A, TOR1) repeats. Rapamycin-sensitive adaptor protein of mTOR (Raptor) in TORC1 and rapamycin-insensitive companion of mTOR (Rictor) in TORC2 function as adaptor proteins (Hay & Sonenberg, 2004; Wullschleger, Loewith, & Hall, 2006). Both mTORC1 and mTORC2 bind to mLST8, which is previously identified as a G protein β subunit like protein (GβL) (Kim et al., 2003). SAPK interacting protein 1 (Sin1), which has a PH domain, stabilizes the mTOR:Rictor complex (Schroder et al., 2007). Sin1 may provide docking sites for mTORC2 substrates while directly interacting with Akt (Jacinto et al., 2006). It has been shown that only mTORC2 phosphorylates Akt on Ser473 (Sarbassov et al., 2005; Sarbassov et al., 2005; Shaw & Cantley, 2006). However, mTORC1 is phosphorylated by active Akt on Thr2446, Ser2448 and Ser2481 (Nave, Ouwens, Withers, Alessi, & Shepherd, 1999; Peterson, Beal, Comb, & Schreiber, 2000; Sekulic et al., 2000). Tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2) are upstream regulators of mTOR. TSC2 is homologous to GTPase-activating proteins (GAPs). Its heterodimerization with TSC1 affects
the activity of Ras homolog enriched in brain (Rheb) via its GAP activity (Inoki, Li, Zhu, Wu, & Guan, 2002). Moreover, Rheb is required for mTORC1 activation. Akt-dependent phosphorylation of TSC2 inhibits TSC1/TSC2 GAP activity. As a consequence, GTP-bound Rheb activates mTORC1 (Castro, Rebhun, Clark, & Quilliam, 2003; Garami et al., 2003).

Another important signaling function of PI3K is the regulation of cytoskeletal dynamics and, by extension, cell migration, which are controlled in large part by the Rho family of small GTPases, such as Rho, Rac, and Cdc42. Certain activators of these GTPases contain PH domains and are thus recruited by 3′-phosphorylated PIs, promoting conversion of Rho, Rac, or Cdc42 to its active, GTP-bound state (Bishop & Hall, 2000; Chong, Tan, Lim, & Manser, 2001; Rameh & Cantley, 1999). Rac- and Cdc42-GTP activate isoforms of the serine/threonine kinase p21-activating kinase (PAK), which plays an important role in a variety of cellular functions including cell morphogenesis, motility, survival, angiogenesis, and mitosis (Bishop & Hall, 2000). PAK can also be activated by direct association with Nckβ (Li, Fan, & Woodley, 2001; Liu et al., 2006; Lu, Katz, Gupta, & Mayer, 1997; Zhao & Manser, 2005; Zhao, Manser, & Lim, 2000).

Figure 1.3 summarizes PI3K-dependent pathways and their interaction with mTOR pathway.
1.3.2 MAPK Pathway

Mitogen-activated protein kinase (MAPK) cascades are highly conserved in eukaryotic cells from yeast to man. The common feature of these pathways is the sequential activation of three kinases; the kinase that phosphorylates the MAPK is generally termed a MAPKK (MAPK kinase), and the kinase that phosphorylates the MAPKK is called a MAPKKK (MAPK kinase kinase). In mammalian cells, the prototypical MAPK is known as extracellular signal-regulated kinase (ERK), and it plays an essential role in cell proliferation, differentiation, and metastasis (Downward, 1992). This pathway is a major downstream target stimulated by all growth factor receptors, such as EGFR, PDGFRs and FGFRs.

The ERK signaling cascade is commonly associated with the small GTPase Ras, of which there are three closely related genes in mammals: H-Ras, N-Ras, and K-Ras. Ras activation is initiated through recruitment of the adaptor protein Grb2, which can bind with its SH2 domain either directly to a specific phosphorylated tyrosine residue on the receptors or indirectly via phosphorylated Shc (Egan et al., 1993; Klint et al., 1995; Rozakisadcock,
The SH3 domains of Grb2 constitute a binding site for the Ras guanine nucleotide exchange factor (Ras-GEF) protein Son of sevenless (Sos) (Buday & Downward, 1993; Chardin et al., 1993; Marte & Downward, 1997). Sos is recruited to the plasma membrane by association with receptor-bound Grb2, which can be stabilized by interaction of the Sos PH domain with lipids (Buday, 1999). Sos localization is important because it affects the proximity of the GEF enzyme to Ras, which is normally associated with the plasma membrane through post-translational lipid modifications. Sos and other Ras-GEFs function by promoting the dissociation of bound GDP from Ras and the subsequent uptake of GTP, which is abundant in the cytosol (Downward, 1992; Hall, 1990). Ras-specific GTPase activating proteins (GAPs) close the activation cycle by stimulating the GTPase activity of Ras and thus the hydrolysis of bound GTP to GDP, and they maintain Ras in a predominantly GDP-bound state in unstimulated cells (Hall, 1990; West, Kung, & Kamata, 1990). This is not to say that Ras-GAPs are not modulated in response to stimulation, as it is established that p120 RasGAP is among the proteins recruited by RTKs and other tyrosine phosphorylated complexes. The phosphatase Shp-2 regulates p120 RasGAP recruitment by dephosphorylating those sites (Kiyatkin et al., 2006; Montagner et al., 2005; Yoo & Hayman, 2006).

In the active, GTP-bound state, Ras recruits the cytosolic serine/threonine kinase Raf to the plasma membrane, allowing it to be activated (Avruch, Zhang, & Kyriakis, 1994; Freed, Symons, Macdonald, McCormick, & Ruggieri, 1994; Michaud, Fabian, Mathes, & Morrison, 1995). A-Raf, B-Raf and c-Raf (also known as Raf-1) are three isoforms of Raf family kinases. B-Raf and c-Raf have been strongly implicated in growth factor-stimulated MEK activation and their modulation by phosphorylation is reasonably well understood. Recruitment of c-Raf by active Ras is the first step of its complex activation process. Inactive c-Raf is found to be bound to 14-3-3 proteins through the cysteine-rich domain (CRD) of c-Raf (Dhillon & Kolch, 2002; Kolch, 2000; Morrison & Cutler, 1997). The phosphorylation of Ser259 and Ser621 mediates binding of the 14-3-3 protein, resulting in c-Raf inactivation (Chang et al., 2003; Zimmermann & Moelling, 1999). In quiescent cells, this interaction maintains c-Raf in an inactive state (Morrison, 2001; Roy & Therrien, 2002). Ras binding
displaces 14-3-3 from Ser259 (Claperon & Therrien, 2007; Kubicek et al., 2002). Therefore, the first function of 14-3-3 is to negatively regulate c-Raf activity (Morrison & Cutler, 1997; Roy & Therrien, 2002). The association of Ras binding domain (RBD) of c-Raf with the Ras effector region is a high-affinity interaction mediated primarily by the residues Gln66, Gln84 and Arg89 of c-Raf (Morrison & Cutler, 1997). The second role of 14-3-3 is as a positive regulator by facilitating and stabilizing the active c-Raf conformation (Michaud et al., 1995; Morrison & Cutler, 1997); however, it is not essential for c-Raf activation and alone is not sufficient to activate c-Raf. It is found that 14-3-3 protects c-Raf from being inactivated by phosphatases (Freed et al., 1994; Michaud et al., 1995). As a hypothesis, 14-3-3 functions to keep c-Raf in an inactive state in quiescent cells, and in response to signaling events may participate in the activation of c-Raf (Dhillon & Kolch, 2002; Michaud et al., 1995). Activation of c-Raf is completed through the phosphorylation of Ser338 and/or Tyr341; inactivating mutations of either site results in a major decrease in c-Raf activity (Dhillon & Kolch, 2002; Mason et al., 1999; Oehrl, Rubio, & Wetzker, 2003; Zang et al., 2008). c-Raf is desensitized through ERK-dependent negative feedback that results in hyper-phosphorylation of c-Raf on Ser289, Ser296 and Ser301 (Dougherty et al., 2005; Hekman et al., 2005). Several other phosphorylation sites have been reported related to activation and deactivation of c-Raf. Phosphorylation of Thr491 and Ser494 has a positive effect on c-Raf activity; however, it is not sufficient to fully activate c-Raf (Chong, Lee, & Guan, 2001). The other negative regulatory phosphorylation sites of c-Raf are Ser29, Ser43, Ser621 and Ser642 (Dougherty et al., 2005; Kubicek et al., 2002; Noble et al., 2008). Activation of B-Raf is similar to c-Raf, the phosphorylation sites on B-Raf, Ser445, Thr598 and Ser601 (residue numbers are from the human proteins), are the corresponding sites on c-Raf, Ser388, Thr491 and Ser494 (Brummer, Shaw, Reth, & Misawa, 2002; Karbowniczek, Robertson, & Henske, 2006; Zhang & Guan, 2000). The B-Raf activation mechanism differs in that Ser445 is constitutively phosphorylated, priming B-Raf for Ras-dependent activation (Tran, Wu, & Frost, 2005). B-Raf is negatively regulated by PKA-dependent phosphorylation of Ser364 (Hmitou, Druillennec, Valluet, Peyssonaux, & Eychene, 2007). Phosphorylation of B-Raf at Ser151 inhibits its binding to active Ras (Ritt, Monson, Specht, & Morrison). In B-Raf, two
ERK-dependent negative feedback sites have been identified, Ser750 and Thr753 (Brummer, Naegele, Reth, & Misawa, 2003; Ritt et al.). Tables 1.1 and 1.2 summarize the phosphorylation sites and their regulatory effects on B-Raf and c-Raf, respectively. Recent studies have been showed that B-Raf and c-Raf both homo- and heterodimerize \textit{in vivo} (Rushworth, Hindley, O'Neill, & Kolch, 2006; Shinkai et al., 1996; Weber, Slupsy, Kalmes, & Rapp, 2001). Active Ras induces the B-Raf:c-Raf heterodimerization through the exposure of 14-3-3 binding site at Ser621 on c-Raf (Weber et al., 2001). This complex is also subject to negative feedback control. ERK-dependent phosphorylation of B-Raf on Thr753 promotes the disassembly of Raf heterodimers (Ritt et al.; Rushworth et al., 2006).

Table 1.1 B-Raf phosphorylation sites.

<table>
<thead>
<tr>
<th>Phosphorylation sites</th>
<th>Regulatory effect</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser151</td>
<td>Negative</td>
<td>Ras binding</td>
</tr>
<tr>
<td>Ser364</td>
<td>Negative</td>
<td>14-3-3 binding</td>
</tr>
<tr>
<td>Ser445</td>
<td>Positive</td>
<td>Constitutive</td>
</tr>
<tr>
<td>Ser598/Ser601</td>
<td>Positive</td>
<td>Activation loop</td>
</tr>
<tr>
<td>Ser750/Thr753</td>
<td>Negative</td>
<td>Feedback loop</td>
</tr>
</tbody>
</table>

Table 1.2 c-Raf phosphorylation sites.

<table>
<thead>
<tr>
<th>Phosphorylation sites</th>
<th>Regulatory effect</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser29</td>
<td>Negative</td>
<td>Feedback loop</td>
</tr>
<tr>
<td>Ser43</td>
<td>Negative</td>
<td>Feedback loop</td>
</tr>
<tr>
<td>Ser259</td>
<td>Positive</td>
<td>14-3-3 binding</td>
</tr>
<tr>
<td>Ser289/Ser296/Ser301</td>
<td>Negative</td>
<td>Feedback loop</td>
</tr>
<tr>
<td>Ser338</td>
<td>Positive</td>
<td>By PAK</td>
</tr>
<tr>
<td>Tyr341</td>
<td>Positive</td>
<td>By v-Src</td>
</tr>
<tr>
<td>Thr491/Ser494</td>
<td>Positive</td>
<td>Activation loop</td>
</tr>
<tr>
<td>Ser621</td>
<td>Negative</td>
<td>14-3-3 binding</td>
</tr>
<tr>
<td>Ser642</td>
<td>Negative</td>
<td>Feedback loop</td>
</tr>
</tbody>
</table>

Activated Raf is a MAPKKK and as such phosphorylates the MAPKK of the ERK cascade, MAPK/ERK kinase (MEK) (Avruch et al., 1994; Diaz et al., 1997). Raf can activate both MEK isoforms, MEK-1 and MEK-2 (Kolch, 2000, 2005). The serine residues Ser218
and Ser222 are the primary sites of MEK-1 phosphorylation by Raf (Xu et al., 1995; Zheng & Guan, 1994).

Another type of MAPK pathway regulator is the scaffold proteins. These proteins nucleate the formation of multi-protein complexes involving multiple kinases of the cascade, enabling the signal transmission to MAPK by facilitating enzyme-substrate interactions. These interactions thus control both spatio-temporal properties and duration of the signaling transduction cascade (Levchenko, Bruck, & Sternberg, 2000; Pouyssegur, Volmat, & Lenormand, 2002; Takahashi, Tanase-Nicola, & ten Wolde).

One of the most studied scaffold proteins in mammalian MAPK signaling is kinase suppressor of Ras (KSR). KSR (105 kDa) can interact with all kinase members of the MAPK pathway. MEK is constitutively bound to KSR, whereas Raf-1, B-Raf and ERK bind in a stimulus-dependent manner (McKay, Ritt, & Morrison, 2009; Ritt et al., 2007). Although KSR contains a kinase domain, its scaffolding function is independent of its kinase activity (Dhanasekaran, Kashef, Lee, Xu, & Reddy, 2007). In quiescent cells, KSR1 is found to be bound with MEK1/2 and the serine-threonine phosphatase PP2A, which positively regulates Ras signaling by dephosphorylating KSR1 and c-Raf (Ory, Zhou, Conrads, Veenstra, & Morrison, 2003). This complex is maintained in the cytosol through its interactions with 14-3-3 and Impedes Mitogenic signal Propagation (IMP), a Ras-sensitive E3-ubiquitin ligase (Chen, Lewis, & White, 2008; Ory & Morrison, 2004). Upon ligand stimulation, activated Ras causes the degradation of IMP, liberating KSR1-MEK1/2 complex. IMP also modulates formation of KSR1-containing complexes, such as KSR1 homodimers, B-Raf/Raf-1 heterodimers, and KSR1/B-Raf and KSR1/MEK complexes (Chen et al., 2008; Matheny et al., 2004; Matheny & White, 2006). This complex translocates to the plasma membrane, where it can bind Raf isoforms and provide a docking site for ERK. Casein kinase 2 (CK2), a component of the KSR1 scaffolding complex, functions as a Raf family N-region kinase. Upon binding of Raf to KSR, CK2 phosphorylates Raf-1 and B-Raf (Ritt et al., 2007). Moreover, active ERK found in KSR1 scaffold complex phosphorylates KSR1 and B-Raf on feedback sites (McKay et al., 2009). Activated ERK is released from the complex and accumulates in the nucleus. In summary, KSR1 acts to both potentiate and attenuate MAPK
signaling cascade during growth factor signaling (McKay et al., 2009). The PH-domain-containing protein, connector enhancer of KSR (CNK), has been identified in a multi-protein complex with c-Raf, and it has been shown that CNK also binds to KSR and stabilizes the KSR:c-Raf interaction in a manner that depends on its PH domain association with PI(3,4,5)P3 (Claperon & Therrien, 2007; Morrison, 2001; Therrien, Wong, Kwan, & Rubin, 1999; Therrien, Wong, & Rubin, 1998).

MEK partner-1 (MP1) is a small scaffold protein that is found constitutively associated with MEK1 and binds to ERK1 to enhance its activation but does not interact with MEK2 or ERK2 (Schaeffer et al., 1998). MP1 is responsible for spatio-temporal regulation of the MAPK pathway via binding to the adaptor protein p14 at late endosomes (Morrison & Davis, 2003; Teis, Wunderlich, & Huber, 2002). Moreover, MP1 associates with active PAK1 and controls PAK1-dependent MEK1 phosphorylation with its binding partner p14 (Pullikuth, McKinnon, Schaeffer, & Catling, 2005).

MAPK organizer-1 (MORG1) is a MP1-interaction partner. It associates with B-Raf, Raf-1, MEK and ERK. The scaffolding function of MORG1 appears to be receptor specific, as it facilitates signal transmission from G-coupled receptors but not from receptor tyrosine kinases (Vomastek et al., 2004).

Paxillin is a scaffold protein found in association with other focal adhesion-specific proteins such as vinculin, FAK, actopaxin and PAK (Brown & Turner, 2004). In quiescent cells, paxillin is contitutively associated with MEK and upon growth factor stimulation, its phosphorylation at Tyr118 results in recruitment of inactive ERK. Binding of Raf to the paxillin complex activates MEK and ERK (Ishibe, Joly, Liu, & Cantley, 2004; Ishibe, Joly, Zhu, & Cantley, 2003).

Figure 1.4 depicts the MAPK/ERK pathway as activated by receptor tyrosine kinases.
1.3.3 PLC-γ Pathway

The phospholipase-C (PLC) pathway plays an important role in propagation and amplification of ligand-induced transmembrane signaling. At least four families of PLCs have been identified: PLC-β, PLC-γ, PLC-δ and PLC-ε. Of these, PLC-γ isoforms associate with receptor tyrosine kinases (RTKs), including EGFR, PDGFRs, and FGFRs via SH2 domain interactions, and its PH domain can stabilize the localization at the plasma membrane (Rhee, 2001; Rhee & Bae, 1997; Rhee, Suh, Ryu, & Lee, 1989).

Multiple tyrosine residues of PLC-γ (Tyr 771, Tyr 783 and Tyr1245) are rapidly phosphorylated by RTKs (Rebecchi & Pentyala, 2000; Rhee, 2001). PLC-γ catalyzes the hydrolysis of phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P$_2$] to generate two second messengers: diacylglycerol (DAG), which remains in the plasma membrane, and soluble inositol (1,4,5)-trisphosphate (IP$_3$) (Di Paolo & De Camilli, 2006; Meisenhelder, Suh, Rhee, & Hunter, 1989; Rhee & Bae, 1997). DAG is required for activation of certain protein kinase
C (PKC) isoforms, while IP₃ induces the release of Ca²⁺ from internal stores such as the endoplasmic reticulum (ER) (Rhee, 2001).

1.3.4 Crosstalk Interactions

The pathways described above also interact with each other through crosstalk, which is important to appreciate because targeting specific molecules involved in signal transduction can have unintended consequences in other pathways. Also, crosstalk may constitute a mechanism by which the inhibition of a therapeutic target might be circumvented. Several modes of signaling crosstalk have been reported, as summarized below; however, there has been no systematic effort to quantify crosstalk interactions or otherwise gauge their relative importance across signaling networks in a given cell type, which is one of the objectives of this project.

It has been reported that Ras can be activated downstream of PLC-γ (Pawson, 2004). One of the results of PLC-γ activation is the increase in DAG and intracellular Ca²⁺ concentrations. This increase stimulates Ca²⁺/DAG-sensitive GEF activity, which localizes to golgi and induces Ras activation at the golgi membrane (Pawson, 2004).

The active Ras-GTP binds and activates the catalytic subunit p110 of PI3K (Jimenez, Hernandez, Pimental, & Carrera, 2002; Kandel & Hay, 1999; Klinghoffer, Duckworth, Valius, Cantley, & Kazlauskas, 1996; Rodriguezviciana et al., 1994). The p85 subunit of PI3K protects the p110 catalytic subunit from Ras-induced activation (Jimenez et al., 2002). The magnitude of the cooperativity is limited by the availability of activated PDGF receptors and PI3K at low and high PDGF concentrations, respectively (Kaur, Park, Lewis, & Haugh, 2006).

Raf is potentially a nexus for crosstalk interactions. The kinase PKC-α, one of the components of the PLC-γ pathway, can activate c-Raf through direct phosphorylation of Ser499 and Ser619 in cells (Carroll & May, 1994; Kolch et al., 1993; Ueda et al., 1996). The serine/threonine kinase Akt antagonizes c-Raf activity by direct phosphorylation of Ser259. This creates a binding site for 14-3-3 at residue Ser259, resulting in negative regulation of c-Raf (Guan et al., 2000; Moelling, Schad, Bosse, Zimmermann, & Schweneker, 2002;
Zimmermann & Moelling, 1999). Finally, PAK acts downstream of the GTPases Rac and Cdc42 to activate c-Raf by phosphorylating Ser338 (Chaudhary et al., 2000; Edin & Juliano, 2005; Kolch, 2000). This interaction also provides resistance to protein kinase A (PKA)-mediated inhibition of the pathway (Edin & Juliano, 2005). PAK also phosphorylates MEK on Ser298 without blocking c-Raf and ERK interaction (Coles & Shaw, 2002; Eblen et al., 2004; Frost et al., 1997; Sundberg-Smith, Doherty, Mack, & Taylor, 2005).

1.3.5 Negative Feedback Interactions in Ras/ERK Pathway

Negative feedback loops are defined as the ability of a system to adjust its output in response to monitoring itself (Freeman, 2000). In the Ras/ERK cascade, three main feedback loops have been identified; desensitization of RasGEF, desensitization of MEK kinase layer and upregulation of phosphatases that specifically dephosphorylate ERK.

The first negative feedback mechanism is ERK-dependent desensitization of Ras-GEFs, which interferes with Ras activation. Upon ligand stimulation, active ERK causes transcriptional up-regulation of four sprouty gene products (Gross, Bassit, Benezra, & Licht, 2001; Wong et al., 2002), which antagonize receptor tyrosine kinase signaling (Casci, Vinos, & Freeman, 1999; Gross et al., 2001; Hanafusa, Torii, Yasunaga, & Nishida, 2002; Ozaki et al., 2001; Reich, Sapper, & Shilo, 1999; Wong et al., 2002). Sprouty proteins are phosphorylated on Tyr55 in response to growth factor stimulation (Hanafusa et al., 2002), a site which functions as a binding site for the SH2 domain of the adaptor protein Grb2 and thus sequesters Grb2, FRS2, and Shp2 (Hanafusa et al., 2002).

Negative regulation of MEK kinases is a complex, multi-step process. Both B-Raf and c-Raf have both positive and negative regulatory phosphorylation sites, which are subject to negative feedback loops (Chong et al., 2001; Hekman et al., 2005) (Tables 1.1 and 1.2). Moreover, regulation of other MEK kinases, such as PAK isoforms, is still unclear. Raf kinase inhibitory protein (RKIP) inhibits c-Raf function by impairing its phosphorylation of MEK by promoting dissociation of c-Raf:MEK complex and acting as a competitive inhibitor of MEK phosphorylation (Corbit et al., 2003; Yeung et al., 2000; Yeung et al., 1999). RKIP has been also found in a complex with c-Raf, MEK and ERK. RKIP binds to either c-Raf or
MEK and prevents association of c-Raf to MEK (Yeung et al., 2000). The protein phosphatase 2A (PP2A), a serine/threonine phosphatase, dephosphorylates both c-Raf and MEK and thus acts as a negative regulator of the pathway (Adams et al., 2005; Bhalla, Ram, & Iyengar, 2002; Chen, Martin, & Brautigan, 1992; Goldberg, 1999; Sun, Charles, Lau, & Tonks, 1993); however, PP2A also shows a positive effect on c-Raf activation by dephosphorylating 14-3-3 and/or c-Raf Ser259 (Abraham et al., 2000; Shin et al., 2009).

Another class of ERK regulators are the dual-specificity MAPK phosphatases (DUSPs or MKPs), which can dephosphorylate both phospho-threonine and phospho-tyrosine residues (Bhalla et al., 2002; Brondello, Brunet, Pouyssegur, & McKenzie, 1997; Hu et al., 2007; Millward et al., 1999). MKP-1 and MKP-3 dephosphorylate both of the ERK sites phosphorylated by MEK (Camps et al., 1998; Muda et al., 1996; Sun et al., 1993). Interestingly, MKP-1 expression is upregulated in an ERK-dependent manner, constituting a negative feedback loop. Both MKP-1 and MKP-3 are subject to proteasomal degradation pathways (Ekerot et al., 2008; Jurek, Amagasaki, Gembarska, Heldin, & Lennartsson, 2009; Marchetti et al., 2005). One important aspect in DUSP regulation is the spatio-temporal regulation of the ERK pathway. MKP-1 and MKP-2 are identified as nuclear phosphatases, whereas MKP-3 and MKP-4 are cytosolic (Caunt, Armstrong, Rivers, Norman, & McArdle, 2008; Keyse, 2000, 2008).

Crosstalk and feedback interactions are shown in Fig. 1.5.
Figure 1.5 Crosstalk and feedback in signal transduction networks. Depiction of a generic network, arranged vertically as follows: receptors, adaptors, receptor-proximal enzymes, membrane-anchored lipids and proteins, and downstream effector kinases. Black arrows indicate recruitment, activation, or production; red arrows indicate negative regulation. (Courtesy of Jason Haugh)
1.4 References


CHAPTER 2

DATA-DRIVEN, MECHANISTIC MODELING
OF BIOCHEMICAL REACTION NETWORKS


Mathematical modeling has emerged as a valuable tool for characterizing and predicting the spatiotemporal dynamics of biochemical reaction pathways and networks in living cells; however, the power of such models is currently limited by the availability of quantitative, kinetic data for comparison and validation. In this chapter, we discuss data-driven modeling of intracellular reaction networks, with a focus on signal transduction in eukaryotic cells. Experimental data types and their limitations, approaches for data processing and normalization, types of models and issues related to model simplification, and parameter estimation methods are covered. To illustrate these principles, we offer two recent examples of data-driven modeling, each dealing with signal transduction through mitogen-activated protein kinases (MAPKs): elucidation of crosstalk between phosphoinositide 3-kinase (PI3K)- and Ras-dependent pathways in mammalian cells.

2.1 Introduction

At a certain level of abstraction, living cells are picoliter-sized reaction vessels in which thousands of biochemical reactions and intermolecular binding processes take place in a dynamic, coordinated, and highly regulated fashion. This is an energy intensive process. Intracellular enzyme activities are modulated by covalent modifications that are rapidly added and removed in a seemingly futile cycle in order to respond to changes in the cell’s external environment. These reactions are responsible for governing cell function, and their
dysregulation and modulation by infectious agents constitute the molecular basis for human disease. From the perspective of chemical kinetics, the inner workings of the cell are fascinating, but we are still a long way from a mechanistic understanding of these reactions and quantitative characterization of their rates. In this chapter, we discuss how mathematical modeling is applied in tandem with biochemical measurements to achieve this goal.

Whether before, during, or after the collection of experimental data, quantitative modeling is a valuable approach for critically assessing and organizing hypotheses that integrate the many processes that might be at play (Mogilner, Wollman, & Marshall, 2006). And, to the extent that a model is trained on a sufficient amount of quantitative data and its mechanistic assumptions are sound, it may be used to predict the outcomes of novel experiments and thus generate new, hypothesis-driven research. Some experiments will inevitably contradict the model predictions, but as with conceptual, ‘arrow diagram’ models, one iteratively refines the model based on new data.

The examples presented here are focused on mechanisms of signal transduction in eukaryotic cells, which are responsible for controlling cell cycle progression, cell motility, responses to stress, programmed cell death, and differentiation of cell function (Hunter, 2000; Pawson, 2004). These reaction pathways transmit information about the cell’s external microenvironment, making them fundamentally distinct from metabolic pathways, which deal in currencies of energy and reducing power. We further narrow our focus on modeling of cell signaling that is both data-driven and rooted in biochemical mechanisms. We distinguish data-driven models from purely theoretical models, where experimental data are either not available or not accessible with current technology, and mechanistic models are distinguished from purely phenomenological and purely statistical/correlative models. To supplement the topics presented here, the reader is referred to a number of reviews on the subject of modeling signal transduction processes (Janes & Yaffe, 2006; Kholodenko, 2006; Ma’ayan, Blitzer, & Iyengar, 2005; Tyson, Chen, & Novak, 2003).

Rather than presenting detailed recipes of experimental or modeling techniques, this chapter aims to shed light on the inherent relationship between the two in data-driven modeling. In Section 2.2 below, the advantages and shortcomings of different experimental
methodologies from the standpoint of modeling and the formulation of models of the appropriate type and level of complexity are discussed. Emphasis is placed on the pressing need for model simplification and more systematic approaches for model parameter specification.

2.2 Principles of Data-Driven Modeling

2.2.1 Types of Experimental Data

Depending on one’s point of view, cell biology is currently either in a data-rich or data-deprived state. There is a wealth of genomic and proteomic data that have yielded mostly qualitative information about the connectivity of pathways, yet there is relatively little in the way of measurements characterizing their dynamics. Here, we briefly discuss the various quantitative experimental methodologies that define the current state of the art and weigh their advantages, caveats, and limitations. We choose to classify measurement techniques in three categories: population endpoint, single-cell endpoint, and single-cell kinetic (Table 2.1). An endpoint measurement is one in which the experiment is stopped at a certain time, and the sample is prepared for analysis, whereas a kinetic measurement is one in which the biochemical readout is monitored in real time. Important considerations include dynamic range (the range of measured values from the lowest limit of detection to the upper limit of assay linearity), throughput (the number of conditions that can be compared in each independent experiment), the ability to multiplex (measure multiple readouts at once), and the ability to assess subcellular localization.
Table 2.1 Capabilities and limitations of common experimental methodologies. Various measurement techniques are rated according to typical performance in four categories: dynamic range/sensitivity, throughput (the number of conditions that can be compared in each independent experiment), the ability to multiplex (measure multiple readouts at once), and the ability to assess subcellular localization. * Immunoblotting using enhanced chemiluminescence and a high-sensitivity, cooled charge coupled device camera for imaging; the traditional method using photographic film for imaging gives a sigmoidal response over a much narrower dynamic range (contributing to the false notion that immunoblotting is generally not quantitative).

<table>
<thead>
<tr>
<th></th>
<th>Dynamic Range</th>
<th>Throughput</th>
<th>Multiplexing</th>
<th>Spatial detail</th>
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<tr>
<td><strong>Population Endpoint:</strong></td>
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<tr>
<td>Immunoblotting *</td>
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<td>+++</td>
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<tr>
<td>Dot blot/ELISA/In-cell</td>
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<tr>
<td>Sandwich ELISA</td>
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<td>In vitro enzymatic assay</td>
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<td>Antibody array</td>
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<td>Mass spectrometry</td>
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<td><strong>Single-cell Endpoint:</strong></td>
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<td>Flow cytometry</td>
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<td>Immunofluorescence</td>
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<td><strong>Single-cell Kinetic:</strong></td>
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<td>Spectral shift</td>
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<td>FRET</td>
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In population endpoint measurements, a large number of cells ($10^3$-$10^8$) are subjected to identical experimental conditions, and a lysate of the cell collective is prepared for analysis. Hence, information about individual cells is lost, and information about subcellular localization is at best indirect; depending on the method of lysis, the preparation can be subdivided based on density and/or detergent solubility into fractions representing different subcellular compartments (cytosol, plasma membrane, endosomes and Golgi, nuclei, etc.). Despite these shortcomings, this approach has several advantages, including potentially high sensitivity and throughput and broad versatility for measuring a variety of molecular readouts; all of these depend critically on the quality of the reagents used. The most common population endpoint measurements, such as immunoblotting, enzyme-linked immunosorbent assays (ELISAs), and in vitro enzymatic assays, involve protein immobilization and the use of antibodies for specific capture or detection. All things being equal, assays that involve an
initial separation step (e.g., gel electrophoresis or the use of a capture antibody) tend to be more specific and therefore have a higher dynamic range. There is also a general trade-off between the ability to multiplex and throughput, as exemplified by antibody arrays (Nielsen & Geierstanger, 2004) and especially current mass spectrometry technology (Domon & Aebersold, 2006; Huang & White, 2008). These methods can also be used in conjunction with co-immunoprecipitation to assess protein-protein interactions; however, because of the work-up time involved, this approach is strongly biased to detect only very stable interactions. From the standpoint of experimental data, the inability to measure intracellular protein-protein interactions quantitatively is arguably the most significant limitation for data-driven modeling.

Single-cell endpoint measurements, which provide information about individual cells, include flow cytometry and immunofluorescence microscopy. Both involve incubation with antibodies, detection of fluorescence, and in the case of intracellular proteins, cell fixation and permeabilization. Flow cytometry offers high throughput in terms of assembling population statistics for each sample and moderate throughput in terms of comparing multiple samples. Immunofluorescence offers information about subcellular localization, but the analysis is tedious and therefore low in throughput.

Single-cell kinetic measurements generally involve microscopic imaging of live cells, in which case information about subcellular localization is obtained. Although this approach suffers from many of the same throughput issues as immunofluorescence, the ability to observe signaling kinetics in real time and in conjunction with cell behavior makes it unique (Giepmans, Adams, Ellisman, & Tsien, 2006; Meyer & Teruel, 2003). The basis for the measurement is the introduction of a biosensor, either genetically encoded or microinjected into the cell; genetically encoded biosensors are fusion proteins comprised of a protein or protein domain of interest, to which a fluorescent protein such as enhanced green fluorescent protein is attached. A limited degree of multiplexing is offered through the use of multiple biosensors labeled with different fluorophores. The dynamic range of the measurement is affected by which particular biosensor and microscopy modality (e.g., wide-field fluorescence, confocal fluorescence, or total internal reflection fluorescence (TIRF)) are
used, and the basis for the measurement (e.g., a shift in spectral properties of the fluorophore, as in calcium imaging, translocation to a particular membrane or intracellular compartment, or changes in Förster resonance energy transfer (FRET)). The most significant limitation of this approach is that there are currently only a small number of biosensors that work well for quantitative studies; another caveat of using biosensors is that they might significantly interfere with or otherwise modulate the signaling processes they were meant to detect.

### 2.2.2 Data Processing and normalization

All data require some form(s) of processing prior to any sort of quantitative analysis. Some of these are obvious and routine, for example the subtraction of assay/image background and the linear rescaling of images for presentation. Typically, quantitative data are also normalized. The purpose of normalization is to adjust for sources of variability, so that the reproducibility of experimentally deduced trends may be compared in a statistically meaningful way. The manner in which this is done varies and is context-dependent (and, in some cases, arbitrary), and hence this topic is worthy of some discussion.

Variability arises because of both the biological system and the assay itself. Biological variability is significant in any measurement involving cells; this is because, no matter how carefully the parameters of the cell culture are controlled, the culture will vary from experiment to experiment. Assay variability arises from heterogeneity within a sample (e.g., from cell to cell in single-cell measurements) and in the preparation of samples, which affects the comparison of conditions within the same experiment, and also from temporal and lot-to-lot changes in the reagents used, which along with biological variability affect the comparison of independent experiments. Sample heterogeneity at the single-cell or population level is generally normalized by dividing the signal by a second measurement that should not be affected by the perturbations being tested. For example, population endpoint measurements are typically normalized by the total amount of cellular protein in the sample or by the amount of an abundant species that should be invariant from sample to sample (e.g., actin or tubulin). This is especially important when comparing samples derived from the same cell line/strain but which have been differentially modified over some period of time,
for example comparing control cells to cells in which over-expression of a wild-type gene or expression of a mutant gene has been introduced. Day-to-day variability of the assay reagents and other assay conditions can be normalized by the measurement of a common standard sample; however, this approach is of little use in the typical case where biological variability is also prominent.

To normalize for biological variability, it is often appropriate to use a negative or positive control sample, acquired in each independent experiment. A pitfall of using a negative control for normalization (e.g., fold-induction) is that it often has the lowest and least reliable signal. For more complex data sets of the sort that is desirable for quantitative modeling, with measurements at multiple time points for a variety of experimental conditions, choosing how to normalize the data by a positive control condition (e.g., maximum stimulation of otherwise unperturbed cells) is subject to some ambiguity. Normalizing by the value at a particular time point is a common practice, but the choice of the time point might be considered arbitrary; normalizing by the maximum (peak) value in each experiment is less arbitrary but nonetheless tends to obscure comparisons between control and non-control conditions at time points other than in the vicinity of the peak. For such data sets, we contend that normalizing in a manner that incorporates all of the time-dependent data for the control condition is more appropriate. Examples include normalizing by the mean value of the control time course, its “area under the curve” (e.g., (Park, Schneider, & Haugh, 2003)), or by normalization factors that minimize its variance across all experiments, e.g., as assessed by the mean coefficient of variation.

Suppose there are \( n \) experiments for which data are collected at \( m \) time points. During each of the \( n \) experiments the same control is run. Let \( X_{ij} \) denote the experimental readout for the control in the \( i \)th experiment at the \( j \)th time point. Often the quantity of interest \( Y_{ij} \) (e.g. the concentration of chemical species) is related to \( X_{ij} \) by an unknown scale factor. That is, \( Y_{ij} = \alpha_i X_{ij} \). Under ideal conditions, the control would not vary from experiment to experiment. Therefore, we seek the set of \( \alpha_i \)'s that minimize a suitable quantity \( F \), for example

\[
F = \sum_{j=1}^{m} \sum_{i=1}^{n} (Y_{ij} - \bar{Y}_j)^2 \quad \text{or} \quad F = \sum_{j=1}^{m} \frac{1}{\bar{Y}_j} \left[ \sum_{i=1}^{n} (Y_{ij} - \bar{Y}_j)^2 \right]^{1/2},
\]
where \( \bar{Y}_j \) is the mean value that results for time point \( j \). The minimization is subject to a constraint that eliminates the trivial solution, \( a_i = 0 \) for all \( i \). Once the \( a_i \) have been found, they are used to scale the experimental time series, allowing the mathematical model to be fit to all the data simultaneously.

### 2.2.3 Suitability of models used in conjunction with quantitative data

In formulating a suitable mathematical description of a system, it is important to cast the model at an appropriate level of abstraction, which should be weighed carefully along with considerations of computational feasibility. While all models of biochemical processes are expected to include fundamentals of chemical reaction kinetics, they are expected to vary along two axes of increasing complexity: from deterministic to stochastic, and from well-stirred to spatially extended (Fig. 2.1). In deterministic models, continuum variables such as species concentrations evolve according to ordinary or partial differential equations (ODEs and PDEs, respectively) and associated initial and boundary constraints, whereas in stochastic models, molecules and molecular complexes are modeled as discrete entities whose states are updated probabilistically (Kepler & Elston, 2001; Li, Cao, Petzold, & Gillepie, 2008). So-called hybrid models incorporate both continuum and discrete variables (Dallon, 2000). On the other axis, well-stirred models assume spatial homogeneity within the domain of interest, and any transport processes in the model (for example, trafficking between intracellular compartments (Lauffenburger & Linderman, 1993)) are incorporated as reaction terms, whereas spatially extended molecules account for spatial gradients and therefore model the underlying transport processes explicitly, according to physicochemical principles (Haugh, 2008).
Figure 2.1 Two axes of mechanistic model complexity. Models can be characterized according to whether they are deterministic or stochastic and whether or not they explicitly account for spatial gradients. Roughly speaking, the degree of computational difficulty increases as one moves from the lower left to the upper right quadrant. In each corner, techniques used to implement such models are listed along with, in parentheses, the type of experimental data that might be described. Abbreviations: ODE, ordinary differential equation; PDE, partial differential equation; SDE, stochastic differential equation; BD, Brownian dynamics.

For data-driven modeling of biochemical systems, the chosen complexity of the model should depend not only on what qualitative information is available in the literature, however reliable, but also in large part on the amount and type of quantitative, experimental data available. For instance, population endpoint measurements tend to be the most versatile and quantitative, yet they do not provide the kind of information that would justify a stochastic or spatially extended description of the model. Therefore, even though more complex models might be formulated, it is most appropriate to cast the model as a set of deterministic ODEs. Data-driven stochastic models generally benefit from single-cell information, which is obtained most quantitatively (albeit without spatial information) from flow cytometry data (Altan-Bonnet & Germain, 2005; Perez & Nolan, 2006; Pirone & Elston, 2004), and spatially extended models must be driven almost exclusively by single-cell kinetic (live-cell microscopy) data (Hirschberg et al., 1998; Janetopoulos, Ma, Devreotes, & Iglesias, 2004; Reynolds, Tischer, Verveer, Rocks, & Bastiaens, 2003; Schneider & Haugh, 2005; Slepchenko, Schaff, Carson, & Loew, 2002).
2.2.4 Issues related to parameter specification and estimation

Another aspect of model complexity that must be carefully considered when making comparisons to data is the amount of molecular detail to include. A comprehensive model, explicitly including all of the “known” biochemistry, comes at the expense of having to identify a large set of parameter values (rate constants and initial concentrations) (Weng, Bhalla, & Iyengar, 1999). Prominent examples of signaling pathway/network models with ~100 or more adjustable parameters have been offered (Bhalla, Ram, & Iyengar, 2002; Hatakeyama et al., 2003; Kiyatkin et al., 2006; Schoeberl, Eichler-Jonsson, Gilles, & Muller, 2002), and in such cases the parameter values are typically culled from published \textit{in vitro} measurements using purified components (or assumed to be similar in magnitude to parameters for related interactions where such data are available) or adjusted by hand to reconcile the sparse biochemical data assembled in various cell types and laboratories. Although models using this approach have proven valuable, it must be recognized that there is a great deal of uncertainty associated with such a parameter specification exercise. Formulation of very detailed models also dictates a qualitative assessment, wherein the model is judged by its ability to correctly produce the gross kinetic features seen in a relatively small collection of measurements (Mogilner et al., 2006).

The other approach is to simplify the model so as to reduce the number of adjustable parameters, to the point where a more direct, quantitative comparison or fit to the data becomes feasible and adequately constrained. Thus, the degree of model simplification is largely determined by the variety of experimental conditions and biochemical readouts in the data set; this, we contend, is the art of data-driven modeling. Simplification of kinetic models is achieved in a number of ways, including the use of scaled, dimensionless variables and through knowledge or assumptions about fast \textit{versus} slow rate processes. Another mode of simplification is the lumping of multiple processes into a single step, which is warranted when quantitative data related to that particular step are absent or unattainable, or when its details are poorly characterized.

Supposing that a model with an appropriate level of granularity has been tailored for a particular set of measurements, how does one fit the model output to the data? This can be
somewhat tricky, because even with appropriate simplification, a pathway/network model is going to have more than a handful of adjustable parameters. Indeed, it is becoming increasingly clear that the values of parameters in models with even modest complexity are not uniquely identifiable, even with near perfect kinetic data (Gutenkunst et al., 2007). With that said, there are efficient methods for identifying a (non-unique) set of parameters that fit the data optimally well. One approach, which has been used to great effect in the modeling of the cell cycle, is the use of global optimization algorithms such as ODRPACK, which implements the Levenberg-Marquardt method with variable step size (Sible & Tyson, 2007; Zwolak, Tyson, & Watson, 2005). Another strategy, which is gaining in popularity, involves Monte Carlo-based or “genetic” algorithms, wherein all of the parameter values are adjusted randomly, according to distributions centered on the current values, and the resulting parameter set is either accepted or rejected with certain probability or based on specified criteria related to the goodness of fit. The classic example of such an approach is the Metropolis algorithm (Metropolis, Rosenbluth, Rosenbluth, Teller, & Teller, 1953) (Fig. 2.2). In this method, parameter sets that improve the goodness of fit are always accepted, whereas sets that yield a poorer fit are accepted with a probability determined by a Boltzmann-like function; the overall error ($\chi^2$) is analogous to the energy, which is compared with a user-specified parameter that is analogous to the thermal energy scale or temperature (the lower the “temperature”, the lower the probability of acceptance). A commonly used variation is simulated annealing, in which the “temperature” is steadily reduced with time, making it more efficient for finding a global optimum (Gonzalez, Kuper, Jung, Naval, & Mendoza, 2007; Hansmann & Okamoto, 1999). Regardless of the method used, it is important to note that the units of the model and those of the measurement are rarely the same, and so a conversion/alignment factor for each data type must usually be assigned or used as a fit parameter.

Faced with the inherent problem of identifying unique parameter values, it might not be fruitful to seek one single, “best” solution to the parameter estimation problem; another approach is the ensemble or collective fitting approach (Brown & Sethna, 2003; Gutenkunst et al., 2007). In this method, one accumulates a large number (potentially $>> 1,000$) of
parameter sets (the ensemble) that fit the data almost equally well. Starting with a single, near-optimal parameter set, the Metropolis algorithm is suitable for collecting the ensemble. At least for ODE models, which are solved with very little computational effort, it is no large task to recompute the model output for each of these parameter sets; the output of the “model”, then, may be taken as the ensemble mean, with its standard deviation yielding a measure of the variability in the model fit or prediction. An advantage of this approach is that one can readily infer whether or not a particular parameter is well constrained by the fit by inspection of the distribution of its values across the ensemble. Arguably, this evaluation is more insightful than the typical sensitivity analysis, which only assesses how the model responds to small changes in the parameter values, made one parameter at a time.
Figure 2.2 Parameter estimation using the Metropolis algorithm. A. Schematic of the algorithm. The values of all model parameters are adjusted at random, according to distributions centered on the previous values, and the resulting quality of fit determines the probability of accepting each successive parameter set. Alignment of the model output to the data is achieved through the assignment of conversion factors, which may be estimated in a separate subroutine. The performance of the algorithm is tuned by adjusting the values of $\alpha$, which characterizes how much the parameters change in each step, and $\beta$, the stringency of the acceptance criterion. B. Illustration of the algorithm run in a highly stringent mode, wherein each accepted move almost always results in a better fit (lower SSD), starting from random guesses of the parameter values. C. After achieving a near-minimum SSD value, the algorithm may be reinitialized with a relaxed stringency, allowing a large number of parameter sets to be collected in an ensemble. The average output of the parameter set ensemble constitutes the output of the model. Quantitative predictions are made through uniform changes (e.g., setting a particular parameter equal to zero) across the ensemble.
2.3 References


CHAPTER 3

SYSTEMATIC QUANTIFICATION OF PI3K-DEPENDENT CROSSTALK INTERACTIONS AFFECTING THE RAS/ERK PATHWAY


Although it is appreciated that canonical signal transduction pathways represent dominant modes 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.
3.1 Introduction

Signal transduction is traditionally characterized in terms of intracellular pathways, which govern outcomes such as cell proliferation, survival, migration, and differentiation. Crosstalk interactions, which couple distinct pathways, are recognized for their importance in cell regulation yet remain poorly defined because of their complex nature (Hunter, 2000). They allow information flow and regulation to be distributed across multiple pathways, and perturbations targeting specific signaling molecules might therefore have unexpected effects (Bray, 1990). Here, we describe the systematic and quantitative characterization of crosstalk between two major signaling pathways, phosphoinositide 3-kinase (PI3K) and Ras/extracellular signal-regulated kinase (Erk), which play prominent roles in signaling networks accessed by many cell surface receptors. Signaling mediated by platelet-derived growth factor (PDGF) receptors, members of the receptor tyrosine kinase class of signal transducers (Claesson-Welsh, 1994; Schlessinger, 2000), is relevant to wound healing, development, and cancer (Heldin & Westermark, 1999) and is distinguished by potent activation of type IA PI3Ks, which control a host of cellular responses through production of specific lipid second messengers (Vanhaesebroeck et al, 2001; Engelman et al, 2006; Hawkins et al, 2006). PDGF receptors also enhance signaling through Ras proteins by promoting Ras guanine nucleotide exchange factor (Ras-GEF) activity and hence an increase in GTP-bound Ras; in this state, Ras activates the canonical Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (Erk) cascade. Erk isoforms are both master integrators of upstream signals and master controllers of transcription factors and other effectors (Kolch, 2000).

Although crosstalk between the PI3K and Ras/Erk pathways has been studied extensively, a definitive pattern of regulation has not yet emerged, because the two pathways apparently affect each other in various ways and in a context-dependent manner. Despite striking evidence that PI3Ks bind to and are activated by Ras-GTP (Rodriguez-Viciana et al., 1994), the effects of blocking Ras signaling on growth factor-stimulated PI3K activation range from partial inhibition (Klinghoffer, Duckworth, Valius, Cantley, & Kazlauskas, 1996; Rodriguez-Viciana et al., 1994) to very little or no inhibition (Kaur, Park, Lewis, & Haugh,
Numerous reports have also implicated PI3K in the activation or regulation of Ras/Erk signaling, but again with disparate conclusions being drawn (discussed in Duckworth & Cantley, 1997). Whereas PI3K inhibitors apparently abrogate Erk activation but not Ras-GTP loading in certain contexts (Cross et al, 1994; Karnitz et al, 1995; Duckworth & Cantley, 1997; King et al, 1997), other reports indicate a role for PI3K upstream of Ras (Hu et al, 1995; DePaolo et al, 1996; Hawes et al, 1996; Wennström & Downward, 1999).

Through a kinetic analysis of PI3K and Ras/Erk signaling in the PDGF receptor system, complemented by quantitative modeling (Tyson et al, 2003; Ma'ayan et al, 2005; Kholodenko, 2006), 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.

3.2 Results

3.2.1 Experimental characterization of PDGF-stimulated Ras/ERK and PI3K Pathways
(work done by Chun-Chao Wang)

To systematically evaluate crosstalk between the PI3K and Ras/Erk pathways, Erk phosphorylation and PI3K-dependent Akt phosphorylation in PDGF-stimulated NIH 3T3 fibroblasts were measured by quantitative immunoblotting for an array of 126 experimental conditions, sampling different combinations of ligand dose, stimulation time, and molecular manipulation. Considering biological replicates and parallel determination of total Erk and
Akt levels, this data set comprises 2,772 total measurements (Experimental work was done by Chun-Chao Wang). We have confirmed on multiple occasions that the immunoblot imaging system used produces a linear response as a function of epitope loaded, over a sufficiently broad dynamic range (unpublished results).

Ras and PI3K promote Erk signaling independently, or PI3K lies upstream of Ras in the same pathway, in which case at least one additional pathway would be responsible for the residual Erk phosphorylation seen when either Ras or PI3K is inhibited. In each of three independent experiments, simultaneous inhibition of Ras and PI3K almost completely abolished PDGF-stimulated Erk phosphorylation, indicating 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 (Fig. 3.1A).

The results reveal that when either Ras or PI3K is inhibited, by expression of dominant-negative (S17N) H-Ras or incubation with LY294002 compound, respectively, PDGF-stimulated Erk phosphorylation is partially inhibited (Fig. 3.1B). Expression of S17N H-Ras sequesters Ras-GEFs and thus affects activation of all Ras isoforms, and we confirmed previously that it prevents PDGF-stimulated Ras-GTP loading in our cells (Kazlauskas & Cooper, 1990). The degree of Erk inhibition depends strongly on both stimulation dose and time.

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 (Kaur et al., 2006; Scheele, Rhee, & Boss, 1995), we measured the kinetics of PDGF-stimulated Ras-GTP loading for selected conditions (Fig. 3.1C). In each of three independent experiments, incubation with 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.
Figure 3.1 Systematic quantification of PDGF-stimulated Erk phosphorylation, RasGTP loading and MKP-1 upregulation. (A) NIH 3T3 fibroblasts were infected with retrovirus produced from empty vector or vector with S17N H-Ras, pretreated with either DMSO control or LY294002, then stimulated with PDGF-BB (dose and time indicated). Whereas inhibition of Ras or PI3K partially blocks Erk phosphorylation, consistent with Fig. 1B&C, blocking both Ras and PI3K abolishes Erk phosphorylation. The results are representative of three independent experiments. (B) Quantification of Erk phosphorylation, comparing either S17N Ras expression (n=6) or PI3K inhibition (n=5). (C) NIH 3T3 fibroblasts were stimulated with PDGF-BB as indicated; pretreatments were control (0.2% DMSO), LY294002 (100 μM), or PD098059 (50 μM). Ras-GTP levels were measured using a quantitative enzymatic assay. (D) Quantification of MKP-1 expression, comparing either S17N Ras expression (n=2) or PI3K inhibition (n=3).
Ras-GTP loading is affected by a known negative feedback loop that destabilizes Ras-GEF recruitment, and accordingly, incubation with PD098059 yields a higher Ras-GTP level (Fig. 3.1C). The net effect of reduced but stable Ras-GEF recruitment would be a lower initial rate and more sustained level of Ras-GTP loading.

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 (Kaur et al., 2006) (Fig. 3.1D). 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. 3.1B), 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. 3.1D); this outcome can be attributed at least in part to the diminished Erk phosphorylation response, integrated over time, when either of the two major pathways is disrupted.

3.2.2 Parsing the magnitudes of PI3K-dependent crosstalk interactions in the PDGF receptor signaling network

Synthesizing the data assembled, a conceptual model of the PDGF receptor signaling network is now clear (Fig. 3.2). 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 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. In the context of the model, the magnitudes of the Ras- and PI3K-dependent 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, and conversely, the observed dynamics can be used to quantify the magnitudes of the inputs.
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; 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 (Brown & Sethna, 2003; Violin et al., 2008) and compared to the corresponding data (Fig. 3.3). The rest of the data used to constrain the algorithm indicates the degree of saturation in the MEK/Erk pathway (Supplementary information, Fig. S2). Taken together, the data force the model to reconcile time- and PDGF dose-dependent features of the network observed under the various experimental conditions tested, including subtle differences between the two control experiments (DMSO vehicle only versus empty pBM-puro vector). Considering the global constraints imposed by the data and the
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. The solid curves represent ensemble means, and the dashed curves are mean ± s.d. \((n = 10,000)\). The data values used to constrain the model (symbols) are also shown.

PDGF concentrations are: red, 1 nM; green, 300 pM; blue, 100 pM; gray, 50 pM; black, 30 pM.

necessarily simplified nature of the kinetic model, it is our assessment that the model performs quite well; however, one feature that it was not able to reconcile well is the sensitivity of the Erk phosphorylation response in S17N Ras-expressing cells stimulated with the lower doses of PDGF (Fig. 3.3; bottom row, second plot from left). We speculate that the apparent sensitivity of the response, relative to the model output, is diminished by cell variability. It is known that variability in the cells’ sensitivity to stimulation (the EC\(_{50}\) of the dose response curve) produces a flatter dose response curve for the population (Altan-Bonnet & Germain, 2005). The model also does not capture the transient nature of the S17N Ras/30 pM PDGF curve, suggesting that ligand depletion or other negative feedback effects might need to be characterized and accounted for in future refinements of the model. Indeed, the transient nature of the Akt phosphorylation response at low PDGF concentrations (data not shown) provides additional evidence for such effects affecting PI3K-dependent signaling.

Parameter statistics 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. 3.4A). The results reveal a consistent ratio of PI3K- and Ras-dependent contributions, as indicated by points clustered around a diagonal line, with the Ras-dependent pathway tending to be more potent under maximal PDGF stimulation. 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). 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 predicted to be intrinsically less potent than the Ras-dependent pathway under maximal PDGF stimulation. Importantly, the MAC quantifies these inputs in a way that uncouples them from negative feedback effects. As shown in Fig. 3.3, feedback desensitization attenuates the maximum Ras-GTP level by roughly two-thirds. Adjusting the MAC to account for this reduction, which presumably affects both Ras-dependent MEK phosphorylation steps equally, yields a median ratio of 1.6 (quartile values of 1.2 and 2.1); that is, Ras-GEF desensitization renders the PI3K-dependent pathway more important. This is consistent with the observation that PI3K inhibition affects Erk phosphorylation somewhat more dramatically than inhibition of Ras-GTP loading.

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. 3.4B). 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, which we attribute to the subtlety of the data constraining it (the effect of PI3K inhibition on Ras-GTP accumulation). Defining the GEF recruitment comparator (GRC) as the ratio of PI3K-dependent/PI3K-independent modes at low PDGF concentrations, the median GRC value for the ensemble is 1.4, with quartile values of 0.7 and 2.0. This analysis suggests that the two Ras activation modes are almost equally important.
3.2.3 Model predictions and potential for model refinement

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. 3.5). 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. 3.5). 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 3.4 Quantitative analysis of PI3K-dependent crosstalk to Ras/Erk. (A) Quantification of Ras- and PI3K-dependent MEK phosphorylation pathways. For each parameter set described under Fig. 7, 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. On the dashed line, the two pathways are equally potent by this measure. (B) 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; when $K_{GP} = K_{GR}/123$ (dashed line), the two modes contribute equally to Ras-GEF recruitment in the limit of low PDGF concentration.
3.3 Discussion

A unique data set was collected to systematically characterize multiple crosstalk interactions between the PI3K and Ras/Erk pathways and regulatory feedback loops in the PDGF receptor signaling network. 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. The basis for these observations lies in the saturability
of the pathways. Half-maximal PDGF receptor phosphorylation is elicited by ~ 0.5-1 nM PDGF-BB (Park, Schneider, & Haugh, 2003), concentrations which yield maximal activation of PI3K and Ras in our cells; downstream of Ras, we also find partial saturation of MEK or Erk. The magnitudes of the inputs to the Erk pathway also affect adaptation of the response imposed by negative feedback regulation.

In contrast, 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 & 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).

To advance this line of inquiry, the PI3K-dependent intermediates that execute the crosstalk to Erk will need to be better and more quantitatively characterized. One candidate pathway is the phosphorylation of Raf-1 on Ser\textsuperscript{338}, which has been implicated in Raf-1 activation. Phosphorylation of Raf-1 on Ser\textsuperscript{338} is catalyzed by isoforms of p21-activated kinase (Pak), which can be activated downstream of PI3K and either Rac or Cdc42 (Chaudhary et al., 2000; Sun, King, Diaz, & Marshall, 2000). Raf-1 mutants that cannot bind Ras-GTP can be activated in cells by co-expression of Pak3 and constitutively active Cdc42, suggesting a Ras-independent pathway (Sun et al., 2000), whereas other evidence suggests a role for Ras in the activation of Raf-1 by Rac/Cdc42 and Pak (Li, Chong, & Guan, 2001; Zang, Hayne, & Luo, 2002). Consistent with this literature, we found that Raf-1 phosphorylation on Ser\textsuperscript{338} strongly depends on both PI3K and Ras in our cells (results not shown); from the standpoint of crosstalk from PI3K to Erk, this finding is inconclusive, because it does not account for the Ras-independent pathway in our cells. Other aspects of Raf-1 regulation might account for the pathway, as there is ample evidence that Ser\textsuperscript{338} phosphorylation is not a strong correlate of Raf-1 activity (Sun et al., 2000; Chiloeches et al., 2001; Oehrl et al., 2003; Beeser et al., 2005); regulation of other Raf isoforms and other putative MEK kinases, such as Pak (Beeser, Jaffer, Hofmann, & Chernoff, 2005; Park, Eblen, & Catling, 2007) and 3-phosphoinositide-dependent protein kinase 1 (Sato, Fujita, & Tsuruo,
2004), might also be considered. It is also worth noting that such PI3K-dependent modes of Erk activation could mask a subtle negative regulation of the Erk pathway, through Akt for example (Zimmermann & Moelling, 1999); however, overexpression of Akt-1 does not affect PDGF-stimulated Erk phosphorylation in our cells, despite a 2-fold increase in phosphorylated Akt (results not shown). Finally, although at least one important mode of PI3K-dependent crosstalk to Erk is Ras-independent, PI3K lipid products apparently contribute upstream of Ras as well, perhaps through direct recruitment of Ras-specific GEF activity or through mediators such as centaurin-α1 (Hayashi et al., 2006).

If such mechanisms are to be tested, mathematical model predictions provide kinetic signatures that could help validate the point of action of a particular pathway from PI3K to Erk. For example, if a particular kinase contributing to the Ras-independent Erk activation pathway were blocked, the model predicts that the time courses of Erk phosphorylation at both low and high PDGF concentrations would be perturbed relative to control (Fig. 9). Furthermore, the extent of perturbation relative to complete PI3K inhibition, especially at high PDGF concentrations, reveals the degree to which the kinase is playing a major role in the pathway. Such an analysis would require quantitative measurements of the type presented here. Importantly, the extent of Erk inhibition in the dominant-negative Ras background would lend further evidence and opportunity for model validation. In turn, characterization of crosstalk mechanisms along these lines allows both conceptual and quantitative models to be refined. The data set generated here, together with new data, will be instrumental in iteratively testing more detailed models of the PDGF receptor signaling network.

Another issue that needs to be confronted is the generality of signal transduction networks across receptor and cell types. Certainly, major disparities in the regulation of signaling in different contexts, even among receptors of the same class, should be expected. Indeed, it is instructive to compare the PDGF receptor network to the proposed regulatory structure ascribed to epidermal growth factor (EGF) receptor, which has been the subject of numerous quantitative modeling efforts (Wiley, Shvartsman, & Lauffenburger, 2003). Recent experimental and modeling work has provided compelling evidence that EGF receptor signaling through PI3K and Erk are coupled primarily through regulation of Gab1 (Kiyatkin
et al., 2006). According to that model, phosphorylated Gab1 recruits PI3K, and Gab1 responds to 3’ phosphoinositides, constituting a positive feedback loop; Gab1 also aids in activation of Ras. Further, Gab1 and therefore PI3K are subject to a negative feedback loop mediated by Erk. The evidence suggests that the inability of EGF receptors to interact directly with PI3K demands the utilization of Gab1 and thus dictates the important features of that network. In contrast, PDGF receptors activate PI3K directly and strongly, perhaps explaining why PI3K is not subject to regulation by Erk in our cells; other evidence suggests that Gab1 is prominently utilized to recruit PI3K in response to EGF, but not PDGF, in the same cell background (Zhang et al., 2002). Clearly, control at the network level is highly context-dependent, and more quantitative approaches will be needed to unravel the mechanisms that distinguish particular signaling systems.

3.4 Appendix: Kinetic modeling and analysis

3.4.1 Kinetic model: PDGF receptor and PI3K activation

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 (Park et al., 2003; Schneider & Haugh, 2005). Definitions and base values of the relevant rate constants are listed in Table 3.1. 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 \( t = 0\),

\[
C_i = \frac{[L]R}{K_{D,L}};
\]

\[
\frac{d(R + C_i)}{dt} = V_s - k_i(R + C_i) + 2\left(k_{-x}C_2 - k_x C_i^2\right);
\]

\[
\frac{dC_2}{dt} = k_x C_2^2 - (k_{-x} + k_r)C_2;
\]

\[
R(0) + C_i(0) = R_0 = \frac{V_s}{k_i}; \quad C_2(0) = 0.
\]
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 $k_x, k_x, k_t, k_e$ 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,

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

$$c_1 = [L]r/K_{D,L}; \quad r(0) + c_1(0) = 1; \quad (\text{Eq. S2})$$

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

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 $k_t$.

**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 (Haugh, 2006; Schneider & Haugh, 2005), 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 (Park et al., 2003; Schneider & Haugh, 2004). Hence, it is justified to assume pseudo-equilibrium for PI3K binding:

$$e_{PI3K}(t) = \frac{2\alpha_{PI3K} c_2(t) - e_{PI3K}(t) \left[1 - e_{PI3K}(t) \right]}{\alpha_{PI3K}}; \quad \alpha_{PI3K} = A_{mem} R_0 E_{PI3K,Tot} / V_{cyt} K_{D,PI3K} / E_{PI3K,Tot}; \quad \kappa_{PI3K} = V_{cyt} K_{D,PI3K} / E_{PI3K,Tot};$$

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 (Haugh, 2006; Park et al., 2003; Schneider & Haugh, 2005):

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

The base values of the parameters for this portion of the model (\( \alpha_{PI3K} \), \( \kappa_{PI3K} \), and \( k_{3PI} \)), listed in Table 3.1, 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 3.1 Kinetic model parameter definitions and values, PDGF receptor/PI3K module.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{D,L} )</td>
<td>PDGF single-site dissociation constant</td>
<td>1.5 nM</td>
</tr>
<tr>
<td>( k_{R0} )</td>
<td>Dimerization rate constant</td>
<td>0.3 min(^{-1})</td>
</tr>
<tr>
<td>( k_x )</td>
<td>Dimer uncoupling rate constant</td>
<td>0.07 min(^{-1})</td>
</tr>
<tr>
<td>( k_e )</td>
<td>Dimer endocytosis rate constant</td>
<td>0.2 min(^{-1})</td>
</tr>
<tr>
<td>( k_t )</td>
<td>Basal receptor turnover rate constant</td>
<td>0.005 min(^{-1})</td>
</tr>
<tr>
<td>( \alpha_{PI3K} )</td>
<td>Receptor/PI3K expression ratio</td>
<td>80</td>
</tr>
<tr>
<td>( \kappa_{PI3K} )</td>
<td>Dimensionless receptor-PI3K dissociation constant</td>
<td>0.3</td>
</tr>
<tr>
<td>( k_{3PI} )</td>
<td>3’ PI turnover rate constant</td>
<td>1.0 min(^{-1})</td>
</tr>
</tbody>
</table>
3.4.2 Kinetic model: 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 Supplement. 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 3.2.

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, \( e_{\text{GEF}}(t) \), is assumed to respond rapidly to changes in the density of receptor dimers, \( c_2(t) \), and 3’ PI lipids, \( m_{3\text{PI}}(t) \), according to the following approximate equilibrium-binding relation.

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

Other functions of \( c_2 \) and \( m_{3\text{PI}} \) on the right-hand side of this equation were evaluated (e.g., adding a dependence on the product of \( c_2 \) and \( m_{3\text{PI}} \)); 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,

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

(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. 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.

The modeling of Ras-GTP accumulation is treated as in past models (Haugh, 2002; Haugh & Lauffenburger, 1997). 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

$$\frac{dm_{Ras}}{dt} = k_{Ras}\left\{[(1 + \Gamma)e_{GEF} - (1 + \Gamma e_{GEF})m_{Ras}\right\}.$$  

$\Gamma = k_{GEF}/k_{Ras}$.

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 (Kaur et al., 2006), it is known that only a small fraction of Ras in our cells is converted to the GTP-bound form, consistent with $\Gamma << 1$ 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 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.

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. We also analyzed a more complex model containing an additional MEK kinase activity, $x_3$, which was both Ras- and PI3K-dependent. In that case, the algorithm generally chose parameters so as to marginalize the influence of $x_3$ downstream; hence, we removed this pathway from the model.

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 (Ferrell, 1996, 2002; Markevich, Hoek, & Kholodenko, 2004; Qiao, Nachbar, Kevrekidis, & Shvartsman, 2007; Wang, Hao, Dohlman, & Elston, 2006), 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 monophosphorylated 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.
\[
(x_1 \cdot y) = \frac{(x_1)_{\text{free}} (y)_{\text{free}}}{K_{M,x1}} = \frac{(x_1)_{\text{free}} y}{K_{M,x1}}; \quad (x_1 \cdot y_p) = \frac{(x_1)_{\text{free}} (y_p)_{\text{free}}}{K_{M,x1}} = \frac{(x_1)_{\text{free}} y_p}{K_{M,x1}};
\]
\[
(x_2 \cdot y) = \frac{(x_2)_{\text{free}} (y)_{\text{free}}}{K_{M,x2}} = \frac{(x_2)_{\text{free}} y}{K_{M,x2}}; \quad (x_2 \cdot y_p) = \frac{(x_1)_{\text{free}} (y_p)_{\text{free}}}{K_{M,x2}} = \frac{(x_1)_{\text{free}} y_p}{K_{M,x2}};
\]
\[
(y_p \cdot z) = \frac{(y_p)_{\text{free}} (z)_{\text{free}}}{K_{M,y1}} = \frac{(y_p)_{\text{free}} z}{K_{M,y1}}; \quad (y_p \cdot z_p) = \frac{(y_p)_{\text{free}} (z_p)_{\text{free}}}{K_{M,y2}} = \frac{(y_p)_{\text{free}} z_p}{K_{M,y2}};
\]
\[
(y_p)_{\text{free}} = \frac{y_p}{1 + z/\tilde{K}_{M,y1} + z_p/\tilde{K}_{M,y2}};
\]
\[
(y_p \cdot y_p) = \frac{(y_p)_{\text{free}} (y_p)_{\text{free}}}{K_{M,yph1}} = \frac{(y_p)_{\text{free}} y_p}{K_{M,yph1}};
\]
\[
(y_p \cdot y_p) = \frac{(y_p)_{\text{free}} (y_p)_{\text{free}}}{K_{M,yph2}} = \frac{(y_p)_{\text{free}} y_p}{K_{M,yph2}};
\]
\[
(y_p)_{\text{free}} = \frac{y_p (1 + z/\tilde{K}_{M,y1} + z_p/\tilde{K}_{M,y2})}{(1 + z/\tilde{K}_{M,y1} + z_p/\tilde{K}_{M,y2}) (1 + y_p/\tilde{K}_{M,yph1}) + y_p/\tilde{K}_{M,yph2}};
\]
\[
(z_p \cdot z) = \frac{(z_p)_{\text{free}} (z)_{\text{free}}}{K_{M,zph1}} = \frac{(z_p)_{\text{free}} z}{K_{M,zph1}}; \quad (z_p \cdot z_p) = \frac{(z_p)_{\text{free}} (z_p)_{\text{free}}}{K_{M,zph2}} = \frac{(z_p)_{\text{free}} z_p}{K_{M,zph2}};
\]
\[
(z_p)_{\text{free}} = \frac{z_p}{1 + z_p/\tilde{K}_{M,zph1} + z_p/\tilde{K}_{M,zph2}}.
\]

The dimensionless parameters \( \tilde{K}_{M,x1} \) and \( \tilde{K}_{M,x2} \) 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

\[
\frac{dx_1}{dt} = k_{d,x1} \left( m_{Ras} \frac{x_1}{1 + y/\tilde{K}_{M,x1} + y_p/\tilde{K}_{M,x2}} \right); \quad x_1(0) = 0; \quad \text{(Eq. S8)}
\]

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

For MEK, the conservation equations are

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

\[
\frac{dy_{pp}}{dt} = \sum_{i=1}^{2} \tilde{V}_{\text{max}, xi} x_i y_p / \tilde{K}_{M, xi}  - \frac{\tilde{V}_{\text{max}, yph2} y_{pp} / \tilde{K}_{M, yph2}}{(1 + z_p/\tilde{K}_{M, yph1} + z_{pp}/\tilde{K}_{M, yph2}) (1 + y_p/\tilde{K}_{M, yph1} + y_{pp}/\tilde{K}_{M, yph2})}; \quad y_{pp}(0) = 0; \quad \text{(Eq. S11)}
\]

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

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 \( \tilde{V}_{\text{max}, xi} \) and \( \tilde{V}_{\text{max}, x2} \) 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

\[
\begin{align*}
\frac{dz}{dt} & = -\frac{\tilde{V}_{\text{max},y} y_{\text{pp}} z}{1 + z/\tilde{K}_{M,y1} + z_p/\tilde{K}_{M,y2}} - \frac{\tilde{V}_{\text{max},z\text{ph}} e_{\text{ph}} z_p/\tilde{K}_{M,z\text{ph}1}}{1 + z_p/\tilde{K}_{M,z\text{ph}1} + z_{\text{pp}}/\tilde{K}_{M,z\text{ph}2}}; \\
n(0) & = 1; & \text{(Eq. S13)}
\end{align*}
\]

\[
\frac{dz_{\text{pp}}}{dt} = \frac{\tilde{V}_{\text{max},y} y_{\text{pp}} z_p}{1 + z/\tilde{K}_{M,y1} + z_p/\tilde{K}_{M,y2}} \left(1 - \frac{\tilde{V}_{\text{max},z\text{ph}} e_{\text{ph}} z_{\text{pp}}/\tilde{K}_{M,z\text{ph}2}}{1 + z_p/\tilde{K}_{M,z\text{ph}1} + z_{\text{pp}}/\tilde{K}_{M,z\text{ph}2}}\right); \\
n_{\text{pp}}(0) & = 0 \text{ (Eq. S14)}
\]

\[
z_p = 1 - z - z_{\text{pp}}, & \quad \text{(Eq. S15)}
\]

The function \(e_{\text{ph}}(t)\) 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.

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

**Negative Feedback Loops Eliciting GEF Desensitization and Up-regulation of MKP-1**

These negative feedback loops are embodied by the functions \(f_{\text{GEF}}(t)\) and \(e_{\text{ph}}(t)\), 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, \(f_{\text{GEF}}(t)\).

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

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 3.2, 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 ~ 15 minutes. Thus, we impose a variable $w$, possibly representing a transcription factor activity, which builds up slowly and switches on MKP-1 expression.

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

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

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

3.4.3 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 3.2 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. * 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).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Minimum</th>
<th>Lower Quartile</th>
<th>Median</th>
<th>Upper Quartile</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{GR}$</td>
<td>Affinity constant, GEFI receptor binding</td>
<td>102</td>
<td>394</td>
<td>495</td>
<td>640</td>
<td>1730</td>
</tr>
<tr>
<td>$K_{GP}$</td>
<td>Affinity constant, 3' PI-dependent GEF binding</td>
<td>0.00266</td>
<td>3.47</td>
<td>5.09</td>
<td>6.51</td>
<td>32.7</td>
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<tr>
<td>$k_{on}$</td>
<td>Characteristic rate constant, Ras-GTP loading</td>
<td>1/min *</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Gamma$</td>
<td>Maximal stimulated GEF/GAP activity ratio</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{d,1}$</td>
<td>MEK kinase deactivation rate constant (Ras-activated)</td>
<td>0.203/min</td>
<td>0.561/min</td>
<td>0.745/min</td>
<td>1.21/min</td>
<td>12.9/min</td>
</tr>
<tr>
<td>$k_{d,2}$</td>
<td>MEK kinase deactivation rate constant (PI3K-activated)</td>
<td>0.305/min</td>
<td>1.77/min</td>
<td>2.35/min</td>
<td>11.2/min</td>
<td>282/min</td>
</tr>
<tr>
<td>$K_{S,1}$</td>
<td>Saturation constant, PI3K-dependent MEK kinase activation</td>
<td>0.251</td>
<td>5.25</td>
<td>6.77</td>
<td>10.2</td>
<td>31.0</td>
</tr>
<tr>
<td>$\frac{V_{m,11}/K_{M,11}}{K_{M,11}}$</td>
<td>Cat. efficiency, MEK --&gt; pMEK (Ras-activated)</td>
<td>0.0579/min</td>
<td>0.516/min</td>
<td>1.18/min</td>
<td>1.62/min</td>
<td>71.3/min</td>
</tr>
<tr>
<td>$\frac{K_{M,11}}{K_{M,11}}$</td>
<td>Michaelis constant, MEK --&gt; pMEK (Ras-activated)</td>
<td>0.343</td>
<td>20.1</td>
<td>30.3</td>
<td>250</td>
<td>2570</td>
</tr>
<tr>
<td>$\frac{V_{m,21}/K_{M,21}}{K_{M,21}}$</td>
<td>Cat. efficiency, MEK --&gt; pMEK (PI3K-activated)</td>
<td>0.0302/min</td>
<td>0.236/min</td>
<td>0.405/min</td>
<td>0.907/min</td>
<td>353/min</td>
</tr>
<tr>
<td>$\frac{K_{M,21}}{K_{M,21}}$</td>
<td>Michaelis constant, MEK --&gt; pMEK (PI3K-activated)</td>
<td>0.0568</td>
<td>13.7</td>
<td>21.6</td>
<td>203</td>
<td>3710</td>
</tr>
<tr>
<td>$\frac{V_{m,pp,1}/K_{M,pp,1}}{K_{M,pp,1}}$</td>
<td>Catalytic efficiency, pMEK --&gt; MEK</td>
<td>5.6x10^{-4}/min</td>
<td>1.65/min</td>
<td>4.40/min</td>
<td>14.7/min</td>
<td>483/min</td>
</tr>
<tr>
<td>$\frac{K_{M,pp,1}}{K_{M,pp,1}}$</td>
<td>Michaelis constant, pMEK --&gt; MEK</td>
<td>0.573</td>
<td>23.0</td>
<td>44.0</td>
<td>978</td>
<td>445</td>
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<tr>
<td>$\frac{V_{m,pp,2}/K_{M,pp,2}}{K_{M,pp,2}}$</td>
<td>Cat. efficiency, pMEK --&gt; pppMEK (Ras-activated)</td>
<td>0.115/min</td>
<td>3.54/min</td>
<td>4.71/min</td>
<td>15.5/min</td>
<td>140/min</td>
</tr>
<tr>
<td>$\frac{K_{M,pp,2}}{K_{M,pp,2}}$</td>
<td>Michaelis constant, pMEK --&gt; pppMEK (Ras-activated)</td>
<td>2.81</td>
<td>18.6</td>
<td>45.5</td>
<td>114</td>
<td>728</td>
</tr>
<tr>
<td>$\frac{V_{m,pp,2}/K_{M,pp,2}}{K_{M,pp,2}}$</td>
<td>Cat. efficiency, pMEK --&gt; pppMEK (PI3K-activated)</td>
<td>0.0318/min</td>
<td>1.09/min</td>
<td>2.41/min</td>
<td>9.45/min</td>
<td>77.6/min</td>
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<tr>
<td>$\frac{K_{M,pp,2}}{K_{M,pp,2}}$</td>
<td>Michaelis constant, pMEK --&gt; pppMEK (PI3K-activated)</td>
<td>0.876</td>
<td>9.59</td>
<td>15.7</td>
<td>31.6</td>
<td>878</td>
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<tr>
<td>$\frac{V_{m,pp,1}/K_{M,pp,1}}{K_{M,pp,1}}$</td>
<td>Catalytic efficiency, pppMEK --&gt; MEK</td>
<td>0.233/min</td>
<td>4.20/min</td>
<td>6.77/min</td>
<td>9.07/min</td>
<td>52.2/min</td>
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<tr>
<td>$\frac{K_{M,pp,1}}{K_{M,pp,1}}$</td>
<td>Michaelis constant, pppMEK --&gt; MEK</td>
<td>1.05</td>
<td>7.99</td>
<td>12.7</td>
<td>42.5</td>
<td>234</td>
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<tr>
<td>$\frac{V_{m,1}/K_{M,1}}{K_{M,1}}$</td>
<td>Catalytic efficiency, Erk --&gt; pErk</td>
<td>0.842/min</td>
<td>0.57/min</td>
<td>11.8/min</td>
<td>52.8/min</td>
<td>&gt;10^4/min</td>
</tr>
<tr>
<td>$\frac{K_{M,1}}{K_{M,1}}$</td>
<td>Michaelis constant, Erk --&gt; pErk</td>
<td>0.0146</td>
<td>9.91</td>
<td>31.9</td>
<td>890</td>
<td></td>
</tr>
<tr>
<td>$\frac{V_{m,pp,1}/K_{M,pp,1}}{K_{M,pp,1}}$</td>
<td>Catalytic efficiency, pErk --&gt; Erk</td>
<td>1.1x10^{-4}/min</td>
<td>0.167/min</td>
<td>0.451/min</td>
<td>1.11/min</td>
<td>63.2/min</td>
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<tr>
<td>$\frac{K_{M,pp,1}}{K_{M,pp,1}}$</td>
<td>Michaelis constant, pErk --&gt; Erk</td>
<td>0.275</td>
<td>5.27</td>
<td>14.0</td>
<td>37.0</td>
<td>167</td>
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</table>
Table 3.2 continued

<table>
<thead>
<tr>
<th>$V_{max1}/K_{M,1}$</th>
<th>Catalytic efficiency, pErk $\rightarrow$ ppErk</th>
<th>0.669/min</th>
<th>8.16/min</th>
<th>31.9/min</th>
<th>66.9/min</th>
<th>7730/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{M,1}$</td>
<td>Michaelis constant, pErk $\rightarrow$ ppErk</td>
<td>0.0481</td>
<td>4.21</td>
<td>8.81</td>
<td>80.7</td>
<td>944</td>
</tr>
<tr>
<td>$V_{max2}/K_{M,2}$</td>
<td>Catalytic efficiency, ppErk $\rightarrow$ pErk</td>
<td>0.0203/min</td>
<td>0.122/min</td>
<td>0.228/min</td>
<td>0.493/min</td>
<td>13.7/min</td>
</tr>
<tr>
<td>$K_{M,2}$</td>
<td>Michaelis constant, ppErk $\rightarrow$ pErk</td>
<td>1.10</td>
<td>9.98</td>
<td>31.5</td>
<td>195</td>
<td>797</td>
</tr>
<tr>
<td>$k_{FB}$</td>
<td>Feedback rate constant, GEF desensitization</td>
<td>0.104/min</td>
<td>0.763/min</td>
<td>0.976/min</td>
<td>1.56/min</td>
<td>23.7/min</td>
</tr>
<tr>
<td>$Z_f$</td>
<td>Dimensionless threshold, GEF desensitization</td>
<td>0.00829</td>
<td>0.146</td>
<td>0.272</td>
<td>0.507</td>
<td>2.50</td>
</tr>
<tr>
<td>$n$</td>
<td>Hill coefficient, GEF desensitization</td>
<td>1.00</td>
<td>1.02</td>
<td>1.05</td>
<td>1.05</td>
<td>1.48</td>
</tr>
<tr>
<td>$K_F$</td>
<td>Gain coefficient, GEF desensitization</td>
<td>1.04</td>
<td>3.16</td>
<td>3.76</td>
<td>5.17</td>
<td>16.6</td>
</tr>
<tr>
<td>$k_u$</td>
<td>Delay rate constant, MKP up-regulation</td>
<td>0.00645/min</td>
<td>0.01187/min</td>
<td>0.03333/min</td>
<td>0.0914/min</td>
<td>0.478/min</td>
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<tr>
<td>$k_{FB,PH}$</td>
<td>Feedback rate constant, MKP up-regulation</td>
<td>0.0373/min</td>
<td>0.998/min</td>
<td>2.34/min</td>
<td>3.69/min</td>
<td>20.0/min</td>
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<tr>
<td>$W_{PH}$</td>
<td>Dimensionless threshold, MKP up-regulation</td>
<td>0.107</td>
<td>0.248</td>
<td>0.385</td>
<td>1.11</td>
<td>12.3</td>
</tr>
<tr>
<td>$p$</td>
<td>Hill coefficient, MKP up-regulation</td>
<td>1.10</td>
<td>1.76</td>
<td>1.98</td>
<td>2.56</td>
<td>24.7</td>
</tr>
<tr>
<td>$K_P$</td>
<td>Gain coefficient, MKP up-regulation</td>
<td>1.35</td>
<td>3.08</td>
<td>4.84</td>
<td>25.1</td>
<td>8055</td>
</tr>
</tbody>
</table>
3.4.4 Parameter estimation

*Metropolis 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 (Metropolis, Rosenbluth, Rosenbluth, Teller, & Teller, 1953). The algorithm was implemented in MATLAB (MathWorks, Natick, MA), adapted from code provided by Tim Elston (Department of Pharmacology, UNC-Chapel Hill) (Violin et al., 2008). 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 Supplementary Fig. S1 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.
Figure 3.6 Saturation of the Erk cascade using phorpol ester. Cells were pretreated for 15 minutes with DMSO only (black symbols) or 200nM 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 ± 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 Erk phosphorylation is close to saturation in PMA treated cells. In the cells not treated with PMA, the PDGF-stimulated response is 80-90% of the apparent saturation level. The solid curves represent the corresponding ensemble means for the kinetic model, and the dashed curves are mean ± s.d. (n = 10,000).

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

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.

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

where \( k_i \) is one of the model parameters, and \( \text{randn} \) 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.

**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.

3.4.5 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. 3.3. To predict the outcomes of certain perturbations, namely the inhibition of PI3K-dependent crosstalk to Ras-GEF or to MEK (Fig. 3.5), the corresponding changes in the parameter values ($K_{GP} = 0$ or $\tilde{V}_{\max,s21} = \tilde{V}_{\max,s22} = 0$, respectively) were made in each of the 10,000 parameter sets, and the mean and standard deviation of the model output were recomputed.

The analysis of MEK phosphorylation presented in Fig. 3.4A 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,
\[ C_{sij} = \frac{\tilde{V}_{\text{max,sij}}}{\tilde{V}_{\text{max,yphj}}} \cdot \frac{\tilde{K}_{M,sij}}{\tilde{K}_{M,yphj}}. \]

Fig. 3.4A shows scatter plots of maximum PI3K-dependent phosphorylation of site 1 \((C_{s11})\) versus maximum Ras-dependent phosphorylation of site 1 \((C_{s11})\) and of maximum PI3K-dependent phosphorylation of site 2 \((C_{s22})\) versus maximum Ras-dependent phosphorylation of site 2 \((C_{s12})\), 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

\[
\begin{align*}
    y_p &= C_{s11} y'_1; \\
    y_{pp} &= C_{s22} y'_1; \\
    y'_1 &= \frac{y_p}{1 + y_p} + y_{pp}; \\
    y_{pp} &= \frac{1}{1 + \frac{y_p}{y_{pp}}}; \\
    \Psi_2 &= \frac{C_{s11} C_{s22}}{1 + C_{s21}}, \\
    \Psi_1 &= \frac{C_{s11} C_{s12}}{1 + C_{s11}}.
\end{align*}
\]

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

\[ \text{MAC} = \frac{\Psi_2}{\Psi_1} = \left( \frac{C_{s21} C_{s22}}{1 + C_{s21}} \right) \left( \frac{C_{s11} C_{s12}}{1 + C_{s11}} \right). \]

The impact of the negative feedback affecting Ras-GEF recruitment is assessed by reducing the values of \(C_{s11}\) and \(C_{s12}\) by the same factor.

The analysis of Ras-GEF recruitment presented in Fig. 3.4B 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

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

For the parameters used here, the proportionality constant is equal to 123. Fig. 3.4B 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,

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


CHAPTER 4

SYSTEMATIC QUANTIFICATION OF NEGATIVE FEEDBACK MECHANISMS IN THE ERK SIGNALING NETWORK

(Adapted from Cirit M, Wang C-C, Haugh JM. Systematic Quantification of Negative Feedback Mechanisms in the Extracellular Signal-Regulated Kinase (ERK) Signaling Network. (Submitted Manuscript))

Cell responses are actuated by tightly controlled signal transduction pathways. Although the concept of an integrated signaling network replete with inter-pathway crosstalk and feedback regulation is broadly appreciated, kinetic data of the type needed to characterize such interactions in conjunction with mathematical models are lacking. In mammalian cells, the Ras/ERK pathway controls cell proliferation and other responses stimulated by growth factors, and several crosstalk and feedback mechanisms affecting its activation have been identified. In this work, we take a systematic approach to parse the magnitudes of multiple regulatory mechanisms that attenuate ERK activation through canonical (Ras-dependent) and non-canonical (PI3K-dependent) pathways. In addition to regulation of receptor and ligand levels, we consider three layers of ERK-dependent feedback: desensitization of Ras activation, negative regulation of MEK kinase (e.g. Raf) activities, and upregulation of dual-specificity ERK phosphatases. Our results establish the second of these as the dominant mode of ERK self-regulation in mouse fibroblasts. We further demonstrate that models of signaling networks, trained on a sufficient diversity of quantitative data, can be reasonably comprehensive, accurate, and predictive in the dynamical sense.
4.1 Introduction

Mammalian cells recognize and respond to chemical stimuli through ligation of specific receptors at the cell surface, which in turn activate highly conserved intracellular signal transduction pathways. These pathways elicit growth and proliferation, polarization and migration, differentiation, and other responses by actuating the cell’s gene-regulatory and cytoskeletal systems. Obviously, signal transduction must be tightly regulated, as spurious intracellular signaling is associated with autonomous cell proliferation, invasive cell migration, and other molecular signatures of cancer progression (Condeelis et al, 2005; Dhillon et al, 2007; Roberts & Der, 2007; Engelman, 2009).

The concept of a signaling pathway provides a useful framework for understanding the flow of information as an ordered series of activation processes, exemplified by the Ras → Raf → mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) → ERK pathway and other MAPK cascades, which control diverse responses in cells stimulated by various growth factors and cytokines (Cuevas et al, 2007; Meloche & Pouysségur, 2007; Whitmarsh, 2007). Our current understanding of signal transduction, however, encompasses the concept of signaling networks, in which the canonical pathways interact with and thus affect one another (crosstalk); the sequential pathway concept is further challenged by the regulation of signaling through negative feedback and, in some cases, reinforcement of signaling through positive feedback (Behar et al, 2007; McKay & Morrison, 2007; Brandman & Meyer, 2008). These complexities of signaling networks have proven difficult to characterize, and most of the data that has accumulated about such mechanisms are qualitative in nature and scattered across different experimental contexts. Indeed, it has not heretofore been demonstrated that one can parse the multiple crosstalk and feedback interactions that modulate signal flow in any given cell type, to the extent of accurately reconciling their contributions as a function of time and dose of stimulus. Although kinetic models of signal transduction processes have steadily appeared over the past decade, and recently published models of the epidermal growth factor (EGF) receptor system in particular have been more tightly integrated with biochemical data to establish quantitative features of signaling networks (Kiyatkin et al, 2006; Birtwistle et al,
2007; Chen et al, 2009), a more comprehensive data acquisition effort is needed to better constrain models at the network scale of complexity.

We previously conducted a systematic analysis of crosstalk in the platelet-derived growth factor (PDGF) receptor network (Wang, Cirit, & Haugh, 2009). The major signaling modes mediated by PDGF receptors are the phosphoinositide 3-kinase (PI3K) pathway and the aforementioned Ras/ERK pathway, which are most closely associated with chemotaxis and cell proliferation, respectively (Meloche & Pouysségur, 2007; Schneider & Haugh, 2006). Through measurements of PDGF-stimulated signaling in mouse fibroblasts, systematically covering a diverse array of stimulation and molecular perturbation conditions and building upon other quantitative studies (Park et al, 2003; Schneider & Haugh, 2004; Schneider & Haugh, 2005; Kaur et al, 2006), we showed that PDGF-stimulated ERK activation requires signaling through either of two pathways: the canonical, Ras-dependent pathway or PI3K-dependent crosstalk. PI3K-dependent signaling positively modulates the ERK pathway both upstream of Ras and independent of Ras, while the PI3K pathway is not significantly affected by endogenous Ras signaling. Through quantitative analysis of a coarse-grained kinetic model, we estimated that the magnitudes of the Ras- and PI3K-dependent contributions to MEK/ERK activation are comparable; the PI3K-dependent pathway was found to be more potent by only a moderate amount (1.6:1 ratio), once negative feedback desensitization of Ras-GTP loading was taken into account (Wang et al., 2009).

We have since refined our kinetic model and acquired additional quantitative data in order to quantify negative feedback regulation of ERK signaling through multiple feedback loops (Fig. 4.1). The three layers of ERK-dependent feedback included in the current model are well documented: 1) desensitization of Ras-GEF recruitment through hyper-phosphorylation of Sos (Langlois et al, 1995; Waters et al, 1995; Klarlund et al, 1996), 2) desensitization of MEK kinases, especially isoforms of Raf (Raf-1, B-Raf, and A-Raf) through phosphorylation on known regulatory sites (Wartmann et al, 1997; Dougherty et al, 2005; Rushworth et al, 2006; Ritt et al, 2010), and 3) transcriptional up-regulation of MAPK phosphatases (MKPs)/dual specificity phosphatases (DUSPs) that dephosphorylate ERK (Owens & Keyse, 2007).
Our analysis shows that the second of these, directly affecting MEK phosphorylation, is in fact the dominant layer of ERK self-regulation in our cells, accounting for > 90% of the signal attenuation. We additionally found significant depletion of growth factor from the extracellular medium, which affects signaling at sub-saturating growth factor concentrations. Support for the refined mathematical model, trained by direct alignment to the superset of old and new data (> 300 distinct experimental measurements), is demonstrated through its ability to predict with reasonable quantitative accuracy the enhancement of PDGF-stimulated MEK phosphorylation in cells with ERK1 and ERK2 expression knocked down. A more surprising model prediction, also confirmed experimentally, is a lack of effect of MKP3/DUSP6 knockdown on ERK phosphorylation.

4.2 Results

4.2.1 MEK phosphorylation kinetics reveal a potent, intermediate layer of negative feedback regulation

We begin our analysis by showing that MEK phosphorylation is regulated in a manner that cannot be explained by feedback loops impinging upstream of Ras or at the level of ERK phosphatases. Whereas our previous model was constrained by quantitative measurements of Ras-GTP loading and ERK phosphorylation, measurements of MEK phosphorylation kinetics in the same cell backgrounds provide critical data and mechanistic insights about the regulation of the pathway (Fig. 4.2). Referring to the diagrams in Fig. 4.1, it is clear why: the activation of both MEK and ERK reflect the integration of Ras- and PI3K-dependent inputs to the pathway, but unlike ERK, MEK is not directly affected by modulation of MKP/DUSP levels.

Samples were obtained from among the same NIH 3T3 cell lysates used previously to quantify ERK and Akt phosphorylation (Wang et al., 2009). Systematic quantification of Ras- and PI3K-dependent contributions to MEK activation was achieved through inhibition
of PI3K and Ras, by incubation with LY294002 compound and expression of dominant-negative (S17N) H-Ras, respectively. The results show that PDGF-stimulated MEK phosphorylation, like phosphorylation of ERK in our cells (Wang et al., 2009), is generally transient and sensitive to ablation of either Ras (emphasizing the PI3K-dependent pathway; Fig. 4.2A) or PI3K (emphasizing the Ras-dependent pathway; Fig. 4.2B) signaling.

Conceptually, the transience of MEK phosphorylation might seem to be consistent with the previously reported ERK phosphorylation kinetics; however, our previous model, using parameter values fit without the benefit of MEK data, predicts sustained MEK phosphorylation with only a small overshoot (Fig. 4.3). Even on a qualitative level, the previous scheme cannot explain how MEK phosphorylation is transient in Ras-inhibited cells stimulated with a high dose of PDGF; in the previous model, partial adaptation of ERK phosphorylation under those conditions had been solely attributed to upregulation of MKP activity (Wang et al., 2009), downstream of MEK. The new results identify desensitization
of MEK phosphorylation, downstream of Ras and PI3K and presumably through ERK-dependent feedback, as a critical regulatory mechanism in the ERK signaling network.

Figure 4.2 PDGF-stimulated MEK phosphorylation is strongly regulated by negative feedback and is sensitive to ablation of Ras or PI3K signaling. PDGF-stimulated MEK1/2 phosphorylation (p-MEK) kinetics in NIH 3T3 cells were assessed by quantitative immunoblotting and normalized by total ERK1 (t-Erk). The blots shown are representative of 3 independent experiments; samples were drawn from lysates used previously to probe phosphorylation of ERK and Akt (Wang et al., 2009). Values are normalized as previously described and are reported as mean ± s.e.m. in arbitrary units (n = 3). A. Comparison of cells expressing dominant-negative (S17N) H-Ras to the empty vector control. B. Comparison of PI3K inhibition (100 μM LY294002) to the 0.2% DMSO vehicle control.
4.2.2 Dual specificity phosphatases MKP3 and MKP1 are modulated with distinct kinetics in PDGF-stimulated cells, but their expression levels do not affect ERK dephosphorylation

We next present evidence that feedback at the level of modulating two DUSP isoforms, MKP1/DUSP1 and MKP3/DUSP6, does not significantly impact ERK phosphorylation kinetics. We showed previously that high doses of PDGF elicit 3- to 5-fold upregulation of MKP1 in our cells (Wang et al., 2009). In the context of the previous model, this negative feedback loop was important for explaining partial adaptation of the ERK phosphorylation response, especially as activated by the PI3K-dependent pathway as explained above; although the potential importance of this feedback has also been emphasized in a number of mathematical models of ERK signaling (Bhalla, Ram, & Iyengar, 2002), it must be acknowledged that MKP1 expression might not be a quantitative indicator of ERK dephosphorylation.

Indeed, other DUSP isoforms, especially MKP3, are thought to be more important in that regard (Owens & Keyse, 2007), and we found that MKP3 and MKP1 expression levels
are modulated quite differently (Fig. 4.4). Whereas MKP1 expression increases sharply (after a time lag) and plateaus in response to high PDGF doses, MKP3 expression rapidly decays and then recovers, as quantified in Fig. 4.4A&B. These kinetics, in stark contrast with those of MKP1 expression under the same conditions, are consistent with ERK-dependent modulation of both synthesis and degradation of MKP3 (Jurek, Amagasaki, Gembarska, Heldin, & Lennartsson, 2009).

Surprisingly, despite the complex regulation of these two DUSPs, we found that ERK phosphorylation is not sensitive to changes in either of their expression levels. Reduction of MKP3 expression by RNA interference (~80% knockdown of the basal MKP3 level) had no significant effect on the kinetics or dose responsiveness of PDGF-stimulated ERK phosphorylation, as compared to cells treated with a scrambled oligonucleotide control (Fig. 4.4C). These results stand in contrast with published data using porcine aortic endothelial cells with heterologous expression of PDGF receptors (Jurek et al., 2009).

Similarly, the expectation that ERK phosphorylation might be negatively correlated with changes in MKP1 expression does not hold in cells treated with MG-132, a proteasome inhibitor that amplifies MKP1 upregulation, or SP600125, an inhibitor of c-Jun N-terminal kinase activity that has the opposite effect (Fig. 4.5). Although these results do not rule out the possibility that ERK phosphorylation is shaped by feedback regulation of other DUSP isoforms, they do further suggest that the primary determinant of ERK adaptation in this system is the transience of MEK activation. Indeed, using our refined mathematical model, we will show that this sufficiently and quantitatively explains the kinetics of the ERK network under all conditions tested.
Figure 4.4 MKP3 expression is regulated in response to PDGF stimulation but does not affect ERK phosphorylation. MKP3/DUSP6 expression in PDGF-stimulated NIH 3T3 cells was monitored by quantitative immunoblotting. The blots shown are representative of 3 independent experiments; samples were drawn from lysates used previously to probe phosphorylation of ERK and Akt (Wang et al., 2009). Values are normalized as previously described and are reported as mean ± s.e.m. (n = 3) in arbitrary units. A. Comparison of cells expressing dominant-negative (S17N) H-Ras to the empty vector control. B. Comparison of PI3K inhibition (100 μM LY294002) to the 0.2% DMSO vehicle control. C. siRNA knockdown of MKP3 does not affect MEK or ERK phosphorylation stimulated by PDGF in our cells. For each immunoblot, control and MKP3 siRNA bands were cropped from the same blot, which is representative of two independent experiments.
Figure 4.5 ERK phosphorylation is not sensitive to molecular perturbations that modulate MKP1 expression. It has been postulated that upregulation of MKP1/DUSP1, a putative ERK phosphatase, serves as a negative feedback that regulates ERK signaling. However, amplification (A) or inhibit (B) of the MKP1 upregulation response, by incubation with 50 μM MG-132 (to block proteosomal degradation of ubiquitinated proteins) or SP600125 (to inhibit c-Jun N-terminal kinase), respectively, do not have the expected effects on ERK phosphorylation stimulated by PDGF. The blots shown are representative of three independent experiments. The plots show quantification for 1 nM PDGF stimulation; values are normalized as previously described and are reported as mean ± s.e.m. in arbitrary units (n = 3).

4.2.3 Sub-saturated PDGF receptor-mediated signaling is affected by PDGF depletion from the extracellular medium

To round out the data needed to accurately quantify the mechanisms that contribute to adaptation of signaling, we sought to ensure that dynamics affecting PDGF receptor activation were sufficiently characterized. Since our previous framework already accounted for endocytosis as a mechanism for PDGF receptor downregulation (Kaur, Park, Lewis, & Haugh, 2006; Park, Schneider, & Haugh, 2003), we speculated that depletion of PDGF from the external medium might significantly temper prolonged responses to low PDGF doses (Fig. 4.6).

To evaluate the significance of PDGF depletion, we measured time courses of PI3K-dependent Akt phosphorylation for low (30 pM) and high (1 nM) doses of PDGF-BB, and prior to the final time point of 75 minutes, the medium was aspirated (at 60 minutes) and
replaced with the same initial PDGF concentration. The results confirm that the Akt phosphorylation level recovers substantially once the low PDGF dose is replenished (Fig. 4.6A). As expected, this is not true of the high PDGF dose, because the PI3K/Akt pathway is maximally activated (saturated) as long as the external PDGF concentration exceeds roughly 0.5 nM (Park et al., 2003).

The kinetics of PDGF-BB depletion were directly quantified by enzyme-linked immunosorbent assay (ELISA) (Fig. 4.6B). The results show that, for initial doses ranging from 0.03-1 nM, the extent of depletion over two hours is 40-50%. To demonstrate the consistency of these data with those of PDGF-stimulated Akt phosphorylation reported
previously (Wang et al., 2009), we achieved a satisfactory global fit of both data types to a minimal sub-model of the pathway kinetics (Fig. 4.6B&C). The two types of experiments were performed using roughly the same cell densities and the same volume of medium, and the fit to the data was highly constrained, as only 5 of the sub-model parameters were adjusted.

4.2.4 The refined mathematical model of the PDGF receptor network reconciles all existing measurements and allows a better fit to previously acquired data

The current model is illustrated conceptually in Fig. 4.1B. It has a total of 22 state variables and 57 adjustable rate parameters; of the parameters, 14 are fixed at constant values, based on previous work and the constrained fit to the ligand depletion and Akt phosphorylation data described in the previous section. The remaining 43 parameters were estimated by direct and global alignment with the rest of the data, which included the kinetics of Ras-GTP loading, ERK phosphorylation, and MKP1 levels reported previously (Wang et al., 2009) and the newly acquired MEK phosphorylation and MKP3 expression data. Thus, whereas our previous analysis required fewer fit parameters, the present analysis further constrains the model fit with a disproportionately higher number of readouts and nearly double the number of data points for comparison (Table 4.1). As in our previous work, the approach is not designed to identify a single set of “best” parameter values but rather a large ensemble \( n = 10,000 \) of parameter sets that perform almost equally well in fitting the data. Analysis of those parameter sets (Table 4.3) indicates which parameters are constrained well by the data and which are less so.

The results show the quality of the fit and the full array of quantitative data used for alignment (Fig. 4.7 and Fig. 4.8). Unlike the previous version, the current model accurately captures the newly quantified MEK phosphorylation kinetics, and it outperforms the previous version in fitting ERK phosphorylation kinetics (Table 4.1). Notably, the current model properly “spreads” the peak ERK phosphorylation levels for the four PDGF doses, and it captures the ERK phosphorylation kinetics of the S17N Ras, 30 pM PDGF time course that was missed by the previous model (Wang et al., 2009).
Table 4.1 Comparison of the current and previous PDGF receptor signaling network models. The fit refers to the Monte Carlo parameter fitting of phosphorylated ERK, Ras-GTP, MKP1 expression, and (in the case of this work) MEK phosphorylation and MKP3 expression readouts. Data points fit refers to the number of distinct experimental conditions; i.e., not considering experimental replicates. It also does not include the PDGF depletion and Akt phosphorylation, which were fit separately. The sum of squared deviations (SSD) for each readout is reported as the mean ± s.d. for the 10,000 parameter sets in each ensemble.

<table>
<thead>
<tr>
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<th>Wang et al., 2009</th>
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<td>Variables</td>
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<td>SSD&lt;sub&gt;ERK&lt;/sub&gt;, n = 104</td>
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<td>4.30 ± 0.25</td>
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<tr>
<td>SSD&lt;sub&gt;Ras&lt;/sub&gt;, n = 21</td>
<td>1.29 ± 0.12</td>
<td>1.13 ± 0.17</td>
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<tr>
<td>SSD&lt;sub&gt;MKP1&lt;/sub&gt;, n = 44</td>
<td>1.37 ± 0.13</td>
<td>1.62 ± 0.18</td>
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<tr>
<td>SSD&lt;sub&gt;MEK&lt;/sub&gt;, n = 84</td>
<td>2.10 ± 0.16</td>
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<tr>
<td>SSD&lt;sub&gt;MKP3&lt;/sub&gt;, n = 84</td>
<td>2.40 ± 0.10</td>
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Among the insights that we can glean directly from the parameter statistics, relevant to feedback regulation of the network, is the tendency of the fit to marginalize the contributions of both MKP1 and MKP3 (parameters $\beta_1$ and $\beta_3$), in relation to a third, time-invariant ERK phosphatase activity. This is a bona fide model prediction, since the experimental data showing the same were not used to constrain the model. Quantitatively, the model accurately predicts the results of the MKP3 knockdown experiment shown in Fig. 4.4C (Fig 4.9).

The lack of sensitivity of ERK phosphorylation to MKP1 and MKP3 dynamics, which are characterized by 11 of the 43 global fit parameters in the current model, together with considerations of fast reactions and enzymatic reactions operating far from saturation, indicate that the model can be simplified without significantly affecting predictions about ERK signaling. Although model simplification is beyond the scope of the present work, we conservatively estimate that less than half of the parameters are needed to achieve essentially the same fit of the Ras, MEK, and ERK data.
Figure 4.7 The refined network model reconciles all experimental data acquired to date. A simulated annealing algorithm was used to align the kinetic model to the data as indicated, thus collecting an ensemble of parameter sets that fit our data set well (Supplementary Text S1). Solid curves are ensemble means, and the dashed curves are mean ± s.d. \( (n = 10,000) \). The mean data values used to constrain the model (symbols) are also shown. PDGF concentrations are: red, 1 nM; green, 300 pM; blue, 100 pM; gray, 50 pm; black, 30 pM.
Figure 4.8 Additional ERK phosphorylation data used to constrain model-data alignment. The plot on the right is for cells pretreated with phorbol ester for 15 min. and then stimulated with PDGF for the times indicated; the corresponding model calculations assume saturated activation of MEK. The plot on the left is the control (DMSO pretreatment). PDGF doses are: red, 1 nM; blue, 100 pM; black, 30 pM. The data used for alignment (symbols) are the same as reported previously [Wang et al., Mol. Syst. Biol., 5: art. 246 (2009)].

Figure 4.9 The current model correctly predicts that MEK and ERK activation are not significantly affected by siRNA knockdown of MKP3. The quantified results of the MKP3 knockdown experiment shown in Fig. 4.4C, aligned with the model output for the scrambled siRNA control, are overlaid with a priori kinetic model predictions of MEK and ERK phosphorylation kinetics, conservatively assuming 90% knockdown of MKP3 in the model. Solid curves represent ensemble means, and dashed curves are mean ± s.d. (n = 10,000). PDGF concentrations are: red, 1 nM; black, 30 pM.
4.2.5 The refined network model successfully predicts the collective strength of ERK-dependent negative feedbacks

To further assess the predictive ability of the current model and thus establish with greater confidence the magnitudes of ERK-dependent negative feedback loops, we abrogated ERK activity by RNA interference and tested the attendant effect on MEK phosphorylation (Fig. 4.10). This experimental test of the model probes the desensitization of MEK phosphorylation almost directly, since both the previous and current models quantitatively account for ERK-dependent desensitization of Ras-GTP loading, based on experiments using a MEK inhibitor (Wang et al., 2009). Such inhibitors function by binding MEK1/2 and preventing their activation, and thus are likely to obscure effects on MEK phosphorylation, motivating the siRNA approach used here.

Using two siRNAs, one targeting each of ERK1 and ERK2, we achieved 80-90% knockdown of both isoforms and confirmed that ERK1/2 depletion yields a dramatic increase in MEK phosphorylation, consistent with relief of ERK-dependent negative feedback. Accordingly, PDGF-stimulated phosphorylation of Raf-1 on known negative regulatory sites (Dougherty et al., 2005) was abrogated in the ERK1/2-depleted cells (Fig. 4.10A). Corresponding a priori predictions of MEK phosphorylation kinetics were generated using our quantitative model, assuming reductions of ERK expression by 80% and 90%, and nice agreement with the experimental data was found (Fig. 4.10B). We note that the calculated enhancement of MEK phosphorylation is sensitive to the extent of ERK knockdown, as the residual ERK retains a certain potency of feedback regulation in the model, and this contributes to the uncertainty of the prediction. Nevertheless, the model could be re-fit, incorporating the ERK siRNA data (assuming 90% knockdown) in the alignment without compromising the overall quality of fit for the rest of the data (Fig. 4.10C and Fig 4.11). We conclude that the current model is refined to the extent that it accurately accounts for the dynamics of ERK-dependent negative feedback at multiple levels of the pathway.
Figure 4.10 siRNA knockdown of ERK1 and ERK2 enhances MEK phosphorylation, as quantitatively predicted by the current model. A. NIH 3T3 cells were transfected with siRNAs directed against ERK1 and ERK2; their pan-ERK expression and PDGF-stimulated MEK1/2 phosphorylation were measured by quantitative immunoblotting in parallel with a scrambled siRNA control. Raf-1 phosphorylation on negative regulatory sites controlled by ERK (Ser^{289}/Ser^{296}/Ser^{301}) and total Akt (as a loading control) were also assessed. The results are representative of two independent experiments. B. The quantified results from A are overlaid with a priori kinetic model predictions of MEK phosphorylation kinetics, assuming 90% or 80% knockdown of ERK in the model; solid curves represent ensemble means, and dashed curves are mean ± s.d. (n = 10,000). PDGF concentrations are: red, 1 nM; black, 30 pM. C. Same as B, except that the model was refit with the additional data included in the alignment, assuming 90% ERK knockdown by siRNA treatment.
Figure 4.11 Model-data alignment incorporating siRNA knockdowns of ERK and MKP3 (Ensemble 2). The model was re-fit after incorporating the data shown in Fig. 4.10A. The results of the fit are shown here (compare to Fig. 4.7) and in Fig. 4.10C.
4.2.6 Desensitization of MEK phosphorylation downstream of Ras and PI3K is the dominant mode of ERK pathway self-regulation

Further analysis of the computational model reveals the relative magnitudes of the negative feedbacks impinging upstream and downstream of Ras (Fig. 4.12), which reconcile the constraints imposed by the experimentally determined Ras-GTP loading and MEK phosphorylation kinetics. We simulated a scenario in which Ras-GEF desensitization is selectively turned off in each of the 10,000 parameter sets, with all other feedbacks intact. This enhances the rates of MEK and ERK phosphorylation through the Ras-dependent pathway; however, the predicted increases in MEK/ERK phosphorylation levels are rather modest (Fig. 4.12A). By comparison, selectively turning off MEK kinase desensitization in the model results in nearly stoichiometric activation of both MEK and ERK (Fig. 4.12B). Taken together, these results indicate that negative feedback at the point of MEK phosphorylation is the dominant mode of ERK pathway self-regulation; that is, relief of this feedback is the primary determinant of the dramatic gain in MEK phosphorylation that is observed in ERK-depleted cells and which was predicted by our model.
Figure 4.12 Feedback desensitization of MEK kinase activities is the dominant mode of ERK self-regulation. Model predictions (ensemble means) of MEK and ERK phosphorylation are shown. PDGF concentrations are: red, 1 nM; black, 30 pM. Solid curves are hypothetical scenarios in which each of the following ERK-dependent feedback loops is selectively turned off: A, Ras-GEF desensitization; B, MEK kinase desensitization. The dashed curves are from the fit to the corresponding data, with all ERK-dependent feedback loops intact.
4.3. Discussion

Adaptation of intracellular signaling has long been recognized as a cornerstone of cell regulation. The concept is well known in the field of chemotaxis, for example, where exact or nearly complete adaptation of the sensory output is thought to enable cells to respond to chemoattractant gradients spanning a broad range of concentrations (Segel et al, 1986; Alon et al, 1999; Parent & Devreotes, 1999; Yi et al, 2000; Levchenko & Iglesias, 2002). Coupled with ultrasensitivity or positive feedback, it is well understood that negative feedback can produce spiking/oscillatory responses, as in calcium signaling and regulation of the cell cycle (Novák & Tyson, 2008; Tsai et al., 2008). At least in principle, the ERK pathway is also capable of oscillations (Kholodenko, 2000; Qiao, Nachbar, Kevrekidis, & Shvartsman, 2007); however, in the context of growth factor receptor-mediated ERK signaling, the more plausible role of negative feedback regulation is that of partial adaptation, modulating what is ultimately a biologically meaningful (quasi-) steady state (Knauer, Wiley, & Cunningham, 1984; Marshall, 1995). Indeed, more than 30 years ago, it was shown that PDGF stimulation renders cells competent for (but not necessarily committed to) DNA synthesis, and that this process requires exposure to PDGF for varying lengths of time, on the scale of hours, depending on the dose of growth factor (Pledger, Stiles, Antoniades, & Scher, 1977).

The topology of a negative regulatory mechanism imposes certain limitations on its kinetic properties. The direct inhibition of upstream signaling components by a MAPK does not readily foster strong adaptation of its output response, because MAPK activation and onset of the feedback are essentially the same process (Behar, Hao, Dohlman, & Elston, 2007). Thus, in our experiments as well as in our mathematical model, ERK phosphorylation exhibits a much less dramatic peak and decline compared with MEK phosphorylation. Some degree of adaptation is attributed to the desensitization of Ras-GTP loading, the magnitude of which was established from our previous experiments (Wang et al., 2009); however, our new results revealed that the predominant level of feedback regulation lies downstream of Ras, for example through ERK-dependent phosphorylation of Raf isoforms (Wartmann et al, 1997; Dougherty et al, 2005; Rushworth et al, 2006; Ritt et al, 2010). This seems to be especially
important in the context of the PDGF receptor network, because signaling through Ras is not the only pathway to ERK, nor is it necessarily the dominant one.

The issue of quantifying crosstalk (PI3K-dependent) and canonical (Ras-dependent) pathways to ERK, which was the primary focus of our previous model, raises a generally important question about model refinement. As additional data come to light, and regulatory mechanisms are added to models, will we find the conclusions drawn from previous analyses to be invalid or obsolete? We have looked into this issue for our models, and the conclusion drawn is that it depends on where one looks. In our previous analysis, the ratio of PI3K-dependent/Ras-dependent signaling inputs – the MEK activation comparator (MAC) – was estimated to be roughly 1.6 once the negative feedback affecting Ras-GEF activity had been taken into account (Wang et al., 2009); for the present model, we calculated an analogous, time-varying quantity, the “dynamic MAC” (Fig 4.13). Under maximal stimulation conditions and with all feedbacks intact, the ensemble-averaged dynamic MAC varies within the range between 1 and 2, suggesting that the PI3K-dependent ERK activation pathway is moderately more potent than the Ras-dependent pathway. This is in accord with the previously estimated, “static” MAC value cited above (Wang et al., 2009); in terms of the overall inputs to MEK, incorporating feedback regulation of upstream components, the models are consistent. With Ras-GEF desensitization turned off, however, the value of the dynamic MAC hovers close to 1 for maximal stimulation, whereas the corresponding static MAC estimate was much lower (median value ≈ 0.2); the discrepancy here reflects the new finding that the feedback regulation of MEK has two distinct layers, of which Ras-GEF desensitization plays a subordinate role.
The dynamic MEK activation comparator (MAC) ratio compares, as a function of time for the current model, the relative potency of PI3K-dependent crosstalk to that of the canonical Ras-dependent pathway in the activation of MEK. First, the ratio of active/inactive MEK, $y_{pp}/\left(1 - y_{pp}\right)$ (i.e., accounting for any partial saturation of MEK activation), was calculated as a function of time for the Ras- and PI3K-inhibited cases. Next, the dynamic MAC is expressed as the ratio of the PI3K- to Ras-dependent values of that metric. A value of 1 indicates that the two pathways contribute equally in the refined kinetic model, once desensitization of the corresponding MEK kinase activity has been taken into account; a value greater than 1 indicates that the PI3K-dependent pathway is the greater contributor and vice-versa. PDGF concentrations are: red, 1 nM; green, 300 pM; blue, 100 pM; black, 30 pM. Solid curves are ensemble means, and the dashed curves are mean ± s.d. ($n = 10,000$). A, all feedbacks intact; B, desensitization of Ras-GTP loading turned off.

Figure 4.13 Assessing the contributions of PI3K- and Ras-dependent inputs to MEK/ERK activation in the current model. The dynamic MEK activation comparator (MAC) ratio compares, as a function of time for the current model, the relative potency of PI3K-dependent crosstalk to that of the canonical Ras-dependent pathway in the activation of MEK. First, the ratio of active/inactive MEK, $y_{pp}/\left(1 - y_{pp}\right)$ (i.e., accounting for any partial saturation of MEK activation), was calculated as a function of time for the Ras- and PI3K-inhibited cases. Next, the dynamic MAC is expressed as the ratio of the PI3K- to Ras-dependent values of that metric. A value of 1 indicates that the two pathways contribute equally in the refined kinetic model, once desensitization of the corresponding MEK kinase activity has been taken into account; a value greater than 1 indicates that the PI3K-dependent pathway is the greater contributor and vice-versa. PDGF concentrations are: red, 1 nM; green, 300 pM; blue, 100 pM; black, 30 pM. Solid curves are ensemble means, and the dashed curves are mean ± s.d. ($n = 10,000$). A, all feedbacks intact; B, desensitization of Ras-GTP loading turned off.

Faced with the many mechanisms by which signaling pathways might be attenuated, it is easy to neglect the most basic of regulatory processes, namely those that affect availability of ligand and receptor molecules. In previous work we carefully quantified PDGF receptor phosphorylation kinetics for stimulation times up to 20 minutes, characterizing the rates of PDGF binding, receptor dimerization, and receptor endocytosis/downregulation (Park et al., 2003). Here we found that, for somewhat longer times (~ 1 hour or more), depletion of PDGF from the extracellular medium also needs to be taken into account, as it clearly affects receptor-mediated signaling at low doses of PDGF (for the number of cells/volume of medium used). At higher PDGF concentrations, Ras and PI3K signaling are both saturated; thus, it requires more time for the effects of ligand depletion to be felt, an example of dose-to duration encoding (Behar, Hao, Dohlman, & Elston, 2008). To the extent that cells effectively integrate growth factor-stimulated signals over a fairly long period of time, ligand depletion ought to affect cell proliferation and other
functional responses. Indeed, under certain conditions it has been observed that the total amount of PDGF added, rather than its initial concentration, dictates the overall extent of cell proliferation in culture (Vogel, Ross, & Raines, 1980). Limitations on cell growth imposed by depletion of EGF-family ligands have also been documented (Reddy, Wells, & Lauffenburger, 1994, 1996).

Mathematical modeling of biological processes has a rich history and has emerged in recent years as a valuable tool for characterizing intracellular signal transduction. In general, the utility of modeling spans a spectrum bracketed by the definition of what is possible and the interpretation of what is (Iyengar, 2009; Mogilner, Wollman, & Marshall, 2006). The present analysis is squarely at the latter extreme, as it is driven by an expanding set of quantitative data, which affords some measure of confidence in model predictions of network dynamics. Looking forward, further data-driven refinements of the model will need to be directed towards a more detailed understanding of the molecular mechanisms that govern network dynamics, especially feedback regulation of Raf isoforms and other MEK kinases.

4.4. Materials and Methods

4.4.1 Reagents

All tissue culture reagents were from Invitrogen (Carlsbad, CA). Human recombinant PDGF-BB was from Peprotech (Rocky Hill, NJ). Antibodies against total ERK1/2 and MKP3 and phospho-specific antibodies against Akt pSer^{473}, ERK pThr^{202}/pTyr^{204}, and MEK pSer^{217}/pSer^{221} were from Cell Signaling Technology (Beverly, MA); antibodies against total Akt1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Human PDGF-BB ELISA kit, with PDGF β-receptor/Fc chimera as the capture reagent, was from R&D Systems (Minneapolis, MN). Pharmacological inhibitors were from Calbiochem (San Diego, CA) or, in the case of MG-132, Sigma-Aldrich (St. Louis, MO); where applicable, cells were pre-incubated with the inhibitor for 30-60 minutes prior to PDGF stimulation. The siGENOME siRNA reagents and siGENOME SMARTpool siRNAs against mouse MKP3 (GeneID: 67603), ERK1 (GeneID: 26417), and ERK2 (GeneID: 26413) and
siGENOME Non-Targeting siRNA Pool #2 were purchased from Dharmaco (Lafayette, CO). Unless otherwise noted, all other reagents were from Sigma-Aldrich.

4.4.2 Cell culture and siRNA transfection

NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, VA) were cultured at 37°C, 5% CO₂ in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and the antibiotics penicillin and streptomycin. Where applicable, NIH 3T3 cells were serially infected with retrovirus bearing empty vector or S17N H-Ras and selected using puromycin prior to each experiment, as described previously (Kaur et al., 2006; Wang et al., 2009). NIH 3T3 cells were transfected with siRNAs according to the manufacturer’s protocol and incubated for three days prior to the experiment.

4.4.3 Lysate preparation and quantitative immunoblotting

Cells were serum-starved for 4 hours prior to stimulation. Detergent lysates were prepared for quantitative immunoblotting, and immunoblots were performed using enhanced chemiluminescence, as described previously (Park et al., 2003). Blots comparing lysates prepared on the same day, representing either different inhibitor treatments or different cell variants and respective control conditions, were performed in parallel and exposed at the same time. The BioRad Fluor S-Max system, which gives a linear response with respect to light output, was used, and band intensity was quantified using local background subtraction. Total cellular protein concentrations were determined by Micro BCA assay (Pierce, Rockford, IL). Immunoblot data were first normalized by an appropriate loading control and then further normalized to evaluate the consistency of relative trends across independent experiments, according to the procedures described in detail previously (Wang et al., 2009).

4.4.4 Kinetic model and computational analysis

The refined mathematical model of the PDGF receptor network is illustrated conceptually in Fig. 4.1B. PDGF receptor binding, dimerization, and endocytosis, and the
production of 3’ PI lipids by receptor-recruited PI3K, are modeled essentially as described previously (Park et al., 2003; Schneider & Haugh, 2005). This portion of the model was supplemented with an equation accounting for depletion of PDGF-BB from the extracellular medium. Our previous coarse-grained model of Ras- and PI3K-dependent MEK kinase/MEK/ERK activation and regulation (Wang et al., 2009) was supplemented with ERK-dependent negative feedbacks affecting the MEK kinase activities and ERK-dependent regulation of MKP3 expression. It also allows that MKP1, MKP3, and/or a constitutive level of dual-specificity phosphatase activity contribute(s) to the dephosphorylation of ERK.

The parameter estimation approach used is related to the algorithm described previously (Wang et al., 2009), with certain modifications; both are Monte Carlo-based and generate a large \( n = 10,000 \) ensemble of “good” parameter sets rather than one “best” fit, but here a modified simulated annealing protocol was designed. 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.

Another difference, prompted by information obtained from the supplier of the phospho-specific antibodies used, was to fit the MEK and ERK phosphorylation data to the sums of the dual- and mono-phosphorylated MEK and ERK species calculated by the model \( (y_{pp} + y_p \) and \( z_{pp} + z_p \), respectively); however, the spectra of antibody specificities in the polyclonal mixtures are of minor concern here, as the model invariably chose parameters such that the calculated mono-phosphorylated forms, \( y_p \) and \( z_p \), were negligible under all conditions tested. In other words, it would not make a difference if one were to fit the phospho-MEK and -ERK data to the weighted sums \( y_{pp} + ay_p \) and \( z_{pp} + bz_p \), where \( a \) and \( b \) are positive constants. To be consistent, this assumption was similarly invoked to obtain the results for the previous model (re-fit using the new simulated annealing protocol) shown in Supplementary Fig. 4.3 and Table 4.1.

The model developed in this chapter is an extension of previous work (Wang et al., 2009). Equations that are exactly the same as used previously are presented in full but explained only briefly; they are marked with an asterisk (*). For extensive explanation of the
associated model formulation and underlying assumptions, see the Supplemental Material of the publication cited above.

4.5 Appendix: Kinetic Modeling Details

4.5.1 PDGF/PDGF Receptor Dynamics and PI3K Activation

The average, dimensionless densities of unbound PDGF receptors \( r \), 1:1 receptor-ligand complexes \( c_1 \), and dimerized receptor complexes \( c_2 \) are conserved by the following equations.

\[
\frac{dr}{dt} = \left(1 + \frac{[L]}{K_{D.L}}\right)^{-1} \left[k_r(1 - r - c_1) + 2(k_{-x}c_2 - k_xR_0c_1^2)\right]; \quad (\text{Eq. S1*})
\]

\[
c_1 = [L]r/K_{D.L}; \quad r(0) + c_1(0) = 1; \quad (\text{Eq. S2*})
\]

\[
\frac{dc_2}{dt} = k_xR_0c_1^2 - (k_{-x} + k_e)c_2; \quad c_2(0) = 0. \quad (\text{Eq. S3*})
\]

The inputs to this part of the model are the PDGF ligand concentration, \([L]\), and five constant rate parameters: \(K_{D,L}, k_r, R_0, k_{-x}, k_e\), and \(k_t\). The output of this model is the fraction of receptors in dimers as a function of time, given by \(2c_2(t)\).

Whereas it was assumed in our previous models that \([L]\) is a constant value, equal to the known concentration added \(([L]_0)\), our new results show that this assumption is not valid for time courses longer than \(\sim 1\) hour. Although it is plausible that receptor-mediated trafficking plays a significant role in PDGF-BB depletion, other effects (e.g., sequestration by extracellular matrix) might contribute or even dominate. Because of this uncertainty, we assume a simplified equation of the Michael-Menten type to describe ligand depletion:

\[
\frac{d[L]}{dt} = -\frac{k_{L,\text{max}}[L]}{1 + [L]/K_{M,L}}; \quad [L](0) = [L]_0. \quad (\text{Eq. S4})
\]

The two new constant rate parameters here are \(k_{L,\text{max}}\) and \(K_{M,L}\). More complicated models, with PDGF depletion explicitly caused by receptor-mediated endocytosis and intracellular
degradation, were also explored; however, they required specification of more adjustable parameters and did not yield a better fit to the data.

As assumed previously, the dimensionless fraction of the PI3K enzyme recruited ($e_{PI3K}$) and the dimensionless 3’ PI messenger density ($m_{3PI}$) respond to the value of $c_2(t)$ according to

$$e_{PI3K}(t) = \frac{1 + \kappa_{PI3K} + 2\alpha_{PI3K}c_2(t) - \sqrt{(1 + \kappa_{PI3K} + 2\alpha_{PI3K}c_2(t))^2 - 8\alpha_{PI3K}c_2(t)}}{2}; \quad \text{(Eq. S5*)}$$

$$\frac{dm_{3PI}}{dt} = k_{3PI}(e_{PI3K} - m_{3PI}), \quad m_{3PI}(0) = 0. \quad \text{(Eq. S6*)}$$

The three constant rate parameters for this part of the model are $\alpha_{PI3K}$, $\kappa_{PI3K}$, and $k_{3PI}$.

For the purpose of specifying missing parameter values, we sought to use our previously published Akt phosphorylation data set (Wang et al., 2009), which we did not previously attempt to fit to a model. As explained in the main text, we do this because PI3K/Akt activation is subject to ligand depletion effects at low PDGF concentrations, and therefore the degree to which the phosphorylated Akt signal decays with time can be used to further constrain the parameter estimation. An additional equation is needed to relate the response of the dimensionless Akt phosphorylation level ($a_p$) to $m_{3PI}(t)$, and the following is the simplest form that allows for saturation of Akt binding to 3’ PI lipids.

$$\frac{da_p}{dt} = k_{d,a}[\frac{(1 + K_a)m_{3PI}}{1 + K_a m_{3PI}} - a_p]. \quad \text{(Eq. S7)}$$

There are two additional rate parameters: $k_{d,a}$ and $K_a$.

Of the 12 constant parameters mentioned above, the values of 7 were the same as used previously (see the table below). The remaining 5 are $k_t$, $k_{L,max}$, $K_{M,L}$, $k_{d,a}$, and $K_a$; although the parameter $k_t$ was included in the previous model, it was determined that its value was not strongly constrained by the previous data, and so we left it open here for reevaluation (as shown below, its fit value is arbitrarily low). The values of the 5 parameters were assigned based on a global, least-squares fit to the PDGF-BB depletion and Akt phosphorylation data sets as shown in Fig. 4.6B&C. With a satisfactory fit to the ligand
depletion and Akt phosphorylation data, we fixed the 10 parameter values that determine $c_2(t)$ and $m_{3PI}(t)$ (all of the parameters listed below except $k_{d,a}$ and $K_a$), the inputs for the rest of the model.

### Table 4.2 Kinetic model parameter definitions and values, PDGF receptor/PI3K module.

The highlighted values are newly fit parameters; the rest are as used in (Wang et al., 2009).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{D,L}$</td>
<td>PDGF single-site dissociation constant</td>
<td>1.5 nM</td>
</tr>
<tr>
<td>$k_x R_0$</td>
<td>Dimerization rate constant</td>
<td>0.3 min$^{-1}$</td>
</tr>
<tr>
<td>$k_e$</td>
<td>Dimer uncoupling rate constant</td>
<td>0.07 min$^{-1}$</td>
</tr>
<tr>
<td>$k_t$</td>
<td>Basal receptor turnover rate constant</td>
<td>$\approx$ 0 min$^{-1}$</td>
</tr>
<tr>
<td>$k_{L,max}$</td>
<td>PDGF depletion rate constant</td>
<td>0.011 min$^{-1}$</td>
</tr>
<tr>
<td>$K_{M,L}$</td>
<td>PDGF depletion saturation constant</td>
<td>1.66 nM</td>
</tr>
<tr>
<td>$\alpha_{PI3K}$</td>
<td>Receptor/PI3K expression ratio</td>
<td>80</td>
</tr>
<tr>
<td>$\kappa_{PI3K}$</td>
<td>Dimensionless receptor-PI3K dissociation constant</td>
<td>0.3</td>
</tr>
<tr>
<td>$k_{3PI}$</td>
<td>3’ PI turnover rate constant</td>
<td>1.0 min$^{-1}$</td>
</tr>
<tr>
<td>$k_{d,a}$</td>
<td>Akt phosphorylation rate constant</td>
<td>$1.02$ min$^{-1}$</td>
</tr>
<tr>
<td>$K_a$</td>
<td>Saturation constant, Akt phosphorylation</td>
<td>21.3</td>
</tr>
</tbody>
</table>

### 4.5.2 Ras/ERK Pathway

The following equations for the dimensionless Ras-GEF recruitment ($e_{GEF}$) and Ras-GTP density ($m_{Ras}$) are exactly as formulated previously.

$$e_{GEF}(t) = \frac{K_{GR} c_2(t) + K_{GP} m_{3PI}(t)}{1 + K_{GR} c_2(t) + K_{GP} m_{3PI}(t)} f_{GEF}(t); \quad \text{(Eq. S8*)}$$

$$\frac{dm_{Ras}}{dt} = k_{Ras} \left[ (1 + \Gamma) e_{GEF} - (1 + \Gamma e_{GEF}) m_{Ras} \right] m_{Ras}(0) = 0. \quad \text{(Eq. S9*)}$$

As explained previously, $f_{GEF}(t)$ is the fraction of Ras-GEF that is freely available, i.e., not desensitized by ERK (see below). The 4 constant parameters here are $K_{GR}$, $K_{GP}$, $k_{Ras}$, and $\Gamma$;
as in (Wang et al., 2009), \( k_{Ras} \) and \( \Gamma \) are assigned fixed values of 1 min\(^{-1}\) and 0.1, respectively.

The Ras- and PI3K-dependent contributions to MEK kinase activity, \( x_1 \) and \( x_2 \), respectively, are governed as follows.

\[
\frac{dx_1}{dt} = \frac{(1 + K_{x_1} m_{Ras})}{1 + K_{x_1} m_{Ras}} \frac{x_1}{1 + y/K_{M_{s11}} + y_p/K_{M_{s12}}}; \quad x_1(0) = 0; \quad \text{(Eq. S10)}
\]

\[
\frac{dx_2}{dt} = \frac{(1 + K_{x_2} m_{3PI})}{1 + K_{x_2} m_{3PI}} \frac{x_2}{1 + y/K_{M_{s21}} + y_p/K_{M_{s22}}}; \quad x_2(0) = 0; \quad \text{(Eq. S11*)}
\]

As explained previously, \( y \) and \( y_p \) are the unphosphorylated and mono-phosphorylated fractions of total MEK, respectively, which appear in Eqs. S10 and S11 to allow for sequestration of active \( x_1 \) and \( x_2 \) by their substrates (this effect was found to be minimal however). Relative to the previous model, there is one modification here: the allowance for saturation of \( x_1 \) activation with respect to Ras-GTP, characterized by the new parameter \( K_{x_1} \) (in effect, \( K_{x_1} = 0 \) in the old model).

For MEK (dual phosphorylated MEK fraction defined as \( y_{pp} \)), the conservation equations are

\[
\frac{dy}{dt} = -\sum_{i=1}^{2} \frac{V_{max,sri} x_i f_{si} y / K_{M_{sir}}}{1 + y / K_{M_{sir}} + y_p / K_{M_{sir2}}} + \frac{V_{max,ph1} y_p / K_{M_{ph1}}}{1 + y_p / K_{M_{ph1}} + y_{pp} / K_{M_{ph2}}}; \quad y(0) = 1 \quad \text{(Eq. S12)}
\]

\[
\frac{dy_{pp}}{dt} = \sum_{i=1}^{2} \frac{V_{max,sri} x_i f_{si} y_{pp} / K_{M_{sir2}}}{1 + y / K_{M_{sir}} + y_p / K_{M_{sir2}}} - \frac{V_{max,ph2} y_{pp} / K_{M_{ph2}}}{(1 + z_p / K_{M_{y1}} + z / K_{M_{y2}}) (1 + y_p / K_{M_{ph1}}) + y_{pp} / K_{M_{ph2}}}; \quad y_{pp}(0) = 0; \quad \text{(Eq. S13)}
\]

\[
y_p = 1 - y - y_{pp}; \quad \text{(Eq. S14*)}
\]

Relative to the previous model, the only differences here are the additions of the variables \( f_{si}(t) \), which account for the fractions of \( x_1 \) and \( x_2 \) that are not desensitized by ERK (see below).
For ERK (non-, mono-, and dual-phosphorylated fractions defined as $z$, $z_p$, and $z_{pp}$, respectively), the conservation equations are exactly the same as in the previous model:

$$
\frac{dz}{dt} = -\frac{V_{\text{max},y_1}y_{pp}z/K_{M,y_1}}{1 + z/K_{M,y_1} + z_p/K_{M,y_2}} + \frac{V_{\text{max},y_2}e_{ph}z_p/K_{M,y_2}}{1 + z_p/K_{M,y_2} + z_{pp}/K_{M,y_2}}; \quad z(0) = 1; \quad \text{(Eq. S15*)}
$$

$$
\frac{dz_{pp}}{dt} = \frac{V_{\text{max},y_2}y_{pp}z_p/K_{M,y_2}}{1 + z_p/K_{M,y_2} + z_{pp}/K_{M,y_2}} - \frac{V_{\text{max},y_2}e_{ph}z_{pp}/K_{M,y_2}}{1 + z_p/K_{M,y_2} + z_{pp}/K_{M,y_2}}; \quad z_{pp}(0) = 0; \quad \text{(Eq. S16*)}
$$

$$
z_p = 1 - z - z_{pp}. \quad \text{(Eq. S17*)}
$$

The function $e_{ph}(t)$ is the dimensionless expression of MAPK phosphatase/dual specificity phosphatase (MKP/DUSP) activity and considers the possible influences of MKP1 and MKP3 isoforms (see below). There are a total of 24 constant parameters invoked in the MEK kinase, MEK, and ERK equations (Eqs. S10-S17): $k_{d,1}$, $K_1$, $k_{d,2}$, $K_2$, and 10 pairs of $V_{\text{max}}$ and $K_M$ values. Of these, only $K_1$ is new.

### 4.5.3 Negative Feedback Regulation of the Ras/ERK Pathway

The model accounts for ERK-dependent desensitization of Ras-GEF as follows (as explained above, $f_{\text{GEF}}$ is the freely available GEF fraction, and therefore $1 - f_{\text{GEF}}$ is the desensitized fraction).

$$
\frac{df_{\text{GEF}}}{dt} = -k_{d,\text{GEF}} \left[ K_{f_{\text{GEF}}} z_{pp} f_{\text{GEF}} - (1 - f_{\text{GEF}}) \right]; \quad f_{\text{GEF}}(0) = 1; \quad \text{(Eq. S18)}
$$

Changes to the mathematical form (and parameter notation) here in relation to the previous model should be noted; the dependence of the desensitization rate on active ERK ($z_{pp}$), which was previously expressed as a Hill function with Hill coefficient $n$, is replaced by a simple proportionality. This simplification was found not to affect the quality of fit, and further it brings the equation in line with an enzymatic mechanism (ERK-catalyzed phosphorylation) far from saturation.

The fractions of freely available $x_1$ and $x_2$, $f_{x_1}$, are modeled in an analogous fashion:

$$
\frac{df_{x_1}}{dt} = -k_{d,x_1} \left[ K_{f_{x_1}} z_{pp} f_{x_1} - (1 - f_{x_1}) \right]; \quad f_{x_1}(0) = 1; \quad \text{(Eq. S19)}
$$
Finally, as in the previous model, the present model accounts for upregulation of MKP activity \( (e_{ph} \text{ in Eqs. S15 and S16})\). In the previous model, it was assumed that the \( V_{\text{max}}'s \) of ERK dephosphorylation are proportional to the expression level of MKP1, which was constrained by experimental data. With new data showing that the expression kinetics of the relevant MKP3 isoform qualitatively differs from those of MKP1, and considering the possibility that still other phosphatase activities might be involved, we relax the previous model assumption by allowing the \( V_{\text{max}}'s \) to be a weighted sum of a) the MKP1 expression level, b) the MKP3 expression level, and c) a constant offset representing constitutive activity. This is implemented in the model as follows. We introduce the variable \( e_{MKP1} \) to represent the dimensionless MKP1 expression level, with the same mathematical form and constrained by the same data as before:

\[
\frac{dw}{dt} = k_{d,w} \left( z_{pp} - w \right); \quad w(0) = 0; \quad \text{(Eq. S21*)}
\]

\[
\frac{de_{MKP1}}{dt} = k_{d, MKP1} \left( 1 + \frac{K_{\text{synth}}^{MKP1}}{W_{MKP1}^p + W^p} - e_{MKP1} \right); \quad e_{MKP1}(0) = 1. \quad \text{(Eq. S22)}
\]

For the dimensionless MKP3 expression level, \( e_{MKP3} \),

\[
\frac{de_{MKP3}}{dt} = k_{d, MKP3} \left( 1 + \frac{K_{\text{synth}}^{MKP3}}{W_{MKP3}^p + W^p} - \left( 1 + K_{\text{deg}}^{MKP3} z_{pp} \right) e_{MKP3} \right); \quad e_{MKP3}(0) = 1. \quad \text{(Eq. S23)}
\]

The form of Eq. S23 as compared with Eq. S22 considers that ERK feedback affects both the synthesis and degradation of MKP3. The function \( e_{ph}(t) \) in Eqs. S15 and S16 for phosphorylated Erk is then related to the variables \( e_{MKP1}(t) \) and \( e_{MKP3}(t) \) as follows.

\[
e_{ph}(t) = \beta_1 e_{MKP1}(t) + \beta_3 e_{MKP3}(t) + 1 - (\beta_1 + \beta_3),
\]

where \( \beta_1 + \beta_3 \leq 1 \). The previous assumption is recovered by setting \( \beta_1 = 1, \beta_3 = 0 \), and the present model also encompasses the possibilities that MKP3 dominates \( (\beta_1 = 0, \beta_3 = 1) \) or ERK dephosphorylation is constitutive \( (\beta_1 = \beta_3 = 0) \).
The negative feedback processes in the current model invoke 17 constant parameters: \( k_{d,G}, K_{G}, k_{d,G1}, K_{G1}, k_{d,G2}, K_{G2}, k_{d,W}, K_{W}, K_{MKP1}^{\text{synth}}, W_{MKP1}, p, k_{d,MKP3}, K_{MKP3}^{\text{synth}}, W_{MKP3}, K_{MKP3}^{\text{deg}}, \beta_{1}, \) and \( \beta_{3}. \) This is 8 more than in the previous model, which did not account for desensitization of the MEK kinase activities nor the regulation and influence of MKP3.

4.5.4 Summary of Model Species and Parameters

The current model is comprised of 23 distinct state variables and has 57 constant parameters. Of the parameters, 14 are assigned fixed values as prescribed above, and 43 are subject to a global fit to identify an ensemble of suitable parameter sets.

4.5.5 Implementing/Predicting Network Perturbations in the Model

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 \( f_{GEP} = 1. \) 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); this imposes a conservative constraint on ERK phosphorylation, stipulating that it should be saturated under those conditions. The perturbations listed above are consistent with our previous paper.

Knockdown of ERK expression levels by RNA interference is modeled as follows. Defining \( \delta_{z} \) as the fractional knockdown of ERK1/2 (e.g., \( \delta_{z} = 0.9 \) corresponds to 90% reduction of intracellular ERK1/2), the prediction is implemented by multiplying or dividing the values of the following parameters by the factor, \( (1-\delta_{z}) \), according to how they are scaled by the intracellular concentration of total ERK:

- Multiplied by \( (1-\delta_{z}) \): \( K_{G}, K_{G1}, K_{G2}, K_{MKP3}^{\text{deg}} \).
- Divided by \( (1-\delta_{z}) \): \( V_{\text{max},y1}, V_{\text{max},y2}, K_{M,y1}, V_{\text{max},zph1}, K_{M,zph1}, V_{\text{max},zph2}, K_{M,zph2}, W_{MKP1}, W_{MKP3} \).

Note that the ratios of \( V_{\text{max}}/K_{M} \) for the reactions in question are not affected.
Knockdown of MKP3 is implemented in an analogous fashion; defining $\delta_{\text{MKP3}}$ as the fractional knockdown of MKP3, we adjust the ERK phosphatase activity as follows.

$$e_{ph}(t) = \beta_1 e_{\text{MKP1}}(t) + (1 - \delta_{\text{MKP3}})\beta_3 e_{\text{MKP3}}(t) + 1 - (\beta_1 + \beta_3).$$

4.5.6 Details of Parameter Estimation Methods

We implemented the following Monte Carlo algorithm:

1. Given an array of parameters $k_i$ for iteration $i$, the model output is computed.

2. Using a branch-and-bound subroutine, we estimate a conversion factor (model output $\rightarrow$ arbitrary experimental units) for each readout $j$ (ppMEK, ppERK, etc.) that minimizes the sum of squared deviations, $SSD_{ij}$. It is noted that the data for the different readouts were renormalized so that the means of the values for the 1 nM PDGF, control (DMSO and empty vector) time courses are all equal to 1; thus, the arbitrary units of the different readouts are set on a common scale.

3. The cumulative sum of squared deviations, $cSSD_i$, is calculated:

$$cSSD_i = \sum_j w_j SSD_{ij}.$$ 

Since the data types were already normalized in a consistent way, equal weighting was used ($w_j = 1$).

4. Each parameter $k_i$ is updated according to $k_{i+1} = k_i (1 + \alpha \text{.randn})$, where randn is a random number drawn from a standard normal distribution. For this study, $\alpha = 0.05$. The step is redone if $k_{i+1}$ is chosen to be less than $10^{-4}$ or greater than $10^4$.

5. For the new set of parameters $k_{i+1}$, repeat steps 1 and 2 and calculate $cSSD_{i+1}$.

6. If $cSSD_{i+1} < cSSD_i$ (improved fit), the new set of parameters is accepted (increment $i$); otherwise, it is accepted with probability

$$p_{i+1} = \exp\left[-\frac{(cSSD_{i+1} - cSSD_i)}{T_i}\right].$$

If the new parameter set is rejected, proceed with the previous parameters $k_i$. 

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7. Go to step 4.

The value $T_i$ is called the “temperature” for iteration $i$, which determines how forgiving the algorithm is when the fit fails to improve. In standard simulated annealing, $T_i$ always decreases with iteration number, according to a defined schedule; thus, a high initial temperature allows the algorithm to easily escape local minima early on and, with steady cooling, approach the global minimum later on. We modify this approach by tying $T_i$ to the current error metric,

$$T_i = \beta \cdot cSSD_i$$

Thus, once the value of $cSSD$ nears its minimum value, the algorithm operates at approximately constant temperature. After some experimentation with our system, a value of $\beta = 0.01$ was used. The algorithm was run for a sufficiently long time so that >50,000 parameter sets were selected in total, and the best 10,000 of these (those with the lowest cumulative SSD values) were taken as the parameter set ensemble used to generate modeling results and predictions.
Table 4.3 Kinetic model parameters, Ras/ERK pathway module. Highlighted values are deemed arbitrarily high (yellow) or arbitrarily low (cyan) and thus do not significantly affect the model output. The Hill coefficient $p$ was constrained to be $\geq 1$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Minimum</th>
<th>Lower Quartile</th>
<th>Median</th>
<th>Upper Quartile</th>
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<td>$K_{GR}$</td>
<td>Affinity constant, GEF/receptor binding</td>
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<td>Maximally stimulated GEF/GAP activity ratio</td>
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<td>$k_{d,1}$</td>
<td>MEK kinase deactivation rate constant (Ras-activated)</td>
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<td>2.40e3 min$^{-1}$</td>
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<td>80.5</td>
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4.6 References


CHAPTER 5

SYSTEMATIC COMPARISON OF RECEPTOR TYROSINE KINASE SIGNALING NETWORKS IN FIBROBLASTS REVEALS QUANTITATIVE DIFFERENCES IN PI3K-AND RAS-DEPENDENT ERK ACTIVATION KINETICS

(Adapted from Cirit M, Haugh JM. Systematic Comparison of Receptor Tyrosine Kinase Signaling Networks in Fibroblasts Reveals Quantitative Differences in PI3K-and Ras-dependent ERK Activation Kinetics. (Written manuscript))

Signal transduction networks in mammalian cells, comprised of a limited set of interacting biochemical pathways, are accessed by various growth factor and cytokine receptors to elicit distinct cell responses. This raises the question as to how specificity of the stimulus-response relationship is encoded at the molecular level. It has been proposed that specificity arises not simply from the activation of unique signaling pathways but also from quantitative differences in the activation and regulation of shared, receptor-proximal signaling proteins; however, experimental measurements of the kind needed to address this hypothesis are currently lacking. Here we present a systematic, comparative analysis of the signaling networks accessed by fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) receptors in mouse fibroblasts, in which the extracellular signal-regulated kinase (ERK) cascade is activated by Ras- and phosphoinositide 3-kinase (PI3K)-dependent pathways. Through the formulation and analysis of data-driven mathematical models, we show that while the FGF stimulation of PI3K signaling is relatively weak, this deficiency is compensated for by a more potent, Ras-dependent activation of ERK. It is envisioned that similar approaches will prove valuable in the elucidation of quantitative differences among other, closely related receptor signaling networks.
5.1 Introduction

Mammalian cells respond to a diverse variety of growth factors, cytokines, and hormones, which are generally recognized through ligation of specific cell surface receptors. Whereas a cell’s receptor repertoire determines the subset of chemical signals to which it can respond, those receptors typically plug into a common set of relatively few, conserved signaling pathways; a question of longstanding interest, then, is how different stimuli or combinations of stimuli might elicit distinct responses (Bray, 1995). Several conceptual and theoretical models of how such specificity is achieved have been offered. For one, specificity might be encoded by the interactions of individual receptor molecules with other cellular proteins. In the case of receptor tyrosine kinases, which include the receptors for various extracellular growth factors and insulin, ligand binding results in self-phosphorylation of tyrosine residues in the receptor cytoplasmic domain (Lemmon & Schlessinger, 2010) and interactions with multiple Src homology 2 domain- and phosphotyrosine binding domain-containing enzymes and protein adaptors (Pawson, 2004). Thus, the phosphorylation stoichiometries of the various receptor sites and their binding affinities for cytoplasmic proteins quantitatively influence the relative activation levels of different signaling pathways, which additionally offers an explanation for how the same stimulus can elicit different responses depending on its concentration. Specificity might also be encoded by the kinetics of activation. In one popular conceptual model, it was proposed that the signaling outcome depends on whether an upstream pathway is activated with sustained versus transient kinetics (Marshall, 1995). Mathematical models have supplemented this concept with the notion that the initial rate of activation, rapid versus slow, might be selective for triggering different downstream pathways (Behar, Dohlman, & Elston, 2007; Komarova, Zou, Nie, & Bardwell, 2005).

The concepts outlined above highlight the need for more quantitative measurements of input-output relationships in signaling networks, accounting for both pathway magnitude and kinetic information. Whereas early investigations sought to establish the relative potencies of growth factors in the activation (Jackson, Stephens, & Hawkins, 1992; Osterop
et al., 1993) and desensitization (Klarlund, Cherniak, & Czech, 1995) of certain signaling pathways, very few studies have been designed so as to vary the stimulation dose and time and manipulate the molecular signaling network in a systematic fashion. This has been rectified to varying extents in recent years through data-driven mathematical modeling, which has been successfully applied to the analysis of cells stimulated with multiple growth factors and cytokines (Borisov et al., 2009; Chen et al., 2009; Janes et al., 2005; Nakakuki et al., 2010; Schoeberl et al., 2009); however, a more concerted data acquisition effort is needed to bring this approach to the level of predicting network dynamics for a variety of stimuli in common cellular backgrounds, which will be important if we are to understand and predict naturally occurring and interventional modes of cell regulation (Iyengar, 2009; Kreeger & Lauffenburger, 2010).

In this work, we build upon systematic analyses of the platelet-derived growth factor (PDGF) receptor signaling network in mouse fibroblasts, resulting in activation of the classical Raf → MEK → extracellular signal-regulated kinase (ERK) cascade, a master regulator of cell fate (Meloche & Pouysségur, 2007; Roberts & Der, 2007). Our previous studies focused on the dynamic contributions of the canonical, Ras-dependent pathway and non-canonical, phosphoinositide 3-kinase (PI3K)-dependent crosstalk (Wang, Cirit, & Haugh, 2009) as well as regulation of the network by multiple, ERK-dependent negative feedback loops (Cirit, Wang, & Haugh, 2010). Here, we offer a quantitative data set of fibroblast growth factor (FGF) receptor-mediated signaling readouts in the same cells and analyses directed towards reconciling the FGF and PDGF receptor networks with a unified kinetic model. One major difference is that FGF is a relatively weak stimulus for the PI3K pathway in these cells, and thus ERK activation is primarily Ras-dependent in this context. We further show that the potencies of Ras-dependent signaling to MEK/ERK mediated by FGF and PDGF receptors cannot be explained by differences in the amounts of Ras-GTP generated on a whole-cell basis; Ras activated in response to FGF is more potent. It is speculated that FGF and PDGF receptor signaling components are differentially localized and thus activate different pools of membrane-associated Ras.
5.2 Results

5.2.1 The FGF receptor signaling network in mouse fibroblasts features relatively weak stimulation of the PI3K/Akt pathway and predominantly Ras-dependent activation of the ERK cascade

In quantitative studies carried out in NIH 3T3 mouse fibroblasts, we previously showed that PDGF receptor-mediated signaling to ERK is channeled through two distinct pathways: the canonical, Ras-dependent pathway and an equally if not more important pathway involving PI3K-dependent crosstalk. In the same cellular background, we find that FGF also stimulates comparable activation of the ERK pathway, assessed quantitatively at the level of MEK1/2 and ERK1/2 phosphorylation (Fig. 5.1A); however, FGF only weakly stimulates PI3K-dependent phosphorylation of Akt (Fig. 5.1A&B). Despite this disparity in PI3K signaling, maximal FGF and PDGF stimulation yield quantitatively similar levels of MEK and ERK phosphorylation after ~30 minutes, although the time courses of FGF-stimulated MEK and ERK phosphorylation exhibit less dramatic adaptation kinetics (Fig. 5.1C). Thus, for FGF in relation to PDGF, it would seem that PI3K-dependent signaling is contributing far less to the activation of MEK and, conversely, that Ras-dependent signaling is contributing more.
Figure 5.1 Quantitative comparison of FGF and PDGF receptor-mediated signaling in mouse fibroblasts under maximal stimulation conditions. A. Phosphorylation time courses of Akt1/2/3 (p-Akt), MEK1/2 (p-MEK), and ERK1/2 (p-Erk) in FGF-2- and PDGF-BB-stimulated NIH 3T3 fibroblasts were measured by quantitative immunoblotting alongside total Akt (t-Akt) and total ERK (t-Erk) as loading controls. Stimulation times were 5, 15, 30, 60, and 120 minutes, and the blots shown are representative of 6 independent experiments. B. Quantitative comparison of FGF- and PDGF-stimulated Akt phosphorylation kinetics. Values are normalized as previously described and are reported as mean ± s.e.m. in arbitrary units (n = 6). C. Quantitative comparison of FGF- and PDGF-stimulated MEK and ERK phosphorylation kinetics. Values are normalized as previously described and are reported as mean ± s.e.m. in arbitrary units (n = 6).

In support of this hypothesis, we found that inhibition of PI3K using LY294002 does not markedly affect MEK and ERK phosphorylation stimulated by high or low doses of FGF (Fig. 5.2A&B), whereas the same inhibitor treatment reduces PDGF-stimulated MEK/ERK phosphorylation substantially (Fig. 5.2C and refs. (Cirit et al., 2010; Wang et al., 2009)). Accordingly, blocking receptor-mediated activation of endogenous Ras through stable expression of dominant-negative S17N H-Ras has a more dramatic effect on FGF-stimulated MEK/ERK phosphorylation; in response to either ligand, simultaneous blockade of both Ras- and PI3K-dependent signaling ablates MEK and ERK phosphorylation almost completely (compare Fig. 5.2B&C).
Figure 5.2 FGF-stimulated MEK/ERK phosphorylation is predominantly Ras-dependent. A. Phosphorylation time courses of Akt1/2/3 (p-Akt), MEK1/2 (p-MEK), and ERK1/2 (p-Erk) in FGF-2-stimulated NIH 3T3 fibroblasts were measured by quantitative immunoblotting alongside total Akt (t-Akt) and total ERK (t-Erk) as loading controls. Cells in which PI3K was inhibited (100 μM LY294002) are compared with cells incubated with 0.2% DMSO vehicle control, and the stimulation times were 5, 15, 30, 60, and 120 minutes. The blots shown are representative of 6 independent experiments. B. NIH 3T3 fibroblasts were infected with retrovirus produced from empty vector or vector with dominant-negative (S17N) H-Ras, pretreated with either DMSO control or LY294002, then stimulated with FGF-2 (dose and time indicated). The panel of immunoblotting readouts is the same as in A, and the blots shown are representative of three independent experiments. C. Same as B except that the cells were stimulated with PDGF-BB (dose and time indicated).
5.2.2 **Computational analysis of Ras- and PI3K-dependent activation of the ERK cascade in the FGF receptor signaling network**

We previously formulated a coarse-grained kinetic model of the PDGF receptor signaling network, accounting for Ras- and PI3K-dependent signaling to ERK and multiple negative feedback mechanisms (Cirit et al., 2010), a related model with 44 adjustable parameters was formulated for the FGF receptor network. The global fit of the model to the available kinetic data, which identifies a large ensemble of suitable parameter sets, shows a satisfactory agreement (Fig. 5.3A-C and Fig. 5.7) and allows for further analysis and predictions of a quantitative nature. For example, we computed the dynamic MEK activation comparator (dMAC), which quantitatively compares the contributions of the PI3K- and Ras-dependent inputs to MEK as a function of time; across the spectrum of PDGF doses assessed previously (0.03-1 nM), the estimated dMAC values level out in the range of 1.5-2 (Cirit et al., 2010). In other words, in the PDGF receptor signaling network, a dMAC value greater than 1 indicates that PI3K-dependent crosstalk is somewhat more potent than the Ras-dependent pathway in activating MEK. By comparison, although a careful quantification of the MEK/ERK phosphorylation data reveals some effect of PI3K inhibition (Fig. 5.3B&C), the estimated dMAC for the FGF receptor network plateaus at ≈ 0.1 (Fig. 5.3D), quantifying the extent to which PI3K-dependent crosstalk plays a subordinate role in this cell/receptor context.
5.2.3 Computational analysis of the FGF receptor signaling network accurately predicts the influence of ERK-dependent negative feedback loops

One of the key regulatory features of the ERK signaling network is negative feedback adaptation, which impinges both upstream and downstream of Ras. In the quantitative fit of the model, feedback regulation upstream of Ras is determined by comparing the Ras-GTP loading data obtained in the presence versus absence of a MEK inhibitor (Fig. 5.3A); with the magnitude of the upstream feedback constrained, the feedback downstream of Ras is left to account for the residual desensitization of the pathway resulting in transient MEK phosphorylation kinetics (Fig. 5.3B). To test the validity of this model, we evaluated its ability to predict a priori the effect of simultaneous siRNA knockdown of ERK1 and ERK2
(≈ 80% reduction in total ERK1/2), which relieves the aforementioned negative feedback loops; accordingly, MEK phosphorylation is markedly enhanced relative to the control (Fig. 5.4A). In the corresponding model prediction, although there is an expected degree of uncertainty across the ensemble of parameter sets, the ensemble mean is almost perfectly aligned with the data (Fig. 5.4B).

To assess the relative contributions of the two layers of negative feedback, we selectively turned off one or the other type of feedback in the FGF receptor network model. Turning off the upstream feedback enhances MEK phosphorylation in the model only modestly in comparison with silencing the downstream feedback, which results in nearly stoichiometric phosphorylation of MEK (Fig. 5.4C). These results strongly suggest that negative feedback impinging downstream of Ras and upstream of MEK plays a dominant role in regulating the pathway, consistent with our previous analysis of PDGF receptor-mediated signaling (Cirit et al., 2010).
Figure 5.4 siRNA knockdown of ERK1 and ERK2 enhances FGF-stimulated MEK phosphorylation, as quantitatively predicted by the current model. A. NIH 3T3 cells were transfected with siRNAs directed against ERK1 and ERK2; their pan-ERK expression and FGF-stimulated MEK1/2 phosphorylation were measured by quantitative immunoblotting in parallel with a scrambled siRNA control. Total Akt (as a loading control) was also assessed. The results are representative of two independent experiments. B. The quantified results from A are overlaid with a priori kinetic model predictions of MEK phosphorylation kinetics, assuming 80% knockdown of ERK in the model; solid curves represent ensemble means, and dashed curves are mean ± s.d. (n = 10,000). FGF concentrations are: red, 1 nM; black, 0.01 nM. C. Feedback desensitization of MEK kinase activities is the dominant mode of ERK self-regulation. Model predictions (ensemble means) of MEK phosphorylation are shown (red, 1 nM FGF; black, 0.01 nM FGF). Solid curves are hypothetical scenarios in which one of the two layers of ERK-dependent feedback is selectively turned off, as indicated. The dashed curves are with all ERK-dependent feedback loops intact.
5.2.4 A single model encompassing the PDGF and FGF receptor networks reveals quantitative differences in the propagation of Ras/ERK signaling

Having demonstrated that kinetic models of the FGF and PDGF receptor signaling networks with similar mathematical structures are capable of generating good fits to all available data and a certain degree of predictive power, we sought to identify minimal parametric requirements that reconcile the two network models. That is, for those processes not directly affected by receptor-level interactions (those which are downstream of Ras-GTP and 3’ phosphoinositide accumulation), we assessed whether or not a common set of model parameters can simultaneously capture FGF- and PDGF-stimulated signaling kinetics. If not, we wish to identify which alterations of the parameters (differences between the two networks as stimulated by FGF versus PDGF) allow a good fit of both data sets.

To address these questions, a series of four model variations were formulated and globally fit to the available data for FGF and PDGF; care was taken to co-normalize the two data sets based on the side-by-side measurements shown in Fig. 5.1. The model variations focus on differences in the phosphorylation kinetics of MEK (which, according to our model, could reflect the levels or/and potencies of the MEK kinase activities mobilized by Ras- and PI3K-dependent signaling) and of ERK (Table 5.1). In the first and least complicated of the model variations, MEK and ERK phosphorylation kinetics as stimulated by FGF and PDGF are identical; that is, the two receptor networks differ only in their propensities for generating Ras-GTP and 3’ phosphoinositides. Although this model is most satisfying from the standpoint of Occam’s razor, it fails to accurately capture the kinetics of FGF-stimulated MEK/ERK phosphorylation (Fig. 5.5A), as reflected in the corresponding error metrics (Table 5.1). By comparison, a model in which MEK phosphorylation kinetics vary between FGF and PDGF offers a demonstrably better fit (Fig. 5.5B), which is somewhat further improved by allowing both MEK and ERK phosphorylation kinetics to vary (Fig. 5.5C). Compared with the first variation, the fit is not improved if only ERK phosphorylation kinetics are varied (Fig. 5.5D).
To reconcile the kinetic models of FGF and PDGF receptor signaling networks, Ras activated in response to FGF stimulation must be more potent in propagating activation of MEK and ERK. A kinetic model encompassing both FGF and PDGF receptor-mediated signaling was formulated with four variations that allowed different levels of the MEK kinase → MEK → ERK cascade to differ between the two receptor networks. Fits of the FGF-stimulated Ras-GTP loading, MEK phosphorylation, and ERK phosphorylation data are displayed as in Fig. 5.3A-C for the FGF only model (Figs. 5.6 and 5.7 and Table 5.1). A. Variation 1: All common parameters the same. B. Variation 2: Distinct MEK activation kinetics; enzymatic parameters of the MEK kinase activities were allowed to have different values for FGF versus PDGF stimulation. C. Variation 3: Distinct MEK and ERK activation kinetics; enzymatic parameters of the MEK kinase and MEK activities were allowed to have different values for FGF versus PDGF stimulation. D. Variation 4: Distinct ERK activation kinetics.
Figure 5.6 Kinetic models of PDGF receptor signaling network fitted along with FGF receptor signaling network. A kinetic model encompassing both FGF and PDGF receptor-mediated signaling was formulated with four variations that allowed different levels of the MEK kinase $\rightarrow$ MEK $\rightarrow$ ERK cascade to differ between the two receptor networks. A. Variation 1: All common parameters the same. B. Variation 2: Distinct MEK activation kinetics; enzymatic parameters of the MEK kinase activities were allowed to have different values for FGF versus PDGF stimulation. C. Variation 3: Distinct MEK and ERK activation kinetics; enzymatic parameters of the MEK kinase and MEK activities were allowed to have different values for FGF versus PDGF stimulation. D. Variation 4: Distinct ERK activation kinetics.
Figure 5.7 Saturation of the ERK cascade in FGF receptor signaling network using phorbol ester. Cells were pretreated for 15 minutes with DMSO only (black symbols) or 200 nM PMA (magenta symbols), prior to stimulation with FGF-2 (1 nM). The solid curves represent the corresponding ensemble means for the kinetic model, and the dashed curves are mean ± s.d. (n =10,000). Top panel: A kinetic model for FGF signaling only (Fig 5.4). Bottom panel: A kinetic model of FGF and PDGF signaling networks (Figs. 5.6 and 5.7). Variation 1: All common parameters the same. B. Variation 2: Distinct MEK activation kinetics; enzymatic parameters of the MEK kinase activities were allowed to have different values for FGF versus PDGF stimulation. C. Variation 3: Distinct MEK and ERK activation kinetics; enzymatic parameters of the MEK kinase and MEK activities were allowed to have different values for FGF versus PDGF stimulation. D. Variation 4: Distinct ERK activation kinetics.
Table 5.1 Comparison of models encompassing FGF and PDGF receptor signaling networks. The various model variations differ according to which level(s) of the MEK kinase → MEK → ERK pathway are allowed to have different kinetic parameters in FGF- versus PDGF-stimulated cells. The sum of squared deviations (SSD) for each readout (Ras, MEK, ERK; \( n \) is the number of measurements fit) serves as a relative error metric and is reported as the mean ± s.d. for the 10,000 parameter sets in each ensemble.

<table>
<thead>
<tr>
<th></th>
<th>FGF only</th>
<th>Var. 1: Same parameters</th>
<th>Var. 2: Distinct MEK activation</th>
<th>Var. 3: Distinct MEK, ERK activation</th>
<th>Var. 4: Distinct ERK activation</th>
</tr>
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<tbody>
<tr>
<td>( SSD_{\text{Ras}} ) (FGF, ( n = 18 ))</td>
<td>1.50±0.09</td>
<td>2.07±0.19</td>
<td>2.27±0.30</td>
<td>2.29±0.28</td>
<td>2.22±0.23</td>
</tr>
<tr>
<td>( SSD_{\text{MEK}} ) (FGF, ( n = 22 ))</td>
<td>0.32±0.07</td>
<td>0.61±0.09</td>
<td>0.46±0.17</td>
<td>0.33±0.10</td>
<td>0.70±0.11</td>
</tr>
<tr>
<td>( SSD_{\text{ERK}} ) (FGF, ( n = 30 ))</td>
<td>0.62±0.07</td>
<td>1.59±0.11</td>
<td>1.32±0.14</td>
<td>1.16±0.11</td>
<td>1.57±0.14</td>
</tr>
<tr>
<td>( SSD_{\text{Ras}} ) (PDGF, ( n = 21 ))</td>
<td>—</td>
<td>1.65±0.21</td>
<td>1.60±0.24</td>
<td>1.64±0.23</td>
<td>1.65±0.23</td>
</tr>
<tr>
<td>( SSD_{\text{MEK}} ) (PDGF, ( n = 84 ))</td>
<td>—</td>
<td>2.81±0.20</td>
<td>2.24±0.78</td>
<td>2.19±0.21</td>
<td>2.40±0.21</td>
</tr>
<tr>
<td>( SSD_{\text{ERK}} ) (PDGF, ( n = 104 ))</td>
<td>—</td>
<td>3.60±0.13</td>
<td>3.29±0.20</td>
<td>3.14±0.15</td>
<td>3.50±0.17</td>
</tr>
</tbody>
</table>

The interpretation of these results taken together is as follows. Relative to maximal PDGF stimulation conditions, FGF generates a comparable level of Ras-GTP but far less PI3K-dependent signaling (on a whole-cell basis), yet the two ligands yield similar levels of MEK phosphorylation at steady state; accordingly, with PI3K inhibited, FGF stimulates higher levels of MEK and ERK phosphorylation than PDGF. Therefore, in the context of the model, the Ras-GTP stimulated by FGF must be more potent in activating MEK phosphorylation. By comparison, the kinetics of ERK phosphorylation by MEK are more consistent between the two networks.

5.3 Discussion

Even structurally related receptors in the same cellular background should be expected to mediate the activation of intracellular signaling networks with distinct magnitudes and kinetics of protein phosphorylation. Unraveling the mechanisms that give rise to this level of complexity will be important if we are to understand how different cell types integrate information about various stimuli and how to perturb those processes to affect cell behavior. In the present comparison of FGF and PDGF receptor-mediated signaling in
mouse fibroblasts, quantitative analysis and modeling were used to elucidate both marked and subtle differences between the two networks.

Their major point of divergence lies in the activation of PI3K signaling, with PDGF stimulating this pathway maximally and FGF weakly so. Activated PDGF receptors directly engage the regulatory subunits of type IA PI3Ks with high avidity and specificity (Kazlauskas & Cooper, 1990; Ottinger, Botfield, & Shoelson, 1998), and so it is likely that potent activation of PI3K signaling is a general feature of PDGF receptor signaling. In contrast, FGF receptors generally rely upon the scaffold proteins FRS2 and Gab1 for PI3K recruitment (Hadari, Gotoh, Kouhara, Lax, & Schlessinger, 2001; Ong et al., 2001), and therefore one might expect PI3K signaling to be highly variable across FGF-responsive cell contexts.

The less obvious difference between the two networks in our cells lies in their common, Ras-dependent pathway to MEK/ERK activation. Whereas both growth factors maximally stimulate Ras-GTP loading and MEK/ERK phosphorylation to similar extents, the level of MEK/ERK phosphorylation elicited by PDGF stimulation relies more on PI3K-dependent signaling, as seen in PI3K-inhibited cells. Our kinetic models encompassing both receptor networks reconcile those quantitative measurements by allowing Ras-GTP generated in response to FGF to yield higher MEK kinase activity, although differential access to MEK phosphatases or differences in feedback regulation provide alternative mechanistic explanations. How might such differences be encoded? The answer, we speculate, lies in the localization of the receptors. Mathematical formulations of the standard type formulated here, which are most appropriate for modeling population data (Haugh et al., 2009), do not readily account for such effects. Depending on the experimental context, growth factors are capable of stimulating Ras-GTP loading and downstream signaling not only at the plasma membrane but also from internal membranes associated with early endosomes, Golgi apparatus, and endoplasmic reticulum (Bivona & Philips, 2003; J.M. Haugh, Huang, Wiley, Wells, & Lauffenburger, 1999; Pol, Calvo, & Enrich, 1998). At the plasma membrane, Ras-GTP might be enriched in the vicinity of activated receptors (Monine & Haugh, 2008) and in membrane microdomains in a Ras isoform-dependent manner.
(Harding, Tian, Westbury, Frische, & Hancock, 2005; Murakoshi et al., 2004; Plowman, Muncke, Parton, & Hancock, 2005). What is clear from these and other indications is that the Ras-GTP level measured on a whole-cell basis only tells part of the story. We find it plausible that differential localization of receptors along with their intracellular binding partners results in activation of distinct Ras pools that vary in their propagation of signaling through MEK. An alternative explanation is that a third, FGF-stimulated pathway, not accounted for here, synergizes with Ras to more potently activate MEK kinases.

This study was intended as a test case for more systematic comparisons of signaling networks that, analyzed no further than the level of molecular connectivity/network structure, might be considered quite similar. In our view it also illustrates the various levels of quantitative scrutiny required, none higher than the level of mathematical model formulation and experimental validation, to achieve different levels of network characterization. The comparable levels of Ras/ERK signaling and the marked disparity in PI3K signaling elicited by FGF and PDGF stimulation in our cells might readily have been ascertained from a rough screen of the sort carried out in recent years (Natarajan, Lin, Hsueh, Sternweis, & Ranganathan, 2006); however, the full picture in the context of pathways that converge upon MEK and ERK phosphorylation and the feedback regulation of those pathways would have been glossed over. Hence, the challenge will be how to sensibly apply more systematic approaches across an array of cellular contexts.

5.4 Materials and Methods

5.4.1 Reagents

All tissue culture reagents were from Invitrogen (Carlsbad, CA). Human recombinant PDGF-BB and murine recombinant FGF-2 were purchased from Peprotech (Rocky Hill, NJ). Antibodies against total ERK1/2 and MKP3 and phospho-specific antibodies against Akt pSer\(^{473}\), ERK pThr\(^{202}/\)pTyr\(^{204}\), and MEK pSer\(^{217}/\)pSer\(^{221}\) were from Cell Signaling Technology (Beverly, MA); antibodies against Ras (Y13-259, agarose-conjugated), Akt-1/2 N terminus, and MKP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Pharmacological inhibitors were from Calbiochem (San Diego, CA) and were
pre-incubated with the cells for 30-60 minutes prior to growth factor stimulation. The siGENOME siRNA reagents and siGENOME SMARTpool siRNAs against mouse MKP3 (GeneID: 67603), ERK1 (GeneID: 26417), and ERK2 (GeneID: 26413) and siGENOME Non-Targeting siRNA Pool #2 were purchased from Dharmacon (Lafayette, CO). Unless otherwise noted, all other reagents were from Sigma-Aldrich.

5.4.2 Cell culture and siRNA transfection

NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, VA) were cultured at 37°C, 5% CO\textsubscript{2} in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and the antibiotics penicillin and streptomycin. Where applicable, NIH 3T3 cells were serially infected with retrovirus bearing empty vector or S17N H-Ras and selected using puromycin prior to each experiment, as described previously (Kaur, Park, Lewis, & Haugh, 2006; Wang et al., 2009). NIH 3T3 cells were transfected with siRNAs according to the manufacturer’s protocol and incubated for three days prior to the experiment.

5.4.3 Lysate preparation and biochemical assays

Cells were serum-starved for 4 hours prior to stimulation. Detergent lysates were prepared for quantitative immunoblotting, and immunoblots were performed using enhanced chemiluminescence, as described previously (Cirit et al., 2010; Wang et al., 2009). Blots comparing lysates prepared on the same day, representing either different inhibitor treatments or different cell variants and respective control conditions, were performed in parallel and exposed at the same time. The BioRad Fluor S-Max system, which gives a linear response with respect to light output, was used, and band intensity was quantified using local background subtraction. To determine the amounts of Ras-bound GTP (from all Ras isoforms), eluted from anti-Ras immunoprecipitates (Y13-259, agarose-conjugated), the coupled nucleoside-5’-diphosphate kinase/luciferase assay was performed as described previously (Kaur et al., 2006; Wang et al., 2009). Both types of data were first normalized by an appropriate loading control and then further normalized to evaluate the consistency of
relative trends across independent experiments, according to the procedures described in
detail previously (Wang et al., 2009).

5.4.4 Kinetic model and computational analysis

Our previously published model of the PDGF receptor network (Cirit et al., 2010)
was modified by removing the feedback modulation of ERK phosphatase expression levels;
as previously shown in that work, this simplification substantially reduces the number of
model parameters without affecting the fit to the PDGF data set. The model of FGF receptor
signaling was adapted from this model with phenomenological equations for the kinetics of
FGF receptor-mediated recruitment of Ras guanine nucleotide exchange factor and PI3K
activities.

The parameter estimation approach used is as described previously (Cirit et al., 2010).
Briefly, it uses a Monte Carlo/simulated annealing-based algorithm to generate a large ($n =
10,000$) ensemble of “good” parameter sets rather than one “best” fit. 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.

5.5 Appendix: Kinetic Modeling Details

5.5.1 Receptor-mediated recruitment of Ras-GEF and PI3K activities

Fractional recruitment of Ras-GEF enzymatic activity ($e_{GEF}$) is potentially saturable
and assumed to be in quasi-equilibrium with receptor activation, which varies with time
according to the kinetics of receptor binding, dimerization, and downregulation. Based on
those considerations and minimizing the number of adjustable parameters, we formulated the
following algebraic equation.

$$e_{GEF}(t) = \frac{A_{GEF} \left(e^{-k_1 t} - e^{-k_2 t}\right)}{1 + A_{GEF} \left(e^{-k_1 t} - e^{-k_2 t}\right)} f_{GEF}(t). \quad (\text{Eq. S1})$$

The phenomenological parameters $A_{GEF}$ (dimensionless), $k_1$ (min$^{-1}$), and $k_2$ (min$^{-1}$) are
determined by data fitting, with different values at each dose of FGF (3 parameters*2 doses =
6 fit parameters here). The fits are constrained so that these parameters are all positive, and \( k_1 < k_2 \). Roughly speaking, \( A_{GEF} \) controls the degree of saturation of the dose response, \( k_2 \) characterizes how quickly the system responds, and \( k_1 \) characterizes the adaptation of the response, e.g. through receptor downregulation and/or ligand depletion. The variable \( f_{GEF}(t) \) is the fraction of Ras-GEF that is freely available, i.e., not desensitized by ERK (see below).

Recruitment of PI3K enzymatic activity is modeled in an analogous fashion as follows.

\[
e_{PI3K}(t) = \frac{A_{PI3K}(e^{-k_1 t} - e^{-k_2 t})}{1 + A_{PI3K}(e^{-k_1 t} - e^{-k_2 t})}.
\]

(Eq. S2)

For each of the two FGF doses, the values of \( k_1 \) and \( k_2 \) are the same as in Eq. S1, and there are different values of the saturation parameter, \( A_{PI3K} \).

### 5.5.2 Accumulation of membrane-associated messengers Ras-GTP and 3’ PI lipids

The dimensionless densities of Ras-GTP (\( m_{Ras} \)) and 3’ PI lipids (\( m_{3PI} \)) respond to the recruitment of Ras-GEF and PI3K activities, respectively, according to the following differential equations of identical mathematical form.

\[
\frac{dm_{Ras}}{dt} = k_{Ras} (m_{Ras,\text{max}} e_{GEF} - m_{Ras}) m_{Ras}(0) = 0.
\]

(Eq. S3)

\[
\frac{dm_{3PI}}{dt} = k_{3PI} (m_{3PI,\text{max}} e_{PI3K} - m_{3PI}) m_{3PI}(0) = 0.
\]

(Eq. S4)

Relative to our previous PDGF receptor signaling models (Cirit et al., 2010; Wang, Cirit, & Haugh, 2009), the parameters \( m_{Ras,\text{max}} \) and \( m_{3PI,\text{max}} \) are new and account for differences in the maximal activation of Ras and PI3K mediated by FGF versus PDGF receptors in our cells (their values are equal to 1 for PDGF signaling). Another minor difference in the \( m_{Ras} \) conservation equation, Eq. S3, is the omission of a saturation parameter, \( \Gamma \), which had already been set to an arbitrarily low value in previous models to reflect the observation that most of the Ras remains in the inactive, GDP-bound form.
5.5.3 Activation of MEK kinase activities and phosphorylation of Akt

As formulated previously, enzymatic activity directed towards the phosphorylation of MEK is comprised of Ras-dependent ($x_1$) and PI3K-dependent ($x_2$) contributions; PI3K-dependent signaling is also responsible for Akt phosphorylation ($a_p$), which is modeled in order to compare with those measurements. The following conservation equations are taken from our previous model (Cirit et al., 2010).

$$\frac{dx_1}{dt} = k_{d,1} \left[ \frac{(1 + K_{x1})m_{Ras}}{1 + y/K_{M,x11} + y_p/K_{M,x12}} \right] x_1; \quad x_1(0) = 0. \quad \text{(Eq. S5)}$$

$$\frac{dx_2}{dt} = k_{d,2} \left[ \frac{(1 + K_{x2})m_{3PI}}{1 + y/K_{M,x21} + y_p/K_{M,x22}} \right] x_2; \quad x_2(0) = 0. \quad \text{(Eq. S6)}$$

$$\frac{da_p}{dt} = k_{d,a} \left[ \frac{(1 + K_a)m_{3PI}}{1 + K_a m_{3PI}} \right] a_p; \quad a_p(0) = 0. \quad \text{(Eq. S7)}$$

$y$ and $y_p$ are the unphosphorylated and mono-phosphorylated fractions of total MEK, respectively, which appear in Eqs. S5 and S6 to allow for sequestration of active $x_1$ and $x_2$ by their substrates; thus, the rate of MEK kinase deactivation would be correspondingly reduced.

5.5.4 Phosphorylation of MEK and ERK

For MEK (dual phosphorylated MEK fraction defined as $y_{pp}$), the conservation equations are as follows (Cirit et al., 2010).

$$\frac{dy}{dt} = \sum_{i=1}^{2} \frac{V_{max,i}x_{i}f_{si}y/K_{M,si}}{1 + y/K_{M,si} + y_p/K_{M,si2}} + \frac{V_{max,lyph1}y_p/K_{M,lyph1}}{1 + y_p/K_{M,lyph1} + y_{pp}/K_{M,lyph2}}; \quad y(0) = 1; \quad \text{(Eq. S8)}$$

$$\frac{dy_{pp}}{dt} = \sum_{i=1}^{2} \frac{V_{max,si2}x_{i}f_{si}y_p/K_{M,si2}}{1 + y/K_{M,si} + y_p/K_{M,si2}} - \frac{V_{max,lyph2}y_{pp}/K_{M,lyph2}}{1 + z/K_{M,y1} + z_p/K_{M,y2}(1 + y_p/K_{M,lyph1}) + y_{pp}/K_{M,lyph2}}; \quad y_{pp}(0) = 0; \quad \text{(Eq. S9)}$$
\[ y_p = 1 - y - y_{pp}. \]  \hspace{1cm} \text{(Eq. S10)}

The variables \( f_{xi}(t) \), account for the fractions of \( x_1 \) and \( x_2 \) that are not desensitized by ERK (see below). MEK phosphatase activity (e.g., PP2A) is taken to be constant (characterized by the parameters \( V_{\text{max},yph1}, K_{M,yph1}, V_{\text{max},yph2}, \) and \( K_{M,yph2} \)).

For ERK (non-, mono-, and dual-phosphorylated fractions defined as \( z, z_p, \) and \( z_{pp}, \) respectively), the conservation equations are as follows.

For \( z \) and \( z_p \):

\[ \frac{dz}{dt} = -\frac{V_{\text{max},y1} y_{pp} z / K_{M,y1}}{1 + z / K_{M,y1} + z_p / K_{M,y2}} + \frac{V_{\text{max},zph1} z_p / K_{M,zph1}}{1 + z_p / K_{M,zph1} + z_{pp} / K_{M,zph2}}; \hspace{1cm} z(0) = 1; \hspace{1cm} \text{(Eq. S11)} \]

For \( z_{pp} \):

\[ \frac{dz_{pp}}{dt} = \frac{V_{\text{max},y2} y_{pp} z_p / K_{M,y2}}{1 + z / K_{M,y1} + z_p / K_{M,y2}} - \frac{V_{\text{max},zph2} z_{pp} / K_{M,zph2}}{1 + z_p / K_{M,zph1} + z_{pp} / K_{M,zph2}}; \hspace{1cm} z_{pp}(0) = 0; \hspace{1cm} \text{(Eq. S12)} \]

Relative to the previous models (Cirit et al., 2010; Wang et al., 2009), the only change here is that the ERK phosphatase activity (dual-specificity phosphatases) is taken to be constant (characterized by the parameters \( V_{\text{max},zph1}, K_{M,zph1}, V_{\text{max},zph2}, \) and \( K_{M,zph2} \)). This is a simplification of the previous models, in which ERK phosphatase activity was potentially affected by ERK-dependent feedback. The justification for this simplification is two-fold: the best fit of the previous PDGF receptor network model was achieved with constant ERK phosphatase activity, and experimentally we found no relationship between the expression levels of MKP1 and MKP3 (dual-specificity phosphatases that respond in different ways to growth factor stimulation) and ERK phosphorylation (Cirit et al., 2010).

5.5.5 Regulation of the network by ERK-dependent negative feedback

There are two distinct layers of negative feedback in the model. The first affects Ras-GEF activity through the aforementioned variable \( f_{GEF} \), and the second affects MEK kinase activities through the aforementioned variables \( f_{x1} \) and \( f_{x2} \). These quantities decrease in response to ERK phosphorylation according to the following, quasi-mechanistic differential equations (Cirit et al., 2010).
\[
\frac{df_{\text{GEF}}}{dt} = -k_{d,G} \left[ K_{fG} z_{pp} f_{\text{GEF}} - (1 - f_{\text{GEF}}) \right] f_{\text{GEF}}(0) = 1. \quad \text{(Eq. S14)}
\]

\[
\frac{df_{x_1}}{dt} = -k_{d,x_1} \left[ K_{f1} z_{pp} f_{x_1} - (1 - f_{x_1}) \right] f_{x_1}(0) = 1. \quad \text{(Eq. S15)}
\]

\[
\frac{df_{x_2}}{dt} = -k_{d,x_2} \left[ K_{f2} z_{pp} f_{x_2} - (1 - f_{x_2}) \right] f_{x_2}(0) = 1. \quad \text{(Eq. S16)}
\]

### 5.5.6 Summary of model parameters and global fitting to FGF data

The equations formulated above invoke a total of 44 constant parameters, of which 4 are assigned fixed values (\(k_{Ras}, k_{3PI}, k_{d,a}, \text{ and } K_a\); see the previous publications) and 40 were subjected to a global fit to the available data set, which included the following 81 measurements for FGF stimulation: Ras-GTP loading kinetics, with and without MEK inhibitor (18 conditions; Fig. 5.3A); MEK phosphorylation kinetics, with and without PI3K inhibitor (22 conditions; Fig. 5.3B); ERK phosphorylation kinetics, with and without PI3K inhibitor (22 conditions; Fig. 5.3C) and with and without phorbol ester (8 conditions; Fig. 5.7); and Akt phosphorylation kinetics (11 conditions; data not shown). The latter do not significantly affect the fitting of the rest of the data and parameters, because the low levels of Akt phosphorylation stimulated by FGF are scaled relative to those stimulated by PDGF (Fig. 5.1B); in other words, for the overall fit it is important only that the Akt phosphorylation levels produced by the model are sufficiently low.

A large ensemble of parameter sets was obtained using a modified simulated annealing algorithm described in detail previously (Cirit et al., 2010). These were sorted according to the lowest sum of squared deviations (SSD, or \(\chi^2\)) to identify the 10,000 “best” parameter sets. As explained previously (Cirit et al., 2010; Wang et al., 2009), each parameter set is associated with a set of conversion factors (one for each of the sets of data listed above) that best align the dimensionless variables of the model to the arbitrary “instrument units” of the data. The overall prediction and uncertainty of the model is expressed as the mean and standard deviation of the aligned outputs (\(n = 10,000\)).
5.5.7 Calculating the dynamic MEK activation comparator (dMAC)

The dMAC is a time-dependent quantity that compares the relative contributions of PI3K- and Ras-dependent signaling converging on MEK (Cirit et al., 2010). For a given experimental condition (here, a particular dose of FGF) and for each of the 10,000 selected parameter sets, the model is run with the Ras pathway silenced ($m_{Ras} = 0$; PI3K-dependent activation of MEK) and then with the PI3K pathway silenced ($m_{3PI} = 0$; Ras-dependent activation of MEK). The quantity $y_{pp}(1 - y_{pp})$, reflecting the rate of MEK activation normalized by the amount of inactive MEK available, is calculated as a function of time, and the dMAC is calculated as the ratio of PI3K-dependent to Ras-dependent $y_{pp}(1 - y_{pp})$ values.

5.5.8 Predicting the effect of siRNA knockdown

This calculation was performed as described previously (Cirit et al., 2010). Defining $\delta$, as the fractional knockdown of ERK1/2 (e.g., $\delta = 0.8$ corresponds to 80% reduction of intracellular ERK1/2), the prediction is implemented by multiplying or dividing the values of the following parameters by the factor, $(1 - \delta)$, according to how they are scaled by the intracellular concentration of total ERK:

- **Multipled by $(1 - \delta)$:** $K_{fG}$, $K_{x1}$, $K_{x2}$.
- **Divided by $(1 - \delta)$:** $V_{max,y1}$, $K_{M,y1}$, $V_{max,y2}$, $K_{M,y2}$, $V_{max,zph1}$, $K_{M,zph1}$, $V_{max,zph2}$, $K_{M,zph2}$.

5.5.9 Co-alignment of PDGF and FGF receptor signaling network models

Model and data set for the PDGF receptor network

The model of PDGF receptor-mediated ERK activation described in detail previously (Cirit et al., 2010), comprised of 57 constant parameters (14 fixed, 43 fit), was used with the following simplification: the modulation of ERK phosphatase activity was neglected, such that ERK species evolve according to Eqs. S11-S13 given above. Together with the modifications prescribed in Eqs. S3 and S4 (removal of the fixed parameter $\Gamma$ and addition of parameters $m_{Ras,max}$ and $m_{3PI,max}$, which are fixed to values of 1 for PDGF), this results in the
elimination of 11 fit parameters and the addition of 1 fixed parameter, leaving a total of 47 constant parameters (32 fit).

The data used for aligning the PDGF receptor network model were presented previously (Cirit et al., 2010; Wang et al., 2009) and include the following 209 measurements: Ras-GTP loading kinetics, with PI3K inhibited, MEK inhibited, or control (21 conditions); MEK phosphorylation kinetics, PI3K inhibited versus control (42 conditions) and with dominant-negative (S17N) Ras versus control (42 conditions); ERK phosphorylation kinetics, PI3K inhibited versus control (42 conditions), dominant-negative (S17N) Ras versus control (42 conditions), and with and without phorbol ester (20 conditions). As explained in the main text of the paper, the PDGF and FGF data are co-normalized with each other based on the side-by-side kinetic data presented in Fig. 5.1 and have common conversion factors for alignment of each experimental readout to the corresponding model output.

Model variations used to simultaneously fit FGF and PDGF stimulation data

In the FGF receptor network model, Eqs. S1 and S2 and their 8 adjustable parameters are unique to FGF receptor-mediated signaling, as are the FGF-specific values of $m_{Ras,max}$ and $m_{3PI,max}$ in Eqs. S3 and S4, respectively. The remaining 34 parameters in the FGF receptor network model, of which 30 are fit, are common with the PDGF receptor signaling model. Taken together, the minimum number of adjustable parameters needed to align both FGF and PDGF stimulation data is 57 (42 fit).

Variation 1: All common parameters the same. The simplest and most restrictive model variation is one in which all 30 of the common fit parameters alluded to above are constrained to have the same values for FGF and PDGF signaling; each parameter set is forced to best reconcile both sets of data.

Variation 2: Distinct MEK activation kinetics. All parameters are constrained to be the same for FGF and PDGF signaling except the following 8 MEK phosphorylation parameters: $V_{max,x11}$, $K_{M,x11}$, $V_{max,x12}$, $K_{M,x12}$, $V_{max,x21}$, $K_{M,x21}$, $V_{max,x22}$, and $K_{M,x22}$. This allows
the Ras- and PI3K-dependent inputs to MEK to be more or less potent depending on where and how they are activated in response to FGF versus PDGF.

**Variation 3: Distinct MEK and ERK activation kinetics.** Here, there are 12 parameters that are again allowed to vary between FGF- and PDGF-stimulated signaling: the 8 parameters listed under Variation 2 and the 4 ERK phosphorylation parameters ($V_{max,y1}$, $K_{M,y1}$, $V_{max,y2}$, and $K_{M,y2}$). This is the most complicated model variation in terms of adjustable parameters and considers the possibility that the two receptors have differential accessibility to scaffold proteins that hold MEKK, MEK, and ERK species in the same complex, for example.

**Variation 4: Distinct ERK activation kinetics.** Here, only the 4 ERK phosphorylation parameters listed under Variation 4 are allowed to vary between FGF- and PDGF-stimulated signaling. This model variation is the least plausible but serves as a control of sorts for the comparisons among Variations 1-3.
5.6 References


CHAPTER 6

REGULATION OF RECEPTOR-MEDIATED ERK AND PI3K PATHWAYS BY UBIQUITINATION/PROTEASOMAL DEGRADATION

In mammalian cells, expression levels of cellular proteins are regulated by two proteolytic mechanisms: lysosomal and proteasomal degradation. Signaling mediated by receptor tyrosine kinases, activated by binding of specific growth factors present in the external milieu, is channeled most prominently through extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) pathways, but whereas downregulation of the receptors by both lysosomal and proteasomal routes is reasonably well understood, the regulated degradation of intracellular pathway components remains far less so. Expression levels of dual-specificity phosphatases (DUSPs), which specifically deactivate ERK and other mitogen-activated protein kinases (MAPks) by dephosphorylation, are known to be down-modulated by a ubiquitination/proteasomal degradation pathway; however, the attendant effects on ERK phosphorylation are difficult to interpret, since pharmacological inhibition of the proteasomal pathway alters the expression levels of many intracellular proteins besides DUSPs.

In this work, we quantitatively and systematically analyzed the effects of proteasome inhibitor (MG-132) treatment on growth factor-stimulated activation of ERK and PI3K signaling pathways. Using a modeling framework that quantitatively accounts for PI3K-dependent crosstalk to the ERK pathway and ERK-dependent feedback regulation of its own activation, we computationally analyzed the sensitivities of the network to perturbations at the various steps of the pathway. The analysis indicates that, while the drug treatment enhances ERK dephosphorylation by a consistent upregulation of DUSPs, it also impacts ERK phosphorylation through perturbations of the upstream pathways.
6.1 Introduction

The rates of biochemical reactions affecting cell function are determined by the intracellular concentrations of enzymes and other regulatory proteins, which in turn depend on their rates of synthesis and degradation. Although gene regulation at the level of transcription and translation are central to cell physiology, so too are the primary pathways by which protein half-lives are modulated: lysosomal and proteasomal degradation.

Lysosomes are organelles containing a class of hydrolases that degrade proteins completely to amino acids and which are active in the acidic lysosomal lumen (pH ≈ 4). A common example of this process is down-regulation of growth factor receptors. The receptors are continuously internalized through the endosomal pathway at a slow, basal rate in quiescent cells, whereas ligand-bound (activated) receptors internalize at a significantly faster rate. The endosomal contents are subject to a sorting process whereby they are either recycled to the plasma membrane or degraded in lysosomes. Most endosomal internalization depends on the characteristics of cytosolic domain of transmembrane protein. Short peptide sequences (tyrosine- and dileucine-based) in the cytoplasmic domains of the receptors act as sorting signals (Bonifacino & Traub, 2003; Pickart, 2001). With the recognition of these peptide motifs, receptors undergo endocytosis by forming lipoprotein-receptor complexes within the plasma membrane that are retained in structures that invaginate to form coated endocytic vesicles.

Another protein destruction mechanism is the ubiquitin-proteasome pathway. This pathway is essential for degradation of short-lived, mislocated, misfolded, and damaged proteins (Lee & Goldberg, 1998; Rock et al., 1994; Sherman & Goldberg, 2001). The conserved polypeptide ubiquitin is composed of 76 amino acid residues (Pickart, 2001). The first step of the ubiquitin conjugation cascade is the activation of carboxyl group of Gly-76 by ubiquitin-activating enzyme (E1) through an ATP-dependent mechanism. Activated ubiquitin is then transferred to ubiquitin-conjugating enzymes (E2) and attached either directly to a protein or via ubiquitin-protein ligase (E3). The specificity of ubiquitination is largely determined by E3 enzymes. Once a protein is mono-ubiquitinated, a polyubiquitin chain may be formed through the same reaction sequence. Poly-ubiquitinated proteins are
recognized by the 26S proteasome (2000 kDa), a multi-subunit, multi-catalytic protease that is found in the cytosol, perinuclear regions and nucleus (McNaught, Olanow, Halliwell, Isacson, & Jenner, 2001). The conserved catalytic core, 20S proteasome (700-kDa), cleaves the peptide bonds of target protein via its proteolytic activity. Another essential structural feature of the 26S proteasome is two 19S regulatory particles, which determine substrate specificity and regulation (Coux, Tanaka, & Goldberg, 1996). In the course of growth factor receptor-mediated signaling, autophosphorylation of the receptors triggers activation of ubiquitination pathway. The activated receptors can be ubiquitinated by Cbl, a E3 ligase, and undergo proteasomal degradation (Joazeiro et al., 1999; Lock et al., 2006), and thus their downregulation is controlled by both proteasomal and lysosomal pathways (Bonita, Miyake, Lupher, Langdon, & Band, 1997; Dikic & Giordano, 2003; Miyake, Mullane-Robinson, Lill, Douillard, & Band, 1999; Sehat, Andersson, Vasilcanu, Girnita, & Larsson, 2007).

Most currently available inhibitors of ubiquitin-proteasome pathway directly target and inhibit the 20S proteasome, rather then the ubiquitination process (Myung, Kim, & Crews, 2001). Peptide aldehydes have been used as reversible serine and cysteine protease inhibitors. Two of the earlier used inhibitors are leupeptin, a serine and cysteine protease inhibitor, and calpain inhibitor, the first potent 20S proteasome inhibitor to be identified (Adams, Palombella, & Elliott, 2000; Lee & Goldberg, 1998). Later, with directed organic chemistry efforts, several potent protease inhibitors have been synthesized to study cellular processes, including MG-115, MG-132, and PSI. The main limitation of these inhibitors is their lack of specificity, owing to the highly reactive aldehyde group (Myung et al., 2001). Other subclasses of synthetic proteasome inhibitors include lactacystin, peptide boronate, vinyl sulfone, and β-lactone.

Growth factor receptors mediate signaling through the mitogen-activated protein kinase (MAPK) and PI3K pathways most prominently. The canonical MAPK pathway is an ordered series of activation processes, exemplified by the Ras → Raf → mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) → ERK pathway. Activation of this pathway is specifically regulated by ERK-dependent feedback loops as discussed in Chapter 4. At least in certain cell contexts, it has been reported that
several components of MAPK and PI3K pathways are subject to proteasomal degradation, including ERK1/2, SH2-containing-phosphatidylinositol-5’-phosphatase (SHIP), and dual-specificity phosphatases (DUSPs) (Jura, Scotto-Lavino, Sobczyk, & Bar-Sagi, 2006; Lin, Chuang, & Yang, 2003; Lornejad-Schafer et al., 2005); however, given the array of targets that might be modulated by the ubiquitin-proteasome pathway, assessing its importance at the pathway/network level presents a complex challenge. In this study, we focused on the effects of the proteasome inhibitor MG-132 as a broad-based perturbation of the signaling network accessed by platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) receptors in mouse fibroblasts. Through a quantitative analysis employing computational modeling, we demonstrate how one might parse the mixed effects of such systematic pharmacological perturbations.

6.2 Results

6.2.1 MG-132 treatment enhances MKP1 and MKP3 levels in serum-starved NIH 3T3 cells with different kinetics

MG-132 is a reversible, cell-permeable proteasome inhibitor. In a number of signaling studies aimed at understanding regulation of MAPK pathways, MG-132 has been to limit degradation of dual specificity phosphatases (DUSPs), such as MKP1 and MKP3, through the ubiquitin-proteasome pathway (Jurek, Amagasaki, Gembarska, Heldin, & Lennartsson, 2009; Lin et al., 2003; Lornejad-Schafer et al., 2005; Moutzouris et al., 2010). To characterize the effects of MG-132 treatment on DUSP protein levels, we pretreated NIH 3T3 fibroblasts with 50 μM MG-132 for 30 minutes prior to stimulation with PDGF-BB. Both at low (30 pM) and high (1 nM) doses of PDGF, MKP1 up-regulation was increased approximately two-fold (Fig 6.1), suggesting that stimulation of MKP1 synthesis is balanced by fast turnover that is partially proteasome-mediated. Surprisingly, the inhibition had no discernible effect on the kinetics of MKP3 expression. Further quantification of certain kinases in the PI3K and MAPK pathways, namely Akt, MEK, and ERK, revealed that 30-minute pretreatment of fibroblasts with MG-132 had only a minor effect on activation of the
ERK pathway. These observations and others suggest that MKP1 is not a major ERK phosphatase activity in our cells (Chapter 4).

Figure 6.1 Quantification of the effects of 30 minute MG-132 pretreatment on PI3K and MAPK pathways with PDGF-treatment. NIH 3T3 fibroblasts were pretreated for 30 minutes with 50 μM MG-132. Phospho-Akt, -MEK, -ERK, total Akt, MEK, ERK, MKP1 and MKP3 levels were measured by immunoblotting (n = 3). Bands were cropped from the same gel.
Given that proteins are differentially susceptible to intracellular degradation mechanisms and thus exhibit various half-lives, the effect of MG-132 is expected to depend on incubation time. To test this, NIH 3T3 fibroblasts were serum-starved for three hours prior to treatment with MG-132 (25 μM) for various times (0.5, 2, or 6 hrs) prior to 1 nM PDGF-BB stimulation. Untreated and PDGF-treated cells were lysed at 0, 15, and 120 minutes (Fig. 6.2). Consistent with the results shown in Fig. 6.1, 30-minute MG-132 pretreatment did not affect total MKP1 and MKP3 protein levels in untreated cells, whereas MKP1 levels upon PDGF stimulation were enhanced by the short drug treatment. In contrast, with 6-hour MG-132 treatment, MKP1 and MKP3 were markedly upregulated in both unstimulated and PDGF-stimulated cells. These findings suggest that proteasomal degradation pathways in fibroblasts control endogenous levels of MKP1 and MKP3 proteins with different kinetics, as reflected in the difference between short and longer MG-132 treatment times.

![Figure 6.2](image)

**Figure 6.2 Quantification of the effects of MG-132 pretreatment time on MKP1 and MKP3 levels with PDGF-treatment.** NIH 3T3 fibroblasts were pretreated for 0.5, 2 or 6 hours with 25 μM MG-132. The maximal effects of MG-132 on MKP1 and -3 were observed at 6 hour pretreatment (Bands were cropped from the same gel).
6.2.2 Sustained treatment with proteasome inhibitor MG-132 reduces PI3K and MAPK pathway activation in PDGF- and FGF-stimulated fibroblasts

In NIH 3T3 fibroblasts, PDGF stimulation elicits robust activation of both PI3K and MAPK pathways, whereas FGF stimulation elicits robust MAPK activation but relatively weak PI3K/Akt signaling (Chapter 5). To systematically investigate the effects of proteosomal degradation inhibition on PI3K and MAPK pathways, we pretreated serum-starved fibroblasts with 25 μM MG-132 for 6 hour and quantified the attendant effects on the signaling network by blotting for phosphorylated (activated) isoforms of Akt, Raf (specifically, cRaf/Raf1), MEK, and ERK.

At both low (30 pM) and high (1 nM) doses of PDGF, the phosphorylation kinetics of these readouts were significantly perturbed by proteasome inhibition (Fig 6.3). Whereas the observed reductions in ERK signaling appear to be consistent with studies implicating the upregulation of MKP1 and especially MKP3 (Jurek et al., 2009; Lin et al., 2003; Lornejad-Schafer et al., 2005; Moutzouris et al., 2010), parallel studies revealed that ERK activation is insensitive to modulation of MKP1/3 levels in NIH 3T3 cells (Chapter 4). More critically, the signaling network upstream of ERK, converging upon activation of MEK, is obviously perturbed even though it is presumably not affected by DUSPs. Indeed, analyzing the relationship between MEK and ERK phosphorylation in a quantitative manner presents seemingly confounding implications. In response to the low PDGF dose, phosphorylation of ERK but not of MEK is perturbed dramatically by the MG-132 treatment, consistent with enhanced ERK phosphatase activity; in contrast, with the high PDGF dose, phosphorylation of both MEK and ERK are markedly reduced. At least qualitatively, this assessment holds for low and high doses of FGF-2 (10 pM and 1 nM, respectively) as well (Fig. 6.4).
Figure 6.3 Quantification of the effects of 6 hour MG-132 pretreatment on PI3K and MAPK pathways with PDGF-treatment. NIH 3T3 fibroblasts were pretreated for 6 hours with 25 μM MG-132. Phospho-Akt, -cRaf, -MEK, and –ERK and total ERK levels were measured by immunoblotting (n = 3). Bands were cropped from the same gel.
Figure 6.4 Quantification of the effects of 6 hour MG-132 pretreatment on PI3K and MAPK pathways with FGF-treatment. NIH 3T3 fibroblasts were pretreated for 6 hours with 25 μM MG-132. Phospho-Akt, -cRaf, -MEK, and –ERK and total ERK levels were measured by immunoblotting (n = 3). Bands were cropped from the same gel.

To further elucidate which nodes in the signaling network were perturbed by proteasome inhibition, we characterized PDGF β-receptor phosphorylation on Tyr751, a known binding site for the regulatory subunit of PI3K (p85) (Cooper & Kashishian, 1993; Panayotou et al., 1993; Ponzetto et al., 1993) and a qualitative proxy for the phosphorylation of other PDGF receptor phosphorylation sites. Despite the prospect of a longer PDGF receptor half life, MG-132 inhibition (6-hour incubation) uniformly reduced the level of receptor phosphorylation by about half in cells responding to the high PDGF dose, whereas
cells stimulated by the lower dose showed little or no effect of MG-132 treatment on this readout (Fig 6.5). The reduction in receptor phosphorylation stimulated by 1 nM PDGF might be attributed to increased activity of protein-tyrosine phosphatases (Chiarugi et al., 2002); however, based on our previous experimental and computational findings (Chapters 3 and 4), activation of downstream signaling is generally saturated under these conditions (Park, Schneider, & Haugh, 2003; Wang, Cirit, & Haugh, 2009).

Figure 6.5 Down-regulation of PDGF receptor upon MG-132 pretreatment. NIH 3T3 fibroblasts were pretreated for 6 hours with 25 μM MG-132. Phospho-PDGFRβ levels were measured by immunoblotting (n = 3). Bands were cropped from the same gel.

Activation of PI3K enzymatic activity results in accumulation of the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP₃) in the plasma membrane, which in turn elicit activation of the serine-threonine kinases Akt and PDK1 (Marte & Downward, 1997; Toker & Cantley, 1997). Src homology 2-containing inositol 5' phosphatase (SHIP) and phosphatase and tensin homolog (PTEN) negatively regulate intracellular levels of PIP₃ (Leslie, Batty, Maccario, Davidson, & Downes, 2008; Rodrigues, Falasca, Zhang, Ong, & Schlessinger, 2000; Rohrschneider, Fuller, Wolf, Liu, & Lucas, 2000). Whereas SHIP function affects cells in a variety of contexts (Aman, Lamkin, Okada, Kurosaki, &
Ravichandran, 1998; Ruschmann et al., 2010; Wang et al., 2009), SHIP is expressed at an undetectable level in NIH 3T3 fibroblasts (Choi et al., 2002; Muraille, Pesesse, Kuntz, & Erneux, 1999). Hence, we assessed the regulation of PTEN levels and found no significant modulation affected by growth factors or proteasome inhibition (Figs. 6.6 and 6.7). The effects of MG-132 treatment on Akt phosphorylation (Fig. 6.3) must be attributed to the modulation of other activities, such as serine-threonine phosphatases that dephosphorylate Akt.

Figure 6.6 Quantification of the effects of 6 hour MG-132 pretreatment on phosphatase levels with PDGF treatment. NIH 3T3 fibroblasts were pretreated for 6 hours with 25 μM MG-132. Total PTEN, MKP1 and MKP3 levels were measured by immunoblotting (n = 3). Bands were cropped from the same gel.

Figure 6.7 Quantification of the effects of 6 hour MG-132 pretreatment on phosphatase levels with FGF treatment. NIH 3T3 fibroblasts were pretreated for 6 hours with 25 μM MG-132. Total PTEN, MKP1 and MKP3 levels were measured by immunoblotting (n = 3). Bands were cropped from the same gel.
6.2.3 Computational analysis of signaling perturbations elicited by MG-132 treatment

To quantitatively characterize PDGF-stimulated PI3K and MAPK pathways in NIH 3T3 cells, we previously acquired an extensive data set on the kinetics of PDGF receptor phosphorylation, ligand depletion, Ras-GTP accumulation, phosphorylation of intracellular kinases, and phosphatase expression levels. This data set was used to "train" a computational model to the extent that it proved to be quantitatively predictive (Chapter 4). That is, once parameterized through a quantitative fit to a large data set, the model can in principle predict the kinetics of activation at various nodes in the network, based on specified initial conditions corresponding to the experimental conditions assumed. Here, we apply this model in a somewhat non-standard way (at least in the context of biological systems) to shed light on the dynamic relationships between pairs of variables in the system. For any pair of dependent variables, this is achieved by constructing a phase plot, wherein the two variables are plotted against one another, with time as an implicit variable. For a specified set of experimental (initial) conditions, such a relationship is plotted as a curve, starting from the initial to the final time, in phase space. Points on such a curve correspond to different times; in comparing a family of such curves (i.e., for different experimental conditions), points corresponding to the same duration in time are connected by what we refer to here as iso-time curves.

In response to stimulation with the high PDGF dose, PDGF β-receptor phosphorylation is reduced by approximately half in MG-132-treated cells (Fig. 6.5 and Table 6.1). To investigate the effect of the reduction in receptor phosphorylation on PI3K and MAPK pathways, we computationally estimated Ras-GTP and Akt phosphorylation using the phase plots (Fig. 6.8); the latter serves as a faithful readout of PI3K signaling, and therefore these two readouts reflect the canonical and non-canonical inputs to the MEK/ERK pathway (Chapters 3 and 4). At all of the experimentally measured time points, Akt phosphorylation is close to saturation, and thus the effects of the reduction in receptor activity are predicted to be negligible (Fig. 6.8A&B). Therefore, the reduction in receptor phosphorylation does not adequately explain the observed reduction in Akt phosphorylation under those conditions (Fig 6.3). Rather, it is speculated that Akt phosphorylation might be affected by upregulation
of a serine-threonine phosphatase activity, such as PP2A (Ugi et al., 2004; Yellaturu, Bhanoori, Neeli, & Rao, 2002).

PI3K signaling, as reflected by the phosphorylation of Akt measured in our cells, engages in crosstalk with the MAPK pathway both upstream and downstream of Ras (Park et al., 2003; Wang et al., 2009). Construction of a phase plot relating Ras-GTP loading to Akt phosphorylation thus reflects the upstream crosstalk; however, this effect is weak for strong PDGF stimulation, since maximal activation of PDGF receptors is sufficient for maximal activation of Ras (Fig. 6.8C). Such insensitivity is generally reflected by mostly flat (horizontal) slopes of the iso-time curves. To explore the contribution of PI3K-dependent crosstalk downstream of Ras, we analyzed a phase plot for the relationship between MEK and Akt phosphorylation kinetics (again, for the high PDGF dose). Based on the phase plots, at high PDGF-BB dose, minor reduction in Akt activity resulted sharp decrease in MEK activity, since the iso-times laid vertically at higher doses (Fig 6.8D). Our experimental data confirmed the dramatic decrease in MEK activity. However, if both phospho-MEK and –ERK data were plotted on the phase plots (Table 6.1), we found out that MEK activity was further suppressed. Thus, we investigated the relationship between RasGTP and MEK activity (Fig 6.8E). MEK activity was strongly dependent on Ras activity. The calculated Ras activity change due to the decrease in Akt activity caused a major reduction in MEK kinases activity. These semi-quantitative findings indicated that proteasomal inhibition affected MEK both as reduction of PI3K-dependent activation of both Ras and MEK.

### Table 6.1 Experimental data from 1nM PDGF-BB and 0.2% DMSO or MG-132 treated NIH 3T3 fibroblasts.
The data in arbitrary units were normalized with experimental and computational results. Numbers in parenthesis show data after MG-132 treated fibroblasts.

<table>
<thead>
<tr>
<th>Stimulation Time</th>
<th>pPDGFR-β</th>
<th>pAkt</th>
<th>ppMEK</th>
<th>ppERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>5 min.</td>
<td>5.5e-2 (3.1e-2)</td>
<td>0.91 (0.79)</td>
<td>7.6e-2 (2.3e-2)</td>
<td>0.77 (0.24)</td>
</tr>
<tr>
<td>15 min.</td>
<td>5.1e-2 (2.8e-2)</td>
<td>1.0 (0.84)</td>
<td>6.8e-2 (2.7e-2)</td>
<td>0.79 (0.36)</td>
</tr>
<tr>
<td>30 min.</td>
<td>4.1e-2 (1.8e-2)</td>
<td>1.0 (0.63)</td>
<td>3.8e-2 (4.6e-3)</td>
<td>0.59 (0.19)</td>
</tr>
<tr>
<td>60 min.</td>
<td>2.0e-2 (7.7e-3)</td>
<td>0.74 (0.34)</td>
<td>2.7e-2 (1.8e-5)</td>
<td>0.42 (0.10)</td>
</tr>
<tr>
<td>120 min.</td>
<td>1.1e-2 (1.8e-3)</td>
<td>0.82 (0.05)</td>
<td>2.0e-2 (0)</td>
<td>0.31 (0)</td>
</tr>
</tbody>
</table>
ERK is activated by direct interaction with MEK and deactivated by dual-specificity phosphatases (DUSPs). To understand the causes for ERK reduction in MG-treated fibroblasts, we first investigated MEK-dependent activation of ERK. The readouts from the phase plots indicated that phospho-ERK levels were lower than computationally predicted levels (Fig 6.8F). Therefore, we measured DUSP levels, such as MKP1 and MKP3, in control and MG-treated cells (Fig. 6.6). Even in unstimulated cells, the observed total MKP1 and MKP3 levels after 6 hour pretreatment were roughly two-fold higher than those of control cells. Although it was shown that the endogenous levels of MKP1 and MKP3 do not apparently affect ERK phosphorylation (Chapter 4), these high levels of ERK-specific phosphatases (or other DUSP isoforms) might be responsible for further deactivation of ERK.

To further validate this analysis, we directly fit the MEK phosphorylation data using a phenomenological, time-dependent function, thus accounting for modulation of all activities upstream of ERK activation (Figure 6.10 and Table 6.2). Hence, using the parameterized equation for MEK activation as an input, we generated a large parameter set ensemble ($n = 10,000$) to fit ERK phosphorylation kinetic data for both control and MG-132 treated cells, assuming that the latter possessed ERK phosphatase activity that was enhanced by a constant factor (Table 6.3). Thus, the analysis shows that an increase in ERK-specific phosphatase activity, by approximately 3.6-fold, is most consistent with all of the PDGF stimulation data.
Figure 6.8 Computational phase plots for the PDGF network. Black and red lines represent PDGF doses and iso-times (5, 15, 30, 60, and 120 minutes), respectively. The time arrow shows the increasing time for iso-times.
6.3. Discussion

Living cells continually interact with their surroundings and must therefore be described and modeled as open systems (Vonbertalanffy, 1950). The quantitative interpretations of these dynamic systems require combined experimental and computational analyses. Qualitative experiments are invaluable in constructing the interaction map within a cascade and/or multiple cascades, whereas quantitative kinetic experiments (dose- and time-dependent) provide additional information about how these networks are regulated in time and space. A key distinction between these two complementary approaches is that kinetic data requires, at least on some level, a mathematical model for interpretation (Kholodenko, 2006; Sauro & Kholodenko, 2004; Wiley, Shvartsman, & Lauffenburger, 2003). Here, the application of data-driven modeling is somewhat different; we sought to characterize and prioritize the mixed effects of a broad-based perturbation of the cell regulatory network, namely the inhibition of proteasomal degradation by MG-132.
In mouse fibroblasts, effects of proteasomal inhibition were manifest at the receptor level and in the activation of downstream signaling pathways. Although it is well understood that receptor tyrosine kinases are downregulated at least in part through direct ubiquitination (Bonita et al., 1997; Chiarugi et al., 2002; Dikic, 2003; Joazeiro et al., 1999; Monsonego-Ornan, Adar, Rom, & Yayon, 2002; Reddi et al., 2007), PDGF-stimulated receptor phosphorylation was reduced, not increased, in MG-132-treated cells in a PDGF dose-dependent manner (Fig. 6.4). This observation might be attributed to an increase in total protein tyrosine phosphatase levels (Chiarugi et al., 2002) and/or an increase in the expression of Sprouty (Spry), which has been shown to sequester the ubiquitin ligase Cbl (Dikic, 2003; Egan, Hall, Yatsula, & Bar-Sagi, 2002; Wong et al., 2002). Despite the dramatic reduction in receptor phosphorylation, our quantitative analysis indicated that the remaining receptor phosphorylation level would still be sufficient to maximally activate both PI3K and MAPK pathways. Therefore, it remains unexplained why Akt phosphorylation was reduced at both doses of PDGF or FGF stimulation (Fig. 6.3 and Fig. 6.4), although it is plausible to speculate that a serine-threonine phosphatase that acts upon Akt (e.g., PP2A) is upregulated in MG-132-treated cells. At a high dose of PDGF in our cells, PI3K-dependent signaling contributes significantly to the activation of the MEK/ERK pathway in parallel with the canonical, Ras-dependent pathway (Wang et al., 2009). Proteasome inhibition only reduced MEK phosphorylation at a high dose of PDGF or FGF, whereas ERK phosphorylation was reduced at both high and low doses of FGF and PDGF. It has been shown that DUSP levels are regulated by the proteasomal pathway (Jurek et al., 2009; Kucharska, Rushworth, Staples, Morrice, & Keyse, 2009; Lin et al., 2003; Lornejad-Schafer et al., 2005). Under maximal stimulation, the reduction in ERK phosphorylation in MG-132-treated cells must be attributed to a combination of increased DUSP levels and also increases in distinct protein activities that reduce the activation of MEK.

Despite the mixed and dose-dependent effects of proteasome inhibition, model-driven analysis revealed that a 3- to 4-fold increase in ERK dephosphorylation rates could be accommodated under both low and high stimulation conditions, i.e., once MEK phosphorylation had been taken into account. This constitutes a quantitative prediction that
we will seek to test in future studies and demonstrates the utility of quantitative modeling in parsing the complexities of pharmacodynamic outcomes.

**6.4. Materials and Methods**

**6.4.1 Reagents**

All tissue culture reagents were from Invitrogen (Carlsbad, CA). Human recombinant PDGF-BB was from Peprotech (Rocky Hill, NJ). Antibodies against total ERK1/2 and MKP3 and phospho-specific antibodies against Akt pSer\(^{473}\), ERK pThr\(^{202}/pTyr\(^{204}\), and MEK pSer\(^{217}/pSer\(^{221}\) were from Cell Signaling Technology (Beverly, MA); antibodies against total Akt1/2 and MKP1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Pharmacological inhibitor MG-132 was Sigma-Aldrich (St. Louis, MO); where applicable, cells were pre-incubated with the inhibitor for 30-480 minutes prior to PDGF stimulation. Unless otherwise noted, all other reagents were from Sigma-Aldrich.

**6.4.2 Cell culture**

NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, VA) were cultured at 37°C, 5% CO\(_2\) in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and the antibiotics penicillin and streptomycin.

**6.4.3 Lysate preparation and quantitative immunoblotting**

Cells were serum-starved for 4 hours prior to stimulation. Detergent lysates were prepared for quantitative immunoblotting, and immunoblots were performed using enhanced chemiluminescence, as described previously (Park, Schneider, & Haugh, 2003). Blots comparing lysates prepared on the same day, representing either different inhibitor treatments or different cell variants and respective control conditions, were performed in parallel and exposed at the same time. The BioRad Fluor S-Max system, which gives a linear response with respect to light output, was used, and band intensity was quantified using local background subtraction. Immunoblot data were first normalized by an appropriate loading
control and then further normalized to evaluate the consistency of relative trends across independent experiments, according to the procedures described in detail previously (Wang, Cirit, & Haugh, 2009).

6.4.4 Kinetic modeling and computational analysis: full model

The mathematical model and parameter ensemble used in this chapter are described in detail in Chapter 4. The parameter ensemble was generated by using the Metropolis algorithm and consisted of 43 fitted parameters and 10,000 sets of equally well fitted parameters. The phase plots were generated by calculating the output of each parameter set and taking the average values of 10,000 unique computational results. Here, instead of fitting data from MG-treated cells to mechanistic model, we semi-quantitatively analyzed the effects of drug treatment on PI3K and MAPK pathways. Then we implicitly fitted MEK data in order to investigate kinetic behaviors of ERK-specific phosphatases in NIH 3T3 fibroblasts.

6.4.5 Kinetic modeling and computational analysis: simplified model

For simplicity, we assumed that the entire single phosphorylated MEK was rapidly phosphorylated to ppMEK, i.e. pMEK ≈ 0 and \( y + y_{pp} = 1 \). A phenomenological, time-dependent function for MEK activation was formulated as follows.

\[
y_{pp}(t) = \frac{A_{ypp,0} + A_{ypp,1} e^{-k_1 t} - (A_{ypp,0} + A_{ypp,1}) e^{-k_2 t}}{1 + A_{ypp,0} + A_{ypp,1} e^{-k_1 t} - (A_{ypp,0} + A_{ypp,1}) e^{-k_2 t}}
\]  

(Eq. 1)

The dimensionless parameters \( A_{ypp,0} \) and \( A_{ypp,1} \) and the rate constants \( k_1 \) (min\(^{-1}\)) and \( k_2 \) (min\(^{-1}\)) were fit to the available data, with different values at each dose of PDGF-BB both for control and MG-treated cells (4 parameters*2 doses*2 treatments = 16 fit parameters). The fits are constrained so that these parameters are all positive, and \( k_1 < k_2 \). Roughly speaking, \( k_2 \) characterizes how quickly the system responds, i.e. through MEK kinase activity, and \( k_1 \) characterizes the adaptation of the response, e.g. through negative feedbacks to MEK kinase layer. The fitted kinetic parameter values and the fit to the data are shown in Table 6.2 and Fig. 6.10, respectively.
Table 6.2 Kinetic model parameter definitions and values, simplified MEK activation model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{ypp,0}$</td>
<td>1nM PDGF-BB, control</td>
<td>0.8999</td>
</tr>
<tr>
<td>$A_{ypp,1}$</td>
<td>1nM PDGF-BB, control</td>
<td>0.0007</td>
</tr>
<tr>
<td>$k_1$</td>
<td>0.1127 min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_2$</td>
<td>0.1228 min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$A_{ypp,0}$</td>
<td>30pM PDGF-BB, control</td>
<td>0.6922</td>
</tr>
<tr>
<td>$A_{ypp,1}$</td>
<td>30pM PDGF-BB, control</td>
<td>0.0008</td>
</tr>
<tr>
<td>$k_1$</td>
<td>0.1077 min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_2$</td>
<td>0.1152 min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$A_{ypp,0}$</td>
<td>1nM PDGF-BB, MG-132 pretreatment</td>
<td>0.0809</td>
</tr>
<tr>
<td>$A_{ypp,1}$</td>
<td>1nM PDGF-BB, MG-132 pretreatment</td>
<td>0.0193</td>
</tr>
<tr>
<td>$k_1$</td>
<td>0.0389 min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_2$</td>
<td>1.033 min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$A_{ypp,0}$</td>
<td>30pM PDGF-BB, MG-132 pretreatment</td>
<td>0.0336</td>
</tr>
<tr>
<td>$A_{ypp,1}$</td>
<td>30pM PDGF-BB, MG-132 pretreatment</td>
<td>0.0069</td>
</tr>
<tr>
<td>$k_1$</td>
<td>0.0581 min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_2$</td>
<td>0.241 min$^{-1}$</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6.10 Quasi-mechanistic model for direct comparison to phospho-MEK experimental data. The solid curves represent computational fit and circles are the experimental data. PDGF concentrations are: red, 1nM; black, 30pM.
For ERK (non-, mono-, and dual-phosphorylated fractions defined as \( z \), \( z_p \), and \( z_{pp} \), respectively), the conservation equations are the same as in the Chapter 4:

\[
\frac{dz}{dt} = -\frac{V_{max,y1}y_{pp} z_p}{K_{M,y1} + z_p} + \frac{V_{max,zph1} e_{ph} z_p}{K_{M,zph1}}; \quad z(0) = 1; \quad (Eq. 2)
\]
\[
\frac{dz_{pp}}{dt} = \frac{V_{max,y2}y_{pp} z_p}{K_{M,y2} + z_p} - \frac{V_{max,zph2} e_{ph} z_{pp}}{K_{M,zph2}}; \quad z_{pp}(0) = 0; \quad (Eq. 3)
\]
\[
z_p = 1 - z - z_{pp}. \quad (Eq. 4)
\]

The function \( e_{ph}(t) \) is the dimensionless expression of MAPK phosphatase/dual specificity phosphatase (MKP/DUSP) activity. Based on our conclusion in Chapter 4, in quiescent NIH 3T3 fibroblasts we assume that \( e_{ph} \) is constant and equal to 1. In MG-treated cells, we also assume that \( e_{ph} \) is constant; however, higher than that of control. Therefore, for MG-132 treatment, we define a new parameter \( C_{eph} \), a coefficient that represents the fold increase in MAPK phosphatase levels due to MG-132 treatment, namely for MG treatment \( e_{ph,MG} = C_{eph} \times e_{ph} \), where \( e_{ph} = 1 \). Using 12 fixed parameters for MEK activation, we fitted ERK data by changing \( V_{max,y1}, K_{M,y1}, V_{max,y2}, K_{M,y2}, V_{max,zph1}, K_{M,zph1}, V_{max,zph1}, K_{M,zph1}, \) and \( C_{eph} \), where \( C_{eph} > 1 \) (9 parameters). The statistical analysis of the parameter ensemble (\( n = 10,000 \)) is given in Table 6.3.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Minimum</th>
<th>Lower Quartile</th>
<th>Median</th>
<th>Upper Quartile</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max,1}}/K_{M,\text{11}}$</td>
<td>Catalytic efficiency, ERK $\rightarrow$ pERK</td>
<td>276 min$^{-1}$</td>
<td>789 min$^{-1}$</td>
<td>1119 min$^{-1}$</td>
<td>1582 min$^{-1}$</td>
<td>33.5e3 min$^{-1}$</td>
</tr>
<tr>
<td>$K_{M,\text{11}}$</td>
<td>Michaelis constant, ERK $\rightarrow$ pERK</td>
<td>0.795</td>
<td>4.51</td>
<td>5.39</td>
<td>7.73</td>
<td>10.2</td>
</tr>
<tr>
<td>$V_{\text{max,ph1}}/K_{M,\text{ph1}}$</td>
<td>Catalytic efficiency, pERK $\rightarrow$ ERK</td>
<td>0.210 min$^{-1}$</td>
<td>0.317 min$^{-1}$</td>
<td>0.365 min$^{-1}$</td>
<td>0.426 min$^{-1}$</td>
<td>0.716 min$^{-1}$</td>
</tr>
<tr>
<td>$K_{M,\text{ph1}}$</td>
<td>Michaelis constant, pERK $\rightarrow$ ERK</td>
<td>2.22</td>
<td>9.28</td>
<td>11.8</td>
<td>14.8</td>
<td>21.5</td>
</tr>
<tr>
<td>$V_{\text{vph1}}/K_{M,\text{vph1}}$</td>
<td>Catalytic efficiency, pERK $\rightarrow$ ppERK</td>
<td>7.2e3 min$^{-1}$</td>
<td>1.9e4 min$^{-1}$</td>
<td>2.4e4 min$^{-1}$</td>
<td>3.3e4 min$^{-1}$</td>
<td>6.8e4 min$^{-1}$</td>
</tr>
<tr>
<td>$K_{M,\text{vph1}}$</td>
<td>Michaelis constant, pERK $\rightarrow$ ppERK</td>
<td>7.5 e-4</td>
<td>1.5 e-3</td>
<td>2.2 e-3</td>
<td>5.8 e-3</td>
<td>7.2 e-3</td>
</tr>
<tr>
<td>$V_{\text{vph1}}/K_{M,\text{vph1}}$</td>
<td>Catalytic efficiency, ppERK $\rightarrow$ pERK</td>
<td>3.00 min$^{-1}$</td>
<td>6.13 min$^{-1}$</td>
<td>11.6 min$^{-1}$</td>
<td>16.6 min$^{-1}$</td>
<td>24.2 min$^{-1}$</td>
</tr>
<tr>
<td>$K_{M,\text{vph1}}$</td>
<td>Michaelis constant, ppERK $\rightarrow$ pERK</td>
<td>4.11</td>
<td>38.1</td>
<td>60.7</td>
<td>82.4</td>
<td>157</td>
</tr>
<tr>
<td>$C_{\text{ph}}$</td>
<td>Fold increase in total ERK phosphatase (MG treatment)</td>
<td>2.84</td>
<td>3.51</td>
<td>3.61</td>
<td>3.71</td>
<td>4.11</td>
</tr>
</tbody>
</table>

Table 6.3 Kinetic model parameters, MEK/ERK module. Highlighted values are deemed arbitrarily high (yellow) and arbitrarily low (cyan). $C_{\text{ph}}$ was constrained to be $\geq 1$. 
6.5 References


expression in h4iie rat hepatoma cells. *Cellular Physiology and Biochemistry*, 16(4-6), 193-206.


