ABSTRACT

RAHMAN, ANISUR. Quantitative Analysis of the Akt/mTOR Signaling Network. (Under the direction of Dr. Jason Haugh).

Cell signaling is a highly complex and conserved process that controls many biological responses, such as cell growth, survival, proliferation and apoptosis. Cells use multiple signaling pathways to regulate their activities, some of which remain poorly defined. Activation of cell signaling is mediated by cell surface receptors that recognize growth factors present in the external environment. Many diseases, including cancers, are caused by defects in these signaling pathways. The mechanistic target of rapamycin (mTOR) pathway is an important mediator of cell growth, cell proliferation, cell survival and protein synthesis, and dysregulation of the components of mTOR pathway contributes to progression of about 50% of all cancers. Therefore, it is of utmost importance to understand how mTOR signaling is regulated. Yet, mTOR regulation remains poorly understood, especially from the standpoint of quantitative analysis. In this study, a systems biology approach with tight coupling between mathematical modeling and biological experiments was applied to gain mechanistic insights about mTOR activation elicited by growth factor stimulation. Many studies have separately implicated the PI3K/Akt and Ras/Raf/MEK/ERK pathways upstream of mTOR complex 1 (mTORC1), but no significant work has been done to quantify the relative contributions of these two pathways. To study these, we used NIH3T3 mouse fibroblasts stimulated with platelet-derived growth factor (PDGF) as our experimental model system and found that the PI3K/Akt pathway plays the dominant role in mTORC1 activation. Focusing on the Akt/mTORC1 signaling axis, we collected detailed kinetic data quantifying phosphorylation of the key signaling proteins (Akt, TSC2, PRAS40 and S6K1). In parallel, simple mechanistic models of the pathway were built based on mass-action kinetics. The models were constrained
by the kinetic data, using an ensemble-based fitting approach (Monte Carlo routine based on Metropolis algorithm) that showed a high level of consistency. Then, the conceptual model was used to generate \textit{a priori} predictions of protein depletion experiments, which showed a semi-quantitative match. The model prediction also indicated the importance of both TSC2- and PRAS40-dependent regulation of mTORC1.

Analysis of the model equations describing the Akt/mTORC1 pathway revealed an indirect activation mechanism constituting a new regulatory motif: deactivation of a negative regulator. Activated Akt activates mTOR by phosphorylating and thus neutralizing TSC2 and PRAS40, which are negative regulators of mTORC1. The indirect mechanism yields a broader range of sensitivity to the input, beyond saturation of regulator phosphorylation, and kinetics that become progressively slower, not faster, with increasing input strength. Another important prediction from the model analysis concerns the status of the mTORC1 complex and suggests how two arms of the pathway, working in a feedforward manner, yield ultrasensitive mTORC1 activation.
Quantitative Analysis of the Akt/mTOR Signaling Network

by
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DEDICATION

To my parents and my wife.
BIOGRAPHY

Anisur Rahman was born in Bangladesh, a south Asian country and raised in Gazipur, a small city located only 25 miles north of the capital Dhaka. He is the eldest son of his parents. He was always interested to read science articles, books, news articles from his childhood. After completing his secondary and higher secondary education, he got admitted to the best engineering university of the country, Bangladesh University of Engineering and Technology (BUET), in Chemical Engineering undergraduate program. He was the top student of his class and as a token of that he received Dr. Ali Karim gold medal and offered a job to work as a lecturer in the same institution. After successfully working for 3 years as a lecturer, he then promoted to assistant professor. He also earned his master’s degree during that time period. The desire to acquire more knowledge brought him to pursue his PhD at North Carolina State University in 2011. He then started working as a research assistant with Dr. Jason Haugh to study cellular signaling pathways in mammalian cells. He has strong desire to continue his research work on cell signaling, computational biology and systems biology.
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I would like to express my sincere thanks to Dr. Jason Haugh (PI) for giving me the opportunity to work in his lab and proposing the research topic. I also would like to express my profound respect to him for his valuable guidance and supervision throughout the entire work. It would not be possible to complete this mammoth task without his cooperation.

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# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................... viii

LIST OF FIGURES ......................................................................................................... ix

CHAPTER 1 Cell signaling, signaling pathways and their role in cancer .................... 1
  1.1 Cell signaling and cancer .................................................................................... 2
  1.2 Ras/Raf/MEK/ERK pathway ............................................................................. 4
  1.3 PI3K/Akt pathway ............................................................................................. 6
  1.4 mTOR pathway .................................................................................................. 8
    1.4.1 mTOR complexes ....................................................................................... 9
    1.4.2 mTORC regulation .................................................................................. 10
    1.4.3 mTORC1 substrates .................................................................................. 14
  1.5 Akt substrates other than TSC2 ....................................................................... 15
  1.6 Mathematical modeling in signal transduction networks .................................. 17
    1.6.1 Why mathematical models are important for studying biological systems 18
    1.6.2 Linear vs. nonlinear and deterministic vs. stochastic models ................. 19
  1.7 Thesis overview ................................................................................................. 21

References .................................................................................................................... 22

CHAPTER 2 Deactivation of a negative regulator: a distinct signal transduction mechanism, pronounced in Akt signaling .................................................. 33
  2.1 Introduction ........................................................................................................ 35
  2.2 Analyses of the two mechanisms ...................................................................... 36
  2.3 Prominence of the mechanism in Akt- and mTOR-mediated signaling ........... 45
  2.4 Conclusions ....................................................................................................... 46

References .................................................................................................................... 47

CHAPTER 3 Pathway specificity of mTOR activation and a consistent kinetic model of the Akt/mTOR pathway ................................................................. 48
  3.1 Introduction ....................................................................................................... 50
  3.2 Materials and Methods ..................................................................................... 52
    3.2.1 Cell culture, inhibitor treatment, growth factor stimulation, and lysate preparation ........................................................................................................ 52
    3.2.2 SDS-PAGE and Western blotting ............................................................... 53
    3.2.3 Data normalization ..................................................................................... 54
3.2.4 Antibodies and other reagents ........................................................................ 55
3.3 Results .................................................................................................................... 56
  3.3.1 PI3K/Akt is the dominant pathway of TSC2 phosphorylation and mTOR activation ............................................................... 56
  3.3.2 Kinetics of mTOR activation and Akt, TSC2 and PRAS40 phosphorylations ...................................................................... 59
3.4 A consistent kinetic model of the Akt/mTOR pathway ........................................ 63
  3.4.1 Model Structure ................................................................................................. 63
  3.4.2 Formulation of the kinetic equations ................................................................ 64
  3.4.3 Acquisition of model parameter sets ensemble ................................................ 68
3.5 Model output ......................................................................................................... 71
3.6 Discussion ............................................................................................................ 75
References .................................................................................................................. 77

CHAPTER 4 mTOR activation dynamics controlled by deactivation of negative regulator motif and Rheb- & PRAS40-mediated seesaw mechanism ......................... 80
  4.1 Introduction ............................................................................................................ 82
  4.2 Materials and Methods ......................................................................................... 84
    4.2.1 Plasmids and lentiviral infections .................................................................. 84
    4.2.2 Predictions based on shRNA-mediated depletion of regulatory proteins .. 85
    4.2.3 Steady-state analysis based on fixed Akt activity .......................................... 86
  4.3 Results ................................................................................................................... 86
    4.3.1 Both Akt/TSC2/Rheb and Akt/PRAS40 substantially regulate mTORC1 86
    4.3.2 Analysis of the model reveals neutralization of negative regulators and seesaw regulation as dynamical motifs of the Akt/mTORC1 signaling axis .......... 88
  4.4 Discussion .......................................................................................................... 94
References .................................................................................................................. 97

CHAPTER 5 Conclusions and future work .................................................................. 99
  5.1 Conclusions .......................................................................................................... 100
  5.2 Future work ......................................................................................................... 101
    5.2.1 Analysis of mTORC1 binding states, guided by model predictions ....... 101
    5.2.2 Elucidate mTORC1 signaling dynamics in dysfunctional cell context .... 101
    5.2.3 Introducing Ras/Raf/MEK/ERK pathway and mTORC2 in the model system ........................................................................ 102
    5.2.4 mTORC1 signaling to cell functional response ........................................... 102
5.2.5 Role of S6K1 negative feedback to IRS-1 .................................................. 104

5.2.6 Evaluate the generality of activation by deactivation of negative regulator motif amongst other Akt substrates ................................................................................ 104

References ................................................................................................................ 106

APPENDICES ........................................................................................................... 108

Appendix A: Development of the models for two mechanisms (chapter-2)........... 109
Appendix B: Variations of the model tested (chapter-3) ......................................... 116
Appendix C: Mathematical analysis of mTORC1 regulation by PRAS40 and Rheb-GTP: Ultrasensitivity (chapter-4) ............................................................... 118
LIST OF TABLES

Table 3.1  Statistics of the parameter set ensemble for the main model of the Akt/mTORC1 signaling axis. All parameters labeled as rate constants have units of min\(^{-1}\); all others are dimensionless. For each parameter, the minimum (Min), first quartile (Q1), median, third quartile (Q3), and maximum (Max) of the 10,000 values in the ensemble are listed........74

Table 3A1  Model comparisons. Cumulative SSD values for each readout (ensemble mean ± SD) and the Akaike Information Criterion (AIC) value is listed for each model. The formula for AIC is \( AIC = N [1 + \ln(2\pi) + \ln(cSSD/N)] + 2k \), where \( N \) is the number of observations and \( k \) is the number of parameters........................................117
LIST OF FIGURES

Figure 1.1  A simple intracellular signaling pathway activated by an extracellular signaling molecule (Albers et al., 2007) .................................................. 3

Figure 1.2  Receptor Tyrosine Kinases (RTKs) mediated activation of Ras/Raf/MEK/ERK pathway (Engelman, 2009) ................................. 6

Figure 1.3  Regulation of PI3K/Akt pathway (Osaki et al., 2004) ......................... 7

Figure 1.4  Components of mTORC1 and mTORC2 (Laplante and Sabatini, 2009) 9

Figure 1.5  mTOR pathway at a glance (Laplante and Sabatini, 2009) ............... 12

Figure 1.6  Possible mechanism of regulation of mTORC1 activation by TSC1/2 and Rheb (Huang and Manning, 2008) ......................... 13

Figure 1.7  Downstream substrates regulated by PI3K/Akt pathway (Engelman, 2009) ...................................................................................... 16

Figure 1.8  Two types of nonlinear saturation a) Hyperbolic b) Sigmoidal ........... 20

Figure 2.1  Multiple example of the indirect activation mechanism downstream of Akt. In each example, the kinase activity of Akt is considered the input, and its substrate is a negative regulator (deactivator) .......................... 36

Figure 2.2  Steady-state properties of mechanisms I and II. a) Schematics of direct (I) and indirect (II) activation. b) Steady-state dose responses, $\rho_{ss}(s)$, of I (blue) and II (red) along with phosphorylation of the upstream regulator, $\phi_{ss}(s)$ (Eq. 1 at steady state); $K = 0.05$, $g = 100$. c) Same as b except with a sigmoidal $\phi_{ss}(s)$ (Hill function with $n = 4$). d) Steady-state output, $\rho_{ss}$, of I vs. $\phi_{ss}$ for $K = 0.05$ and indicated values of the gain constant, $g$. e) Same as d but for II ......................................................... 39

Figure 2.3  Illustration of the steady-state sensitivities of mechanisms I and II as phosphorylation of the regulator approaches saturation. In both cases, progressive increases in the input signal eventually result in phosphorylation of most of the regulator (indicated by –P) at steady state. In the case of mechanism I, the regulator has a positive influence on the downstream response, and the phosphorylated form is more active. Near saturation, a further increase in the input results in only a slight fractional gain in the activity of the positive regulator. Conversely, in the case of mechanism II, the regulator has a negative influence on the downstream response, and the phosphorylated form is less active. Near saturation, a further increase in the input results in a dramatic fractional change (reduction) in the remaining activity of the negative regulator .......... 40
Figure 2.4  Kinetic properties of mechanisms I and II.  
a) Response of I to a step change in \( s \) from zero to the indicated \( s(0) \). Time is given in units of \( k_p t \); parameters are \( K = 0.05, \ g = 10, \ k_{d,0} = 0.1 k_p \).  
b) Same as a but for II.  
c) Same as a but for a transient input, \( s(t) = s(0) \exp(-0.03 k_p t) \).  
d) Same as c but for II.  

Figure 2.5  Identification of activation mechanism I (green) and II (red) in the Akt/mTOR signaling network.  

Figure 2.6  Serial and parallel schemes incorporating mechanism I or/and II.  
a) Steady-state outputs of two response elements, \( \rho_1 \) and \( \rho_2 \), activated by I in series. At each level, \( K = 0.05, \ g = 100 \).  
b) Same as a but for II in series.  
c) Incoherent feedforward loop (FFL) in which I and II are activated in parallel to activate and inhibit, respectively, the terminal output. For both I and II, \( K = 0.05, \ g = 100 \). The parameters for Eq. 5 are \( \alpha = 2.5, \ \beta = 50 \).  

d) Same as c but for II.  

Figure 2.7  Temporal response of an incoherent feedforward motif with direct activation of an activator by mechanism I and indirect activation of an inhibitor by mechanism II. Parameter values are the same as in figure 2.6c, and \( k_{d,0} = 0.1 k_p \) for both I and II. Values of the input, \( s \), are as indicated for each curve, and time is expressed in units of \( k_p t \). Note that high values of \( s \) yield an adaptive response due to the inherent disparity in time scales for I and II.  

Figure 2.8  Analysis of a coherent feedforward loop (FFL) with direct and indirect activation of two activators in parallel.  
a) Hypothetical circuit diagram.  
b) Signaling in the mTOR network that loosely maps to the hypothetical circuit.  
c) Steady-state response of the coherent FFL, assuming additive contributions to the output, according to  
\[
\text{Output} = \frac{(a \rho_I + \beta \rho_{II})}{1 + a \rho_I + \beta \rho_{II}} 
\]

Parameter values are \( K = 0.05 \) and \( g = 100 \) for both I and II; the parameters for the output function are \( \alpha = 0.5, \ \beta = 1 \). Note that the output shows a broad sensitivity, over several logs of \( s \), due to the disparity between the saturation of I and II.  
d) Kinetics of the system, with the same parameter values as in c and \( k_{d,0} = 0.1 k_p \) for both I and II. Values of the input, \( s \), are as indicated for each curve, and time is expressed in units of \( k_p t \). Note that high values of \( s \) provoke an initial rapid increase due to activation of I, followed by slower increase due to delayed activation of II.  

Figure 3.1  The mTORC1 signaling network.  

Figure 3.2  PI3K/Akt is the dominant mode of mTORC1 activation. NIH3T3 cells were pretreated with either DMSO or 10µM U0126, 10µM Akt inhibitor VIII and 100nM rapamycin for 40 minutes then either left unstimulated or stimulated with 1nM PDGF for 15 minutes. Phosphorylation of Akt,
ERK, MEK, TSC2 and S6K1, along with corresponding total proteins, were measured by immunoblotting.

Figure 3.3 Inhibition of Akt ablates PDGF-stimulated phosphorylation of PRAS40 Thr246. NIH3T3 cells were pretreated with either DMSO or 10µM Akt inhibitor VIII for 40 minutes then either left unstimulated or stimulated with 1nM PDGF for 15 minutes. Phosphorylation of Akt and of PRAS40, along with total proteins, were measured by immunoblotting, confirming that phosphorylation of PRAS40 Thr246 is Akt-dependent.

Figure 3.4 PDGF dose and time response on phosphorylation of Akt, TSC2, PRAS40 and S6K1 for NIH3T3 cells.

Figure 3.5 Normalized Akt, TSC2, S6K1 and PRAS40 phosphorylation time courses for 0.01, 0.03, 0.3 and 1 nM PDGF. The data are normalized by loading controls and then by mean value of the 1nM time course for each phosphor-protein. The normalized data are reported as mean ± SE (n=5 independent experiments).

Figure 3.6 Rapamycin treatment does not affect upstream signaling, indicating a lack of mTORC1-dependent feedback. Immunoblots showing PDGF-stimulated phosphorylation of various proteins in cells pretreated with 0.2% DMSO (control) or 100nM rapamycin. NIH 3T3 cells were incubated with either DMSO or rapamycin for 40 minutes and then either left unstimulated or stimulated with PDGF for various doses and durations as indicated. Right: quantification of Akt phosphorylation (dashed lines: DMSO; solid lines: rapamycin) for n=1 experiment.

Figure 3.7 Schematic of the kinetic model.

Figure 3.8 Identification of a large ensemble of parameter sets that fit the data near optimally. A) Block flow diagram of the Monte Carlo algorithm used to generate and choose the parameter sets. B) cSSD values (orange) for each accepted parameter set, in the order accepted. Those with cSSD values below the green line were the best 10,000 parameter sets, constituting the ensemble.

Figure 3.9 Ensemble averaged model output fit to the kinetic data. Each quadrant shows the fit for a different dose of PDGF as indicated; note that y-axes are scaled differently to allow the quality of fit to be more fully assessed. The ensemble averaged (mean) model output (green curves) may be compared to the experimental data (red circles). Black dashed lines indicate mean ± SD for the model output, indicating the level of variation in the fit.

Figure 3.10 Distributions of parameter values across the ensemble. A) For each parameter of the model, the distribution given in Table 3.1 is plotted as a box (1st and third quartiles, with the red line marking the median value).
and whiskers (minimum and maximum). All rate constants have units of \text{min}^{-1}; all other parameters are dimensionless.

B) Same as A, except the parameters are sorted according to the ratio of the third quartile to the first quartile (low to high). A smaller ratio indicates a more tightly constrained parameter.

Figure 4.1 mTORC1 regulation by Akt-mediated feedforward loop with two arms: a) TSC2-Rheb and b) PRAS40.

Figure 4.2 Perturbation of the mTORC1 regulation. A) Stable shRNA-expressing cell lines were established to perturb key regulatory proteins in the Akt/mTORC1 axis: TSC2, PRAS40, and Rheb. For each target, two hairpins targeting different sequences were selected, based on the extent of protein knockdown (% KD, as indicated), along with a non-targeting control line (shNEG). Representative immunoblots (left) and quantification (right) of target protein knockdown and S6K1 phosphorylation for the indicated PDGF stimulation conditions are shown. Phospho-S6K1 data are normalized by total S6K1 and then by the mean value of each shNEG time course. The normalized data are reported as mean ± SE (n = 3 independent experiments). B) The corresponding model predictions are the means of the model ensemble (n = 10,000) for each condition, normalized in the same manner as the data.

Figure 4.3 Neutralization of negative regulators. Ensemble predictions of the model at steady state (mean ± SD, n = 10,000) show that phosphorylation of the negative regulators TSC2 and PRAS40 approach saturation at low stoichiometries of Akt activation. This offers maximal sensitivity of Rheb-GTP loading and mTORC1 activation, because TSC2 and PRAS40 regulate those responses in their unphosphorylated states.

Figure 4.4 Seesaw regulation of mTORC1. A) Steady-state analysis of the model predicts the status of the mTORC1 complex (mean ± SD, n = 10,000), with a transition from largely PRAS-inhibited and largely Rheb-induced states. As shown for 10 randomly selected parameter sets (right), this mode of enzyme regulation encodes an ultrasensitive, nonlinear input-output relationship over the relevant range of Akt activity (i.e., with Akt activity scaled as it was in the alignment to phospho-Akt data, where a value of 1 corresponds to the mean of the 1 nM PDGF time course). B) Depiction of the seesaw mechanism and mathematical expression of mTOR sensitivity to Akt.

Figure 4.5 Predicted effects of regulatory protein depletion on the status of the mTORC1 complex. The results for the control (ensemble mean ± SD) are from figure 4.4A. In the other panels, results for the ensemble mean of the control are reprised (dashed curves) for comparison to that of 80% knockdown (KD) of TSC2, PRAS40, or Rheb (solid curves). As a measure of pathway potency, the bar plot at right compares the Akt
activity required to achieve 50% of mTORC1 in the active, Rheb-induced state for the control and knockdown conditions .................................................. 91

Figure 4.6  Detailed view of the ensemble fit to kinetic data, with the parameter constraints $\alpha = \beta = 1$. Each quadrant shows the fit for a different dose of PDGF as indicated; note that y-axes are scaled differently to allow the quality of fit to be more fully assessed. The ensemble averaged (mean) model output (green curves) may be compared to the experimental data (red circles). Black dashed lines indicate mean ± SD for the model output, indicating the level of variation in the fit. The quality of fit may be visually compared to that shown in figure 4.3, and numerical comparisons are given in Table 3A1 .................................................................................................. 92

Figure 4.7  Model predictions with the parameter constraints $\alpha = \beta = 1$. The experimental data are reprised from figure 4.2A. The corresponding model predictions, with the parameters $\alpha$ and $\beta$ set equal to 1, are the means of the model ensemble (n = 10,000) for each condition, normalized in the same manner as the data ........................................................................ 94

Figure 5.1  mTORC1 control of protein synthesis, lipid synthesis and autophagy via S6K1, 4E-BP1, SREBP1 and ULK1/ATG13 ......................................................... 103
CHAPTER 1

Cell signaling, signaling pathways and their role in cancer
1.1 Cell signaling and cancer

Cell signaling is a process by which cells respond to their surrounding environments, controlling basic cellular activities as well as cell fate through a complex system of intracellular reactions (Alberts et al., 2007). Cells respond to signals in the extracellular milieu or associated with other cells through surface receptors and three major classes of these receptors are G-protein-coupled receptors, enzyme-coupled receptors and ion-channel-couple receptors (Blume-Jensen and Hunter, 2001; Dibb et al., 2004; Eswarakumar et al., 2005; Lemmon and Schlessinger, 2010). The number of different biological signals is large and different cells respond differently to different signals. Chemical factors (ligands) that bind receptors include insulin and growth factors such as isoforms of platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin like growth factor (IGF), and fibroblast growth factor (FGF) (Ravandi et al., 2003). Upon binding of these ligands, the receptors may undergo different chemical or structural changes. Some receptors form dimers or tetramers (Clayton et al., 2005), some receptors undergo autophosphorylation (Cobb et al., 1989), whereas others may show a conformational change (Favelyukis et al., 2001). These changes in receptor state allow intracellular proteins to be recruited to the plasma membrane, and some of these are phosphorylated while in complex with the activated receptors (Bae et al., 2009; Reynolds et al., 2003). These receptor-proximal signaling proteins can initiate a series of modifications involving other intracellular protein and lipid molecules. Intracellular proteins can either activated or deactivated by phosphorylation. Among these intracellular signals, some signal leads to the nuclear localization and protein translations; others may end up at the cytoplasm.
As shown in figure 1.1, the extracellular ligand usually binds to a receptor protein embedded in the plasma membrane of the target cell. The receptor protein usually has extracellular, transmembrane, and intracellular domains. Binding of the ligand to the extracellular domain of the receptor activates one or more intracellular signaling pathways involving a host of signaling proteins. Finally, one or more of the intracellular signaling proteins alters the activities of effector proteins and thereby the behavior of the cell (Alberts et al., 2007).

Figure 1.1: A simple intracellular signaling pathway activated by an extracellular signaling molecule (Alberts et al., 2007)
Stimulation with a single growth factor can elicit activation of several downstream pathways (McCubrey et al., 2000), with points of intersection (crosstalk) among the pathways. In a particular pathway, there may be feedback loops in which a downstream protein imposes either a positive or negative effect on the upstream pathway (Amit et al., 2007). Dysregulation of these complex signaling pathways plays a prominent role in cancer. Cancer is widely considered a genetic disease, and the ability of the cancer cells to adapt to different microenvironments is encoded in the structure and spatiotemporal dynamics of signal transduction networks (Choudhary et al., 2009; Kolch et al., 2015; Ladbury and Arolf, 2012; Perkins, 2007). Most cancers arise as a result of genetic mutations of cellular genes, which cause constitutive activation of oncogenes or inactivation of tumor suppressor genes. Genetic mutations can result from DNA damage or replication errors. Oncogenic mutations can manifest as dysregulated signaling pathways or networks that behave abnormally and promote uncontrolled cell growth, or neoplasia (Sever and Brugge, 2015). Currently, signal transduction pathways are being targeted for the next generation of cancer therapies (Shimizu and Nakagawa, 2015).

Understanding the complexity of signaling networks is a challenging goal at the forefront of cell signaling research, yet some cell signaling pathways are relatively well characterized. Among the best known are the Ras/Raf/MEK/ERK and PI3K/Akt pathways.

1.2 Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK or extracellular signal regulated kinase (ERK)/MAPK pathway is perhaps the most studied and most important pathway characterized to date. This pathway is activated by most cell surface receptors and is a master regulator of cell cycle progression, cell adhesion and migration, cell survival, differentiation, metabolism, and proliferation.
Deregulation of the MAPK pathway leads to certain cancers and other human diseases (Peyssonnaux and Eychene, 2001; Roberts and Der, 2007).

Figure 1.2 is a simple diagram of the Ras/Raf/MEK/ERK pathway as activated by receptor tyrosine kinases (RTKs). Upon activation of the receptors by ligands (growth factors) binding, the adaptor proteins Shc and Grb2 are recruited to the plasma membrane, in complex with the guanine nucleotide exchange factor, Sos. Activated Sos converts GDP-bound Ras to GTP-bound Ras (Ras-GTP) by inducing a transient conformational change in Ras (Mandal et al., 2016). Ras-GTP binds and recruits the serine/threonine kinase Raf to the plasma membrane and Raf gets activated by homodimerization or heterodimerization. Activated Raf then activates dual specificity kinase MEK1/2 by phosphorylating on two serine residues 217 and 221. Active MEK1 and MEK2 activate ERK by phosphorylation on activation loop residues T202/Y204 and T185/Y187 (Chin and D'Mello, 2005; McCubrey et al., 2007; Shaul and Seger, 2007). The components of the ERK pathway have isoforms. Raf includes A-Raf, B-Raf and C-Raf. MEK includes MEK1 and MEK2 (Roskoski, 2012) which differentially control the duration and amplitude of the ERK cascade response (Kocieniewski and Lipniacki, 2013). Similarly, ERK family has ERK1 and ERK2 (Roskoski, 2012). Activated ERK phosphorylates more than 150 substrates, with about half of them located in nucleus and half of them in the cytoplasm (Cirit and Haugh, 2012). Activated ERK shuttles between the cytosol and nucleus (Ahmed et al., 2014; Chuderland et al., 2008). MEK1 is subject to negative feedback from activated ERK, which is further conferred to MEK2 by hetero-dimerization (Kocieniewski and Lipniacki, 2013). In the negative feedback loop, ERK phosphorylates MEK1 at threonine 292. Some components of the Raf/MEK/ERK pathway are mutated or aberrantly expressed in human cancer (Chang et al., 2003; Roberts and Der, 2007). Several types of kinase inhibitors
are used to block signaling through this pathway, of which the frequently used are MEK inhibitors (Friday and Adjei, 2008). MEK inhibitors are widely used in research and therapeutics to study the role of this pathway in cancer.

![Figure 1.2: Receptor Tyrosine Kinases (RTKs) mediated activation of Ras/Raf/MEK/ERK pathway (Engelman, 2009)](image)

1.3 PI3K/Akt pathway

PI3K-Akt pathway is important for many aspects of cell growth, proliferation and survival. In human cancer this pathway is targeted by genomic aberration more frequently than any other pathway (Guo et al., 2015). Genomic aberrations include mutation, amplification and rearrangement. Deregulated PI3K/Akt pathway affects transcription and translation inside the
cell (Bader et al., 2005). It has been reported that PI3K signaling is linked to many forms of human cancer including breast cancer, lung cancer, melanoma and leukemia (Osaki et al., 2004). The PI3K/Akt pathway is stimulated by many growth factors and regulators.

![Figure 1.3: Regulation of PI3K/Akt pathway (Osaki et al., 2004)](image)

Figure 1.3 shows how the PI3K/Akt pathway is activated downstream of RTKs. PI3K is a heterodimeric complex of 85 and 110 kDa subunits. Tyrosine-phosphorylated receptors recruit PI3K to the plasma membrane by binding to the SH2 domain of the PI3K regulatory subunit, p85. Thus, the heterodimer is targeted to the membrane (Vara et al., 2004). The interaction between these two subunits is necessary for p110 enzymatic activity. The regulatory subunit p85 inhibits the activity of p110 until p85 binds to a cognate phospho-tyrosine motif (Hennessy et al., 2005; Luo et al., 2003). At the inner leaflet of the plasma membrane, PI3K
phosphorylates the glycerophospholipid phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P₂) at the 3-hydroxyl group of the inositol ring, converting it to PtdIns(3,4,5)P₃ (Cantrell, 2001). Phosphatases such as phosphatase and tensin homolog deleted in chromosome 10 (PTEN) dephosphorylate PtdIns(3,4,5)P₃ to regenerate PtdIns(4,5)P₂. Another phosphatase, SH2-domain-containing 5-inositol phosphatase (SHIP), hydrolyzes PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂ (Lam et al., 2012). However, PtdIns(4,5)P₂ is the more abundant form of PIP₂ in mammalian cells (Insall and Weiner, 2001). PtdIns(3,4,5)P₃ recruits and activates signaling proteins containing a pleckstrin homology (PH) domain such as the serine-threonine protein kinase, Akt, which regulates many of the downstream events controlled by PI3K (Calleja et al., 2009). Each of the three isoforms of Akt (Akt1, Akt2 and Akt3) has an N-terminal PH domain and a C-terminal kinase catalytic domain (Lawlor and Alessi, 2001). Following the activation of PI3K, Akt is recruited to the membrane, where it is phosphorylated on the residue T308. The full activation of Akt requires phosphorylation at site S473 (Laplante and Sabatini, 2009; Park et al., 2003; Toschi et al., 2009). Activated Akt influences diverse cellular processes, including cell survival, cell growth, metabolism, tumorigenesis and metastasis by phosphorylating a wide array of downstream substrate proteins. The PI3K/Akt pathway has been a prominent target for cancer therapy, and inhibitors of this pathway have been developed (Hennessy et al., 2005; Porta et al., 2014).

1.4 mTOR pathway

Downstream of both the Ras/Raf/MEK/ERK and PI3K/Akt pathways is mechanistic target of rapamycin (mTOR), a 289 kDa serine-threonine protein kinase that belongs to the PI3K-related protein kinase family. Its ortholog TOR was discovered when studying rapamycin-resistant mutants of the common yeast, Saccharomyces cerevisiae (Hall, 2016; Heitman, 2015;
Seto, 2012). In mammalian cells, mTOR is a central regulator of cell growth, cell metabolism, proliferation and survival. Deregulation of mTOR has been implicated in progression of 50% of all types of cancers (Guertin and Sabatini, 2007; Zoncu et al., 2011). Together, the Akt and mTOR signaling axis is frequently altered in ovarian tumors (Dobbin and Landen, 2013). This pathway is considered a promising therapeutic target for many cancers including those of the cervix, prostate, and others (Chang et al., 2015). It has been discovered recently that Zika viruses deregulate Akt-mTOR signaling in human fetal neural stem cells to inhibit neurogenesis and induce autophagy (Liang et al., 2016).

1.4.1 mTOR complexes

In mammalian cells, mTOR is present in two multi-protein complexes: mTOR complexes 1 (mTORC1) and 2 (mTORC2) (figure 1.4).

mTORC1 consists of five proteins: mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8), proline-rich Akt substrate 40 kDa (PRAS40) and DEP-domain-containing mTOR interacting protein (Deptor). mTOR is the catalytic subunit of mTORC1. Deptor and PRAS40 negatively regulate mTORC1 activity (Peterson et al., 2009; Wang et al., 2007). Raptor regulates assembly of the complex and
recruits substrates to mTORC1 (Hara et al., 2002; Kim et al., 2002). The function of mLST8 in the complex is still unclear, but it has been shown that depletion of mLST8 had no effect on normal cell growth, whereas overexpression enhanced cell growth; mLST8 is also highly expressed in human colon and prostate cancer cell lines and tissues (Kakumoto et al., 2015). When mTORC1 is activated, it directly phosphorylates Depto and PRAS40, causing release of these components from the complex, resulting in further activation of mTORC1.

mTORC2 has six components, several of which are in common with mTORC1. The components are mTOR, Depto, mLST8, rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein 1 (mSIN1) and protein observed with Rictor-1 (Protor-1). Rictor and mSIN1 stabilize each other and are the structural foundation of mTORC2 (Frias et al., 2006; Jacinto et al., 2006). Rictor also interacts with Protor-1, but this interaction is not well understood. Depto negatively regulates the activity of mTORC2. mLST8 is required for mTORC2 function, but how it affects mTORC2 function is not clear (Guertin et al., 2006). There remains much to discover regarding mTORC2 regulation.

1.4.2 mTORC regulation

Currently, mTORC1 regulation is far better understood relative to mTORC2 regulation. mTORC1 integrates four major signals: growth factors, energy status, oxygen, and amino acids (Kim et al., 2013; Laplante and Sabatini, 2009). Figure 1.5 depicts activation of the mTOR pathway. Growth factor stimulation elicits receptor-mediated activation of the Ras/Raf/MEK/ERK and PI3K/Akt pathways, both of which promote mTORC1 activation (Winter et al., 2011). ERK and Akt phosphorylate tuberous sclerosis complex 2 (TSC2) on T1462 to inactivate the activity of TSC1/2 complex (Ma et al., 2005a; Manning et al., 2002).
TSC1/2 complex is a critical negative regulator of the mTORC1 pathway, acting as a GTPase-activating protein (GAP) for the small, Ras-related GTPase, Ras homolog enriched in brain (Rheb). As a GAP, TSC1/2 promotes conversion of Rheb to its inactive, GDP-bound state (Inoki et al., 2003). The 140 kDa TSC1 and 200 kDa TSC2 proteins share no homology with each other or with other proteins. The functional GAP domain is at the C-terminus of TSC2, and TSC1 is required for stabilization of TSC2 by preventing ubiquitin-mediated degradation (Huang et al., 2008; Huang and Manning, 2008). GTP-bound, active Rheb interacts with mTORC1 to facilitate its activation (Long et al., 2005). Figure 1.6 illustrates one possible mechanism of activation of mTORC1 by Rheb (Huang and Manning, 2008). Rapamycin is an mTOR inhibitor that binds to FK506-binding protein of 12 kDa (FKBP12) and interacts with the FKBP12-rapamycin binding domain (FRB) of mTOR. As shown in figure 1.6, rapamycin thus inhibits the activity of mTORC1 (Guertin and Sabatini, 2007). Under unfavorable growth conditions, mTORC1 is bound to FKBP38 (a FKBP12 homologue) and is in the off state (Bai et al., 2007). Upon growth factor stimulation, Rheb-GTP directly associates with FKBP38 and triggers its release from mTORC1. Thus, FKBP38 apparently inhibits mTORC1, and this inhibition is counteracted by Rheb-GTP (Proud, 2007). Addition of rapamycin causes FKBP12-rapamycin binding to mTOR, rendering mTORC1 inactive. However, this proposed mechanism is also debated by many researchers (Parmar and Tamanoi, 2010; Uhlenbrock et al., 2009), underscoring the complexity of mTORC1 activation.
Upon activation, mTORC1 phosphorylates many downstream substrates, such as p70 ribosomal S6 kinase 1 (S6K1), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and sterol regulatory element binding protein 1 (SREBP1). mTORC1 positively regulates cell growth by promoting anabolic processes and limiting catabolic processes. mTORC1 promotes cell growth by enhancing both protein synthesis, through its downstream substrates S6K1 and 4E-BP1 (Dazert and Hall, 2011), and lipid synthesis, through SREBP1 (Duvel et al., 2010).
Compared with mTORC1, relatively little is known about mTORC2 regulation. Some reports suggest that mTORC1 directly regulates the activity of mTORC2, but this mechanism is not well established (Dibble et al., 2009; Julien et al., 2010; Treins et al., 2010). There is also evidence that mTORC2 is activated by associating with ribosomes in response to growth factor stimulation (Betz and Hall, 2013; Zinzalla et al., 2011). Evidence for direct activation of mTORC2 kinase activity by PtdIns(3,4,5)P3 is also documented (Gan et al., 2011). Other work implicates TSC1/2 complex in positive, rather than negative, regulation of mTORC2 activity, by a mechanism that is independent of TSC2 GAP activity (Huang et al., 2008). Like mTORC1, mTORC2 apparently modulates various cellular processes, with mTORC2 affecting actin cytoskeleton organization, cell survival, and other functions (Goncharova et al., 2011; Rosner et al., 2009). The protein substrates phosphorylated by mTORC2 include SGK1, Akt, and PKC (Cybulski and Hall, 2009; Garcia-Martinez and Alessi, 2008). mTORC2 is among the kinases that directly phosphorylate Akt on its hydrophobic motif, S473, which is required
for full activation of Akt (Facchinetti et al., 2008; Hresko and Mueckler, 2005; Sarbassov et al., 2005).

1.4.3 mTORC1 substrates

To date, many proteins are known to be directly phosphorylated by mTORC1. The best-known substrates are S6K1 and 4E-BP1, and others include SREBP1, ULK1/ATG13, PRAS40, STAT3 and CLIP-170 (Laplante and Sabatini, 2009).

**S6K1:** Activated mTORC1 phosphorylates S6K1 on T389. S6K1 is involved in mRNA translation and protein synthesis, and it acts in a rapamycin-sensitive manner. Active S6K1 has been shown to phosphorylate about a dozen different substrates. These include ribosomal protein S6 (rpS6), eukaryotic translation initiation factor 4E (eIF4E), eIF4B, programmed cell death 4 (PDCD4) and eukaryotic elongation factor 2 kinase (eEF2K). The contributions of S6K1 towards the overall control of protein synthesis is still unclear (Wang and Proud, 2011). In certain contexts, S6K1 can negatively regulate Akt activity by phosphorylating and thus inhibiting insulin receptor substrate 1 (IRS1), constituting a negative feedback loop that might affect cell survival or protein synthesis (Dann et al., 2007; Zhang et al., 2008).

**4E-BP1:** The protein 4E-BP1 is phosphorylated by active mTORC1 on sites T37, T46, S65 and T70 (Gingras et al., 1999). In quiescent cells, 4E-BP1 binds and inactivates the protein eIF4E, blocking its function in mRNA translation. Phosphorylation of 4E-BP1 neutralizes its ability to sequester eIF4E, allowing eIF4E to take part in mRNA translation (Choo et al., 2008; Wang and Proud, 2011).

**PRAS40:** PRAS40 is both a substrate and a regulator of mTORC1. PRAS40 is phosphorylated on site S183 by active mTOR, which helps shift PRAS40 binding from mTORC1 to 14-3-3
protein. Dissociation of PRAS40 enhances mTORC1 activity, and thus there is potential for a positive feedback loop (Nascimento et al., 2010; Oshiro et al., 2007; Wang et al., 2007; Wang et al., 2008).

**ATG13/ULK1:** The proteins ATG13 and ULK1 are involved in regulating autophagy. Autophagy is an intracellular process by which cytoplasmic constituents are degraded by proteolytic degradation at the lysosome (Glick et al., 2010). Inhibition of mTORC1 activates autophagy, whereas activation of mTORC1 inhibits autophagy and promotes cell survival by phosphorylating and thus inhibiting the function of ATG13 and ULK1 (Wong et al., 2015). ULK1 is phosphorylated on sites S637 and S757 (Kim et al., 2011a), and ATG13 is phosphorylated on site S318 (Alers et al., 2012; Jung et al., 2010).

### 1.5 Akt substrates other than TSC2

The roles of Akt signaling in cell survival have been intensely studied for the past two decades. Apoptosis, or programmed cell death, is a normal process that occurs during metazoan development. Akt promotes cell survival by phosphorylating and thus inhibiting many pro-apoptotic and glycogen synthase proteins (Kim and Chung, 2002). Figure 1.7 depicts some of the known pro-apoptotic and glycogen synthase proteins regulated by the PI3K-Akt pathway. The important Akt substrates include, but are not limited to, BAD, PRAS40, BIM, FoxO1a/3a/4, and GSK3α/β (Engelman, 2009).

**BAD:** Bcl-2-associated death promoter (BAD) was the first pro-apoptotic protein found to be regulated by Akt (Datta et al., 1999). BAD is a member of Bcl-2 family and promotes apoptosis by binding to Bcl-XL, which facilitates release of cytochrome c from mitochondria to the cytosol. Active Akt phosphorylates BAD on S136 (Datta et al., 1997; Polzien et al., 2009),
causing BAD to dissociate from Bcl-XL and associate with 14-3-3 proteins (Kim and Chung, 2002). 14-3-3 binding sequesters BAD and thus prevents cytochrome c release.

![Diagram of downstream substrates regulated by PI3K/Akt pathway](image)

**Figure 1.7: Downstream substrates regulated by PI3K/Akt pathway (Engelman, 2009)**

**PRAS40:** As described earlier, PRAS40 is part of the mTORC1 complex and is phosphorylated by mTORC1 on S183. Efficient phosphorylation of S183 requires prior phosphorylation of PRAS40 on T246 by Akt. Phosphorylation of PRAS40 by Akt and mTORC1 disrupts the binding between mTORC1 and PRAS40 and thus relieves the inhibition of mTORC1 activity. T246 and S183 phosphorylation promotes PRAS40 binding to 14-3-3 proteins (Nascimento et al., 2010; Oshiro et al., 2007; Wang et al., 2012).

**FoxO:** FoxO transcription factors are members of the Forkhead family of proteins that play vital roles during development (Zhao et al., 2010). FoxO members FoxO1a, FoxO3a, and
FoxO4 are well characterized (Greer and Brunet, 2005). In the absence of growth factors, FoxO proteins translocate to the nucleus and induce transcription of various cell death-related genes, causing cell cycle arrest or apoptosis. Upon growth factor stimulation, these proteins are phosphorylated by Akt, and thus they are excluded from the nucleus and ultimately degraded via the ubiquitin-proteasome pathway (Brunet et al., 1999). Akt phosphorylates FoxO3 on three predicted sites: T32, S253 and S315; FoxO1 is phosphorylated on S256, and FoxO4 on T32 and S197 (Crossland et al., 2008; Kops et al., 1999; Matsuzaki et al., 2005; Rena et al., 2001; Tzivion et al., 2011).

**GSK3α/β**: GSK3α/β are serine-threonine protein kinases that phosphorylate and inactivate glycogen synthase in most cell types (Doble and Woodgett, 2003). Active Akt negatively regulates GSK3α and GSK3β kinase activities by phosphorylation of residues S21 and S9, respectively (Cross et al., 1995; Fang et al., 2000; Phyu et al., 2016).

### 1.6 Mathematical modeling in signal transduction networks

The direction of scientific research has changed over the past 50 years because of major discoveries in biology and medicine. Interest in applying knowledge of physics, chemistry, mathematics, and computer science to study biology has steadily grown; conversely, mathematical sciences have been inspired by biological concepts (Noble, 2015; Robeva et al., 2010).

Biologists typically represent a biological system as a conceptual model, in which interactions among entities (molecules, commonly) are represented in a diagram. This sort of model description allows certain ambiguities regarding the system behavior. In a mathematical description of the system, it is possible to eliminate those ambiguities, because the forms of
the constituent equations are explicitly tied to assumptions about the system. Mathematical models may also be called mechanistic models when they describe the mechanisms that drive the system dynamics; this approach requires knowledge and application of physicochemical principles. Often the resulting model consists of a set of differential equations that describe how the system changes with time i.e., the system’s dynamic behavior (Kreeger and Lauffenburger, 2010). Like all models, mathematical models are simplified versions of the real system. It does not account for every minute details of the system rather only the key aspects. The minor aspects are either assumed unaffected or lumped together in a form of model parameters (Bender, 1978).

A mathematical model can be used in distinct ways. Model analysis describes the application of mathematical techniques to extract general insights into a system’s potential behavior. On the other hand, model simulation may be performed to generate predictions about how the system is expected to behave after being perturbed in a prescribed way. There are numerous commercially available software packages that can perform simulation. The combination of model analysis and simulation can reveal non-intuitive connections between the structure of a system and its consequent behavior (Ingalls, 2013).

1.6.1 Why mathematical models are important for studying biological systems

Vast experimental data indicates the complexity of eukaryotic signal transduction pathways, which were initially framed as linear and independent sequences of enzymatic reactions. It has since become evident that signaling pathways are embedded in branched networks with a substantial degree of crosstalk. The inherent complexity of signal transduction networks and their importance for a wide range of cellular functions have motivated the development of computational models to represent, characterize and elucidate the underlying
mechanisms (Brown et al., 2004; Cirit and Haugh, 2011; Noble, 2015; Resat et al., 2009).
Mathematical models allow us to analyze biological systems both qualitatively (does a molecular readout, such as protein phosphorylation, increase or decrease?) and quantitatively (by how much does the measurement change?), and they may be used to generate predictions and suggest appropriate experiments to test how those systems function (Conzelmann et al., 2004). As biological systems are very complex, a fully detailed mathematical model is not feasible, and therefore simplifying assumptions must be imposed. In kinetic models, each reaction or protein-protein interaction step is defined by a function (rate law) containing parameters (rate constants) that characterize the kinetics of binding or reaction (Cirit and Haugh, 2011).

Complex signaling pathway modeling imposes three key challenges: a) the model must likely contain a large number of parameters, whose values are unknown (Bailey, 2001); b) most kinetic models are incomplete, because they capture the essential protein-protein interactions while ignoring others (Golikeri and Luss, 1974); and c) as new interactions are discovered, models must be flexible so that one can easily incorporate the new information (Brown et al., 2004; Vojtek and Der, 1998).

1.6.2 Linear vs. nonlinear and deterministic vs. stochastic models

A model is considered linear if all the dependent variables exhibit linearity with respect to the independent variables. This is a highly restricted condition, and therefore linear models show only a limited range of system behavior.

All models that do not satisfy the condition of linearity are nonlinear models. Nonlinear systems and models are more difficult to study compared to linear models. They show a diverse
range of patterns and are therefore difficult to generalize. The form of nonlinearity that appears most in biochemical or genetic interactions is saturation, in which one variable monotonically increases with another, but with diminishing sensitivity. Two kinds of saturation are depicted in figure 1.8. Figure 1.8A depicts hyperbolic saturation, where the sensitivity of y with respect to x decreases from a linear relationship until saturation is reached as the value of x increases. Figure 1.8B shows sigmoidal saturation, in which the slope of y with respect to x first increases as x increase (i.e., showing ultrasensitivity), then it decreases as saturation is approached (Gershenfeld, 1998; Goldbeter and Koshland, 1984; Kim and Ferrell, 2007).

![Figure 1.8: Two types of nonlinear saturation a) Hyperbolic b) Sigmoidal](image)

The saturation phenomenon is represented by Hill equation, where \( n \) represent Hill coefficient (a cooperativity or sensitivity parameter) with \( n = 1 \) for hyperbolic saturation and \( n > 1 \) for sigmoidal saturation. \( K_d \) is dissociation constant.

\[
y = \frac{[x]^n}{K_d + [x]^n}
\]

Deterministic models are those that produce output that is defined by the model equations and associated constraints (e.g., initial conditions). In other words, there is no randomness that affects the future state of the system. Continuous systems described
by differential equations are deterministic systems, even though the state of the system at a
given location and time may be difficult to describe explicitly.

On the other hand, a stochastic model allows randomness in the system behavior. The
behavior of a stochastic model is affected by the specified conditions and also unexpected
disturbances or forces. Repeated run of a stochastic model will thus give distinct results.
Chemical systems are comprised of discrete entities (molecules and molecular complexes)
engaging in discrete events (binding interactions and chemical transformations); the discrete
nature of chemical systems can only be captured by stochastic modeling, but stochastic
simulations can be rather complicated relative to deterministic ones (Muller and Kuttler, 2015;
Vries et al., 2006).

In Chapter-3 of this thesis, a model of the PI3K/Akt/mTOR pathway is presented and
may be characterized as mechanistic, nonlinear, and deterministic.

1.7 Thesis overview

In the following chapters, an elaborative study of the Akt/mTOR pathway is presented in a
quantitative manner. An overview of the results presented in this thesis is as follows:

1. Deactivation of a negative regulator: a distinct signal transduction mechanism,
   pronounced in Akt signaling (Chapter-2)

2. Pathway specificity of mTOR activation and a consistent kinetic model of the
   Akt/mTOR pathway (Chapter-3).

3. mTOR activation dynamics controlled by deactivation of negative regulator motif and
   a Rheb- and PRAS40-mediated seesaw mechanism (Chapter-4).

Chapter-5 concludes the thesis with closing remarks and recommendations for future work.
References


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CHAPTER 2

Deactivation of a negative regulator: a distinct signal transduction mechanism, pronounced in Akt signaling

Kinase cascades, in which enzymes are sequentially activated by phosphorylation, are quintessential signaling pathways. Signal transduction is not always achieved by direct activation, however. Often, kinases activate pathways by deactivation of a negative regulator; this indirect mechanism, common in Akt signaling, has yet to be systematically explored. Here, we show that the indirect mechanism has properties that are distinct from direct activation; with comparable parameters, the indirect mechanism yields a broader range of sensitivity to the input, beyond saturation of regulator phosphorylation, and kinetics that become progressively slower, not faster, with increasing input strength. These properties can be integrated in network motifs to produce desired responses, as in the case of feedforward loops.
2.1 Introduction

Phosphorylation of proteins and lipids, catalyzed by specific kinase enzymes, is ubiquitous in intracellular signal transduction. A classic example in eukaryotes is the canonical structure of the mitogen-activated protein kinase (MAPK) cascades, in which three kinases are sequentially activated by phosphorylation (Dhillon et al., 2007). Another example is the phosphoinositide 3-kinase (PI3K)/Akt pathway, which (like the mammalian MAPKs) is prominently dysregulated in human cancers (Yuan and Cantley, 2008). Type I PI3Ks phosphorylate a lipid substrate to produce the lipid second messenger, PIP$_3$, which recruits the protein kinase Akt and mediates its activation by phosphorylation (Fayard et al., 2010; Manning and Cantley, 2007). In no small part because of these important pathways, we typically think of phosphorylation as a direct means of activating molecular interactions and reactions in signal transduction. This is not the only way to increase the ‘flux’ through a signaling pathway, however. Consider signaling downstream of Akt, which phosphorylates a host of protein substrates to affect diverse functions. A survey of the Akt signaling hub shows that many of these reactions result in a decrease, rather than an increase, in activity/function of the substrates (Manning and Cantley, 2007). And, among those substrates, the four shown in the following figure are considered negative, not positive, regulators of downstream signaling (figure 2.1). Whereas negative regulators are appreciated for their roles in feedback adaptation of signaling, the implications of deactivating a negative regulator as an indirect mechanism of pathway activation has yet to be explored.
Here, we use simple kinetic models to elucidate the basic properties of pathway activation by deactivation of a negative regulator (hereafter referred to as mechanism II), as compared with the standard activation of a positive regulator (mechanism I).

2.2 Analyses of the two mechanisms

The analysis is presented in the context of protein phosphorylation, but the conclusions may be generalized to other reversible modifications or to allosteric binding interactions. The common first step is phosphorylation of the regulatory molecule by the kinase. The activity of the upstream kinase such as Akt may be represented by a dimensionless, time \((t)\)-dependent ‘input signal’ function, \(s(t)\). We assume that the total amount of regulator is constant and define its phosphorylated fraction as \(\phi(t)\). Neglecting concentration gradients and saturation of the upstream kinase and of the opposing (constitutively active) phosphatase(s), the conservation of phosphorylated regulator is expressed as follows (see Appendix).

Figure 2.1: Multiple example of the indirect activation mechanism downstream of Akt. In each example, the kinase activity of Akt is considered the input, and its substrate is a negative regulator (deactivator).
\[ \frac{d\phi}{dt} = k_p [s (1 - \phi) - \phi]; \quad \phi(0) = 0 \quad \text{Eq.1} \]

The parameter \( k_p \) is the pseudo-first-order rate constant of protein dephosphorylation.

In the case of \( s = \text{constant} \) (i.e., subject to a step change at \( t = 0 \)), the properties of this simplified kinetic equation are well known (Lauffenburger and Linderman, 1993) and may be summarized as follows. As the magnitude of the signal strength \( s \) increases, the steady-state value of \( \phi, \phi_{ss} \), increases in a saturable fashion; when \( s \gg 1, \phi_{ss} \) approaches its maximum value of 1 and is insensitive to further increases in \( s \). The kinetics of \( \phi(t) \) approaching \( \phi_{ss} \) become progressively faster as \( s \) increases, however.

Next we model the influence of the regulator on a downstream response. Defining the fractional response as \( \rho \) and following analogous assumptions as above, we formulate equations for mechanisms I and II as follows.

\[
\frac{d\rho}{dt} = \begin{cases} 
[k_{a,0} + (k_{a,\text{max}} - k_{a,0})\phi](1 - \rho) - k_{d,0}\rho & \text{(I)} \\
(k_{a,0}(1 - \rho) - [k_{d,0} - (k_{d,0} - k_{d,\text{min}})]\phi)\rho & \text{(II)}
\end{cases}
\quad \text{Eq.2}
\]

In each equation, the first term on the right-hand side describes activation, and the second, deactivation. In I, the effective rate constant of activation increases linearly with \( \phi \), from a minimum value of \( k_{a,0} \) when \( \phi = 0 \) up to a maximum value of \( k_{a,\text{max}} \) when \( \phi = 1 \); the deactivation rate constant is fixed at \( k_{d,0} \). Conversely, in II, the effective rate constant of deactivation decreases linearly with \( \phi \), from a maximum value of \( k_{d,0} \) when \( \phi = 0 \) down to a minimum value of \( k_{d,\text{min}} \) when \( \phi = 1 \); in this mechanism, the activation rate constant is fixed at \( k_{a,0} \). The initial condition is assigned so that \( \rho \) is stationary when \( \phi = 0 \). To further set the two mechanisms on a common basis, we define dimensionless parameters such that the maximum steady-state value of \( \rho \) (with \( \phi_{ss} = 1 \)) is the same for both I and II.
\[
g = \frac{k_{a,\text{max}}}{k_{a,0}} = \frac{k_{d,0}}{k_{d,\text{min}}}; \quad K = \frac{k_{a,0}}{k_{d,0}} \quad \text{Eq.3}
\]

With these definitions, each conservation equation is reduced to the following dimensionless form.

\[
\frac{1}{k_{d,0}} \frac{d\rho}{dt} = \begin{cases} 
K[1 + (g - 1)\phi](1 - \rho) - \rho & \text{(I)} \\
K(1 - \rho) - [1 - (1 - g^{-1})\phi]\rho & \text{(II)} 
\end{cases} \quad \text{Eq.4}
\]

Mechanisms I and II (figure 2.2a) are compared first at the level of their steady-state solutions, \(\rho_{ss}\), for stationary \(s\). Eq. 1 yields the familiar hyperbolic dependence of \(\phi_{ss}\) on \(s\), and \(\rho_{ss}(s)\) has the same shape for both mechanisms; however, whereas \(\rho_{ss}\) of I shows saturation at a lower value of \(s\) than \(\phi_{ss}\), the opposite is true of II (figure 2.2b). Thus, II retains sensitivity to the input even while phosphorylation of the upstream regulator shows saturation. This is perhaps more readily seen when \(\phi_{ss}(s)\) is replaced with a sigmoidal Hill function (i.e., with \(s\) replaced by \(s^n\) in Eq. 1) (figure 2.2c). The key parameter that affects the relative sensitivities of I and II and the disparity between them is the gain constant, \(g\) (see Appendix). As this parameter is increased, \(\rho_{ss}\) of I becomes increasingly saturable with respect to \(\phi_{ss}\) (figure 2.2d), whereas \(\rho_{ss}\) of II gains sensitivity as \(\phi_{ss}\) approaches 1 (figure 2.2e). As an illustrative example, consider that when \(\phi_{ss}\) is increased from 0.90 to 0.95, or from 0.98 to 0.99, the amount of the negative regulator in the active state is reduced by a factor of 2 (figure 2.3).
Figure 2.2: Steady-state properties of mechanisms I and II. 

a) Schematics of direct (I) and indirect (II) activation. 

b) Steady-state dose responses, $\rho_{ss}(s)$, of I (blue) and II (red) along with phosphorylation of the upstream regulator, $\phi_{ss}(s)$ (Eq. 1 at steady state); $K = 0.05$, $g = 100$. 

c) Same as b except with a sigmoidal $\phi_{ss}(s)$ (Hill function with $n = 4$). 

d) Steady-state output, $\rho_{ss}$, of I vs. $\phi_{ss}$ for $K = 0.05$ and indicated values of the gain constant, $g$.  
e) Same as d but for II.
Figure 2.3: Illustration of the steady-state sensitivities of mechanisms I and II as phosphorylation of the regulator approaches saturation. In both cases, progressive increases in the input signal eventually result in phosphorylation of most of the regulator (indicated by $-P$) at steady state. In the case of mechanism I, the regulator has a positive influence on the downstream response, and the phosphorylated form is more active. Near saturation, a further increase in the input results in only a slight fractional gain in the activity of the positive regulator. Conversely, in the case of mechanism II, the regulator has a negative influence on the downstream response, and the phosphorylated form is less active. Near saturation, a further increase in the input results in a dramatic fractional change (reduction) in the remaining activity of the negative regulator.

The two mechanisms also show distinct temporal responses. In the response of I to a step increase in $s$, $\rho(t)$ approaches $\rho_{ss}$ with a time scale that generally becomes faster as $s$ increases; unless the kinetics of $\phi(t)$ are rate-limiting, the time scale is $\sim k_{d,0}^{-1}(1-\rho_{ss})$ (figure 2.4a). Conversely, the response of II generally becomes slower as $s$ increases, as the frequency of deactivation decreases while that of activation is constant; the time scale is $\sim k_{a,0}^{-1}\rho_{ss}$ (figure 2.4b).
Figure 2.4: Kinetic properties of mechanisms I and II. 

*a*) Response of I to a step change in $s$ from zero to the indicated $s(0)$. Time is given in units of $k_pt$; parameters are $K = 0.05$, $g = 10$, $kd,0 = 0.1k_p$. 

*b*) Same as a but for II. 

*c*) Same as a but for a transient input, $s(t) = s(0)\exp(-0.03k_pt)$. 

*d*) Same as c but for II.

To approximate a transient input, we model $s(t)$ as a step increase followed by a decay. For I, the response $\rho(t)$ is such that the variation in the time of the peak, as a function of the step size, is modest; the subsequent decay is prolonged when $\phi(t)$ hovers close to saturation (figure 2.4c). Such kinetic schemes have been analyzed in some detail previously (Behar et al., 2008; Haugh, 2012). In contrast, the response of II to the transient input is such that the system retains sensitivity and consistent decay kinetics beyond the saturation of $\phi(t)$; the distinctive feature is that $\rho(t)$ peaks noticeably later in time as the magnitude of the peak increases (figure 2.4d).

Having established the basic steady-state and kinetic properties of mechanism II as compared with the ‘canonical’ I, we considered what outcomes could be achieved by linking
these motifs in series or in parallel. Such schemes are identified in the Akt/mTOR signaling network (figure 2.5).

Figure 2.5: Identification of activation mechanism I (green) and II (red) in the Akt/mTOR signaling network.

In a standard kinase activation cascade, it is understood that saturation and sensitivity are compounded with each step of the cascade (Kholodenko et al., 1997). Thus, two sequential steps of I yield progressive saturation of the steady-state output at lower $s$ (figure 2.6a), and the desaturating effect of II is likewise compounded (figure 2.6b). By corollary it follows that a sequence of I and II will show an intermediate dose response; that is, the II step offsets the saturation effect of I.
Figure 2.6: Serial and parallel schemes incorporating mechanism I or/and II. \( a \) Steady-state outputs of two response elements, \( \rho_1 \) and \( \rho_2 \), activated by I in series. At each level, \( K = 0.05, g = 100. \) \( b \) Same as \( a \) but for II in series. \( c \) Incoherent feedforward loop (FFL) in which I and II are activated in parallel to activate and inhibit, respectively, the terminal output. For both I and II, \( K = 0.05, g = 100. \) The parameters for Eq. 5 are \( \alpha = 2.5, \beta = 50. \)

A more complex scheme is to combine the two mechanisms in parallel, as in an incoherent feedforward loop (FFL) connected to an AND NOT output as follows.

\[
\text{Output} = \frac{\alpha \rho_I}{1 + \alpha \rho_I + \beta \rho_{II}} \quad \text{Eq. 5}
\]

Given the differential saturation properties of I and II, this scheme readily yields the expected biphasic dose response (Kaplan et al., 2008) without the need for disparate values of the parameters (figure 2.6c). Regarding the kinetics, the analysis shown in figure 2.4 makes it clear that II naturally introduces time delays in cascades or network motifs. Thus, for the incoherent FFL at high, constant \( s \), activation of inhibition by II would tend to yield a dynamic response marked by a peak followed by adaptation (figure 2.7). Analogous calculations were carried out for a coherent FFL as well (figure 2.8).
Figure 2.7: Temporal response of an incoherent feedforward motif with direct activation of an activator by mechanism I and indirect activation of an inhibitor by mechanism II. Parameter values are the same as in figure 2.6c, and $k_{d,0} = 0.1k_p$ for both I and II. Values of the input, $s$, are as indicated for each curve, and time is expressed in units of $k_pt$. Note that high values of $s$ yield an adaptive response due to the inherent disparity in time scales for I and II.
Figure 2.8: Analysis of a coherent feedforward loop (FFL) with direct and indirect activation of two activators in parallel. 

a) Hypothetical circuit diagram. 

b) Signaling in the mTOR network that loosely maps to the hypothetical circuit. 

c) Steady-state response of the coherent FFL, assuming additive contributions to the output, according to

\[ \text{Output} = \frac{\alpha \rho I + \beta \rho II}{1 + \alpha \rho I + \beta \rho II} \]

Parameter values are \( K = 0.05 \) and \( g = 100 \) for both I and II; the parameters for the output function are \( \alpha = 0.5, \beta = 1 \). Note that the output shows a broad sensitivity, over several logs of \( s \), due to the disparity between the saturation of I and II. 

d) Kinetics of the system, with the same parameter values as in c and \( k_{d,0} = 0.1 k_p \) for both I and II. Values of the input, \( s \), are as indicated for each curve, and time is expressed in units of \( k_p t \). Note that high values of \( s \) provoke an initial rapid increase due to activation of I, followed by slower increase due to delayed activation of II.

2.3 Prominence of the mechanism in Akt- and mTOR-mediated signaling

As shown in figure 2.5, multiple instances of the deactivation of a negative regulator motif are found in mTOR-mediated regulation of protein and lipid synthesis and Akt-mediated regulation of apoptotic pathways. Upstream of mTOR, Akt phosphorylates and deactivates two negative regulators TSC2 and PRAS40, which cause activation of mTORC1. These two parallel pathways work together to activate mTOR and can be compared to a coherent
feedforward loop as discussed above. Downstream of mTORC1, the proteins EIF4E and EIF4A are liberated via the indirect activation mechanism (see Chapter-1, section 1.4).

Akt is a general mediator of growth factor-induced survival and has been shown to suppress apoptosis induced by a variety of stresses, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion, and DNA damage (Ahmed et al., 1997). Akt promotes cell growth, proliferation, and survival by phosphorylating protein substrates such as BAD, FoxO and GSK3 (Engelman, 2009). Akt-mediated phosphorylation inhibits the activities of these proteins, and we hypothesize that the deactivation of a negative regulator concept applies generally to these Akt substrates.

2.4 Conclusions

A distinct, indirect signal transduction mechanism characterized by deactivation of a negative regulator is described in this chapter. This motif shows steady-state sensitivity beyond saturation, and therefore the activity of the upstream kinase, such as Akt, can be relatively high. By comparison, the direct activation of signaling by phosphorylation requires that activity of the kinase be regulated, or specifically countered by high phosphatase activity, to maintain sensitivity and avoid saturation of the response. The mechanism described here also introduces relatively slow kinetics (for comparable parameter values). This property, together with its extended range of sensitivity, would allow the motif to be incorporated in signaling networks to yield desired steady and unsteady responses in a robust manner. Considering that key signaling processes mediated by Akt (notably activation of the mammalian target of rapamycin (mTOR) pathway) are achieved by deactivation of negative regulators, we assert that greater recognition of this mechanism and of its distinct properties is warranted.
References


CHAPTER 3

Pathway specificity of mTOR activation and a consistent kinetic model of the Akt/mTOR pathway
It has been shown that both the upstream pathways, Ras/Raf/MEK/ERK and PI3K/Akt, contribute to the activation of mTORC1. But the relative contributions of these two pathways was not investigated explicitly. In this study, we have shown that PI3K/Akt is the dominant pathway for activating mTORC1 in PDGF stimulated NIH3T3 cells. Detailed kinetic phosphorylation data were collected and a consistent kinetic model of the pathway has been built. The model takes into account the necessary protein activation and deactivation steps with simplifying assumptions.
3.1 Introduction

mTORC1 and mTORC2 signaling networks are shown in figure 1.5. A more simplified version of only mTORC1 regulation is shown in figure 3.1. The primary positive regulator of mTORC1 kinase activity is the small GTPase, Ras homology enriched in brain (Rheb), which is negatively regulated by the GTPase activating protein (GAP) activity of the tuberous sclerosis complex, TSC1/2 (Garami et al., 2003; Inoki et al., 2003; Li et al., 2004; Tee et al., 2003). The active, GTP-bound form of Rheb accumulates when TSC2 is deactivated by phosphorylation on regulatory sites. This is achieved by isoforms of the serine-threonine kinase Akt, a central signaling hub activated downstream of type IA phosphoinositide 3-kinases (PI3Ks) (Dibble and Cantley, 2015; Manning and Cantley, 2007). On the other hand, several studies link the MAPK/ERK pathway to inactivating phosphorylation of TSC2 (by ERK1/2 and by the ERK substrate, RSK) (Ballif et al., 2005; Huang and Manning, 2008; Ma et al., 2005b; Ma et al., 2007; Rolfe et al., 2005). The contribution of this crosstalk, relative to the canonical Akt-mediated pathway, has yet to be quantified.
Figure 3.1: The mTORC1 signaling network

Although much has been done to understand how the mTORC1 pathway is regulated and to identify substrates of mTORC1 and their functions in cell growth, proliferation, and survival, quantitative studies and mathematical modeling related to mTOR signaling are lacking. Mathematical modeling of signaling networks is presently limited by the availability of quantitative data (Cirit and Haugh, 2011). To provide such data for the mTOR pathway, one of the primary objectives of this study is to measure the kinetics of TSC2 phosphorylation as an important intermediate step linking Akt to mTOR activation. With that data, we seek to evaluate the consistency of a mathematical model accounting for mTOR activation by deactivation of a negative regulator.

For this study, the model cell line we used is NIH3T3 mouse embryonic fibroblast (ATCC) as a commonly available cell line. Platelet-derived growth factor (PDGF)-BB is used
as the primary physiological stimulus. This system is well-suited because the kinetics and spatiotemporal dynamics of PI3K/Akt activation was previously characterized in this context (Cirit et al., 2010; Park et al., 2003; Schneider and Haugh, 2004). Moreover, PDGF stimulation consistently elicits robust activation of PI3K/Akt in cells expressing the conjugate receptors, which recruit type IA PI3Ks directly and with high avidity (Escobedo et al., 1991; Kazlauskas and Cooper, 1990). Other growth factor receptors, such as those for insulin, IGF-1, and EGF, rely on adaptor proteins IRS1 and Gab1 for even modest activation of PI3Ks, and negative feedback at the level (e.g., via disruption of IRS-1) is a complicating factor for mTORC1 (Harrington et al., 2004; Manning et al., 2005). As outlined in the following sections, we have collected a new data set for this system, composed of quantitative immunoblotting measurements, which canvas an array of conditions suitable for constraining a kinetic model of mTORC1 activation.

3.2 Materials and Methods

3.2.1 Cell culture, inhibitor treatment, growth factor stimulation, and lysate preparation

NIH3T3 mouse embryonic fibroblast were acquired from American Type Culture Collection (Manassas, VA). NIH3T3 cells were cultured in 100 mm cell culture dishes. Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/glutamine solution (PSG) was used as the growth medium. All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). For lysate collection, cells were transferred to 60mm dishes. When the cells reached 80-90% confluency, they were serum starved for 4 hrs. Then the cells were treated with various pharmacological inhibitors (rapamycin, U0126, Akt-inhibitor-VIII) or with Dimethyl Sulfoxide (DMSO)
solvent vehicle control for approximately 40 minutes prior to growth factor stimulation. Cells were lysed in 50 mM HEPES, 100 mM NaCl, 10% Glycerol, 1% Triton X-100, 1 mM Na3VO4, 50 mM β-glycerophosphate, 5 mM NaF, 1mM EGTA, 10mM sodium pyrophosphate, and 10 μg/ml each of aprotinin, leupeptin, pepstatin A, and chymostatin. The cell lysates are then stored in -80°C incubator if not used for western blotting.

3.2.2 SDS-PAGE and Western blotting

Detergent lysates were prepared by mixing them with 4X sample buffer (Tris-Hcl 250mM, SDS 8%, Glycerol 40%, bME 8% and Bromophenol 0.02%). Self-casted polyacrylamide gels were prepared using a pre-defined protocol. For the experiments described here 7.5, 10 and 15% resolving gels were used for high, medium and low molecular weight proteins respectively. For electrophoresis, a molecular weight marker was run in one of the lanes. The running buffer was composed of 3.03% Tris-HCl, 14.4% Glycin and 1% SDS. After SDS-PAGE, the proteins were transferred from the polyacrylamide gel to a polyvinylidene fluoride (PVDF) membrane for western blotting using a transfer buffer (Tris-HCl 0.3% w/v, Glycine 1.43% w/v and MeOH 20% v/v). The PVDF membranes were then blocked with 5% milk solution in TBST (Tris-HCl 0.12% w/v, NaCl 0.87% w/v and Tween-20 0.1% v/v) overnight at 4°C. The membranes were next incubated with corresponding primary antibodies for 1.5 hrs. Then they were washed three times, 5 minutes each with TBST. Next the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for another 1.5 hrs. After secondary incubation, the membranes were washed three times with TBST and two more times with TBS lacking Tween-20. After the final wash, the membranes were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher), which reacts with HRP and produces light. Multiple 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. Chemiluminescence was measured using a SYNGENE G:BOX Chemi XRQ digital imaging system. Densitometry analysis was performed with local background subtraction using QuantityOne software (Bio-Rad Laboratories Inc.).

At this point, we address the use of SDS-PAGE/Western blotting as our workhorse measurement approach, in case there is a misconception that it is inherently ‘not quantitative’. This view is based historically on the old method of using film to develop blots and is fueled by commonly used protocols that absolutely sacrifice data quality for time and reagent savings. Current CCD-based imaging systems rival the sensitivity of film and provide an ample linear range; we routinely confirm assay linearity with respect to the amount of analyte loaded. And we eschew approaches that render immunoblotting unreliable, namely semi-dry transfer and blotting with minimal volumes of antibody solutions. Performed the right way and with appropriate data normalization across experiments as previously described (Ahmed et al., 2014; Cirit et al., 2010; Wang et al., 2009), we see consistent and reproducible trends in immunoblot measurements. We also contend that the PAGE separation yields superior signal-to-background, which we are not willing to compromise for higher throughput (e.g., by ELISA, Luminex, In-cell Western, etc.).

3.2.3 Data normalization

Immunoblot data were first normalized by appropriate loading controls measured for the same lysates. The phospho-protein amount was normalized by the total amount of the same protein, except in the case of phospho-TSC1; blotting of total TSC2 yielded unacceptable variability, and hence total ERK1/2 was used instead. To evaluate the consistency of relative trends across
independent experiments, the data were further normalized so that the mean of 1 nM PDGF
data equals 1.

3.2.4 Antibodies and other reagents

Human recombinant PDGF-BB was purchased from Peprotech (cat#100-14B). Antibodies acquired from Cell Signaling Technology are against ERK1/2 (cat#9107), MEK1/2 (cat#9126), Akt (cat#9272), S6K1 (cat#2708), TSC2 (cat#4308), PRAS40 (cat#2691), β-Actin (cat#4970), ERK pThr202/pTyr204 (cat#9101), MEK pSer217/pSer221 (cat#9121), Akt pSer473 (cat#9271), S6K1 pThr389 (cat#9234), TSC2 pThr1462 (cat#3617), PRAS40 pThr246 (cat#2997), and PRAS40 pSer183 (cat#5936). Antibodies against Rheb were purchased from Santa Cruz Biotechnology (cat#sc-271509). The pharmacological inhibitors U0126 and Akt inhibitor VIII were from Calbiochem (cat#662005, 124017), and rapamycin was purchased from Sigma-Aldrich (cat#R8781). Cells were pre-incubated with the indicated inhibitor or DMSO control for 40 minutes prior to growth factor stimulation. The concentrations of the inhibitors used were 10 μM U0126, 10 μM Akt inhibitor VIII, and 100 nM Rapamycin. Lipofectamine and PLUS transfection reagents were purchased from Invitrogen (cat#18324012, 11514-015). Except where noted otherwise, all other reagents were purchased from Sigma-Aldrich.
3.3 Results

3.3.1 PI3K/Akt is the dominant pathway of TSC2 phosphorylation and mTOR activation

Experiments were performed to quantify the contribution of Ras/Raf/MEK/ERK and PI3K/Akt pathways on the phosphorylation of TSC2 (T1462) and activation of mTORC1 in NIH3T3 fibroblasts. NIH3T3 cells were left untreated, stimulated with PDGF, or PDGF-stimulated in the presence of inhibitors that block MEK (U0126), or/and Akt (Akt inhibitor VIII), and mTORC1 (rapamycin). Quantitative immunoblotting was performed to assess PDGF-stimulated phosphorylation of S6K1, TSC2, ERK, MEK and Akt. The phosphorylation status of ribosomal S6 kinase 1, S6K1, is a readout of mTORC1 activity (figure 3.2). When the cells are left untreated, minimal phosphorylation of S6K1, TSC2, ERK, MEK, or Akt is detected, whereas PDGF stimulation clearly elicits phosphorylation of all these proteins. The mTORC1 inhibitor rapamycin completely blocks phosphorylation of S6K1, confirming that phosphorylation of S6K1 is mTORC1-dependent. Rapamycin treatment does not have significant effect on phosphorylations of ERK, MEK, Akt and TSC2 proteins. This also confirms the lack of a negative feedback in our system from S6K1 to insulin receptor substrate 1 (IRS-1) which has been reported in many studies. The MEK inhibitor U0126 blocks MEK kinase activity toward ERK, so there is no phosphorylation of ERK as expected, but it does not significantly perturb S6K1 or TSC2 phosphorylation at T389 and T1462 respectively. The phosphorylation of MEK is enhanced due to absence of negative feedback from ERK to MEK (Cirit et al., 2010). Akt-Inhibitor-VIII blocks the phosphorylation of Akt by antagonizing the pleckstrin homology domain of Akt and thereby affecting the binding of Akt to the plasma membrane.
Figure 3.2: PI3K/Akt is the dominant mode of mTORC1 activation. NIH3T3 cells were pretreated with either DMSO or 10 µM U0126, 10 µM Akt inhibitor VIII and 100 nM rapamycin for 40 minutes then either left unstimulated or stimulated with 1nM PDGF for 15 minutes. Phosphorylation of Akt, ERK, MEK, TSC2 and S6K1, along with corresponding total proteins, were measured by immunoblotting.

Unlike U0126, Akt inhibitor-VIII blocks S6K1 and TSC2 phosphorylation completely. This finding indicates that TSC2 phosphorylation on T1462 and mTORC1 activation (i.e., S6K1 phosphorylation) are predominantly PI3K-Akt dependent. One thing to note is that Akt inhibitor-VIII treatment reduces PDGF-stimulated phosphorylation of ERK and MEK. This is expected based on previous work showing that PI3K signaling positively regulates the Raf/MEK/ERK pathway (Aksamitiene et al., 2012; Cirit et al., 2010). Finally, combined
application of U0126 and Akt inhibitor-VIII completely blocks TSC2 and hence S6K1 phosphorylation. PRAS40, an essential negative regulator of mTORC1, is also phosphorylated by Akt on T246, which is blocked by Akt-In-VIII treatment (figure 3.3).

Figure 3.3: Inhibition of Akt ablates PDGF-stimulated phosphorylation of PRAS40 T246. NIH3T3 cells were pretreated with either DMSO or 10µM Akt inhibitor VIII for 40 minutes then either left unstimulated or stimulated with 1nM PDGF for 15 minutes. Phosphorylation of Akt and of PRAS40, along with total proteins, were measured by immunoblotting, confirming that phosphorylation of PRAS40 T246 is Akt-dependent.

This experiment was designed with 1 nM PDGF stimulation for 15 minutes, because it has been shown that the observed $K_d$ of PDGF binding to PDGF receptors ranges from 0.1~1nM. Also, MEK, ERK and Akt phosphorylation peaks at 5-15 minutes after PDGF stimulation. The IC$_{50}$ values of rapamycin, U0126 and Akt-In-VIII are in the nM to sub-µM range (Cirit and Haugh, 2012; Kelly et al., 1991; Park et al., 2003; Walker et al., 1992; Wang et al., 2015). The experiment was also tested under submaximal PDGF concentration (0.03 nM), showing similar qualitative patterns of S6K1 phosphorylation with rapamycin, U0126 and Akt Inhibitor-VIII (results not shown).
3.3.2 Kinetics of mTOR activation and Akt, TSC2 and PRAS40 phosphorylations

The Akt/mTOR was probed further at the level of system dynamics, considering phosphorylated Akt as the input, phosphorylation of S6K1 as the output, and Akt-dependent phosphorylation of TSC2 and of PRAS40 as key, intermediate steps. PDGF dose and stimulation time were systematically varied. Cells were pre-treated with 0.2% DMSO vehicle control and then either unstimulated or PDGF-stimulated for various time points as indicated in figure 3.4. We measured the following phosphorylation kinetics: Akt S473, TSC2 T1462 (the regulatory site phosphorylated by Akt) and S6K1 T389 (substrate of mTORC1). We have also measured phosphorylation of PRAS40 on both T246 (the regulatory site phosphorylated by Akt) and on S183 (which stabilizes 14-3-3 binding). The total amounts of corresponding proteins were also probed. This unique data set canvasses an array of time points and doses of PDGF; in our experience, assessing both the time- and stimulus-dependence, at multiple steps of the pathway, is essential for constraining kinetic models. Beta-actin was used as a loading control.

Figure 3.5 shows the quantification of the blots for five biological repeats. The level of phosphorylated protein is normalized by the total for each sample and then further normalized so that the average of 1nM PDGF data is 1. It is clear from the plots that the phosphorylation level of Akt, TSC2, S6K1 and PRAS40 reach saturation at [PDGF] ~ 1nM, whereas minimal phosphorylation is elicited by the lowest [PDGF] ~ 0.01nM. Consistent with previous work, Akt phosphorylation is transient, which has been attributed to PDGF depletion and downregulation of PDGF receptors, showing dose saturation at ~ 0.3 nM PDGF-BB (Cirit et al., 2010). S6K1 phosphorylation shows a similar pattern, except with a noticeable kinetic delay and a less dramatic adaptation of the maximal response compared to Akt. By comparison,
it is curious that both phospho-TSC2 and -PRAS40 (T246) exhibit greater sensitivity to PDGF dose, judging from the responses to 0.03 nM vs. 1 nM PDGF-BB. PRAS40 S183 shows only a modest increase in PDGF stimulation. These distinct kinetics suggest that Akt controls mTORC1 activation via mechanisms with complex or counterintuitive properties.

Figure 3.4: PDGF dose and time response on phosphorylation of Akt, TSC2, PRAS40 and S6K1 for NIH3T3 cells.
Figure 3.5: Normalized Akt, TSC2, S6K1 and PRAS40 phosphorylation time courses for 0.01, 0.03, 0.3 and 1 nM PDGF. The data are normalized by loading controls and then by mean value of the 1nM time course for each phospho-protein. The normalized data are reported as mean ± SE (n=5 independent experiments).
It was also confirmed that the upstream signaling pathways of mTORC1, Raf/MEK/ERK and PI3K/Akt pathways are not significantly affected by rapamycin treatment (figure 3.6).

Figure 3.6: Rapamycin treatment does not affect upstream signaling, indicating a lack of mTORC1-dependent feedback. Immunoblots showing PDGF-stimulated phosphorylation of various proteins in cells pretreated with 0.2% DMSO (control) or 100nM rapamycin. NIH 3T3 cells were incubated with either DMSO or rapamycin for 40 minutes and then either left unstimulated or stimulated with PDGF for various doses and durations as indicated. Right: quantification of Akt phosphorylation (dashed lines: DMSO; solid lines: rapamycin) for n=1 experiment.
3.4 A consistent kinetic model of the Akt/mTOR pathway

mTORC1 is a signaling hub that controls cell metabolism and growth. Along with the upstream kinases, PI3K and Akt, mTOR has been increasingly implicated in cancer biology and therapy (Polivka and Janku, 2014). Despite its functional significance, little in the way of quantitative analysis or mechanistic modeling has been brought to bear on mTOR signaling. After collecting detailed kinetic phosphorylation data, the next step is to build a kinetic model of the pathway to do system level analysis of the pathway.

We built a mathematical model of the mTORC1 subnetwork, which includes the essential species and processes needed to describe the network. Judicious assumptions are invoked to minimize the number of adjustable parameters. This model is fit to the kinetic and dose response data (figure 3.5) using a proven method that generates a large ensemble of suitable parameter sets, and the model is refined as needed to capture all features of the data.

3.4.1 Model Structure

The model structure is represented as a block flow diagram (figure 3.7).

![Figure 3.7: Schematic of the kinetic model](image)
Growth factor stimulation elicits PI3K recruitment to the membrane and PI3K-catalyzed generation of 3’PI. 3’PIs then mediate phosphorylation and activation of Akt. Active Akt mediates parallel phosphorylation of TSC2 and PRAS40, which inactivates the GAP function of TSC2 on Rheb and neutralizes the inhibitory effect of PRAS40 on mTORC1. The active/unphosphorylated TSC2 acts as a GTPase activating protein for Rheb and converts Rheb-GTP to Rheb-GDP. The active Rheb-GTP interacts with mTOR to stimulate its activity whereas PRAS40 inhibits the activity of mTOR. Activated mTOR then phosphorylates many downstream substrates (4E-BP1, S6K1 etc.). Here, S6K1 phosphorylation was measured to monitor mTORC1 activity.

3.4.2 Formulation of the kinetic equations

After formulating the model structure, simple kinetic equations are formulated representing activation or inactivation of the various proteins. Considering the granularity and emphasis of the model we aimed to develop, we chose not to model the PDGF receptor dynamics (ligand binding, receptor dimerization, receptor trafficking and ligand depletion). There is no evidence of any negative or positive feedback affecting PDGF-stimulated PI3K/Akt activation, other than receptor endocytosis (Cirit et al., 2010; Park et al., 2003; Schneider and Haugh, 2004; Schneider and Haugh, 2005). Therefore, consistent with the prior publications we model receptor dynamics using a dose-dependent double exponential function, \( S(t) \) (Haugh and Lauffenburger, 1998). We assume that the reversible recruitment of PI3K enzyme by the receptor (fractional activation, \( e \)) is relatively rapid (and therefore near equilibrium) and saturable (Cirit and Haugh, 2012).
**PI3K enzyme, e:**

\[ e = \frac{S(t)}{1 + S(t)}; \quad S(t) = P(1 - e^{-k_1 t}) + A(e^{-k_1 t} - e^{-k_2 t}) \]

*S* represents the transient nature of receptor activation and *e* represents recruitment of PI3K to the plasma membrane. The *P* and *A* parameters represent the plateau (steady-state) and amplitude (peak) of receptor activation, respectively. Rate constant *k*₁ describing the decay of \( S(t) \) and rate constant *k*₂ > *k*₁ describing the initial increase of \( S(t) \). These equations have four adjustable parameters *P*, *A*, *k*₁ and *k*₂. For each of the four PDGF doses, *P* and *A* are assigned different values, whereas the values of *k*₁ and *k*₂ were the same for all doses; thus there are 10 adjustable parameters overall.

Next are the dynamic equations for 3’phosphoinositides formation and recruitment and activation of Akt. We assume that PI3K reaction is insensitive to the availability of the substrate (the amount of phosphatidylinositol 4,5-biphosphate (PIP2) is in excess), and the phosphatase PTEN is constitutively active and far from saturated. Phosphorylation of Akt is modeled as a bimolecular reaction with rate constant *k*ₐ, and therefore the activities of the kinases involved are implicit. This is reasonable if one considers binding of 3’PI by the pleckstrin homology domain of Akt as the rate-determining step. A pseudo-first-order rate law, with rate constant *k*₋ₐ, describes dephosphorylation of Akt.

**3’PI lipid, l:**

\[ \frac{dl}{dt} = k_{3PI}(e - l); \quad l(0) = 0 \]

**Phosphorylated Akt, a:**

\[ \frac{da}{dt} = k_a l(1 - a) - k_-a a; \quad a(0) = 0 \]
In the differential equations, \( l \) represents the density of 3'PI in the membrane and \( a \) is the fraction of Akt that is phosphorylated. These equations have 3 adjustable rate constants: \( k_{3PI} \), \( k_a \) and \( k_a \).

TSC2 and PRAS40 are phosphorylated by Akt, resulting in the neutralization of these regulators. In the model, \( g \) and \( p \) are the fractions of TSC2 (GAP) and PRAS40, respectively, in the phosphorylated state. To account for basal phosphorylation, both Akt-dependent and constitutive phosphorylation terms are included.

**Phosphorylated TSC2 (GAP), \( g \):**

\[
\frac{dg}{dt} = (k_{g0} + k_ga)(1 - g) - k_{-g}g; \quad g(0) = \frac{k_{g0}}{k_{g0} + k_{-g}}
\]

**Phosphorylated PRAS40, \( p \):**

\[
\frac{dp}{dt} = (k_{p0} + k_pa)(1 - p) - k_{-p}p; \quad p(0) = \frac{k_{p0}}{k_{p0} + k_{-p}}
\]

These equations thus introduce 6 more adjustable rate constants: \( k_{g0}, k_g, k_{-g}, k_{p0}, k_p \) and \( k_{-p} \). The initial conditions are set such that the system is stationary at \( a = 0 \).

The model assumes that the small GTPase Rheb is converted to the active, GTP-bound state (fraction \( r \)) by a constitutive exchange reaction with pseudo-first-order rate constant \( k_r \). Control is imposed by deactivation of TSC2 GAP activity. The interaction between unphosphorylated GAP (fraction \( 1 - g \)) and Rheb-GTP is assumed to be far from saturation (catalytic efficiency \( k_{-r} \)). The initial condition is set such that the system is stationary for \( g = g_0 \). The resulting equation has 2 more adjustable rate constants: \( k_r \) and \( k_r \).

**Rheb-GTP, \( r \):**

\[
\frac{dr}{dt} = k_r(1 - r) - k_{-r}(1 - g)r; \quad r(0) = \frac{k_r}{k_r + k_{-r}(1 - g(0))}
\]
At this point, the model needs to describe mTORC1 activity; although there is some uncertainty as to the precise mechanism (Wang and Proud, 2011), it is a great opportunity for modeling to help elucidate it. In the model equation, the dimensionless mTORC1 activity, \(m\), is affected positively by Rheb-GTP and negatively by unphosphorylated PRAS40 [dimensionless \(r\) and \((1-p)\), respectively]. To model this regulation in a compact manner, we consider mTOR to be in pseudo-equilibrium with those two entities, noting that the ‘interaction’ with Rheb-GTP could represent physical binding of Rheb-GTP or an indirect yet proportional effect of Rheb exerted on the complex, e.g. by affecting substrate binding. Taking \(M_0, M_r, M_p,\) and \(M_{rp}\) as the fractions of mTORC1 in the ‘free’, Rheb-associated, PRAS40-bound, and both Rheb- and PRAS40-associated states, and assuming that Rheb-GTP and PRAS40 are in excess, the governing equations are as follows.

\[
\begin{align*}
M_r &= K_r r M_0; \\
M_p &= K_p (1-p) M_0; \\
M_{rp} &= \beta K_r r M_p = \beta K_p (1-p) M_r = \beta K_r K_p r (1-p) M_0
\end{align*}
\]

\[M_0 + M_r + M_p + M_{rp} = 1\]

The parameters \(K_r\) and \(K_p\) are the associated equilibrium constants, and \(\beta\) is a parameter characterizing the cooperativity of the two interactions. Thus, the model allows for the nature of these interactions to range from mutually exclusive (\(\beta = 0\)) to completely independent (\(\beta = 1\)) to positively cooperative (\(\beta > 1\)). Finally, assuming different contributions to the mTOR activity, with \(m = \alpha M_r + (1-\alpha) M_{rp}\) (constant parameter \(0 \leq \alpha \leq 1\)), we obtain the following expression which has 4 adjustable parameters: \(\alpha, \beta, K_r\), and \(K_p\).

**mTORC1 activity, \(m\):**

\[
m = \frac{\alpha K_r r + (1-\alpha) \beta K_r K_p r (1-p)}{1 + K_r r + K_p (1-p) + \beta K_r K_p r (1-p)}
\]
Lastly, the kinetic equation for S6K1 phosphorylation, fraction $s$, is as follows, introducing the rate constants $k_s$ and $k_{s}$. The initial condition is such that the equation is stationary for $m = m_0$, where $m_0$ is the value of $m$ calculated with $r = r_0$ and $p = p_0$.

**Phosphorylated S6K1, $s$:**

$$\frac{ds}{dt} = k_s m(1 - s) - k_{s\cdot}s; \quad s(0) = \frac{k_s m(0)}{k_s m(0) + k_{s\cdot}}$$

This final equation has 2 more adjustable rate constants: $k_s$ and $k_{s\cdot}$.

### 3.4.3 Acquisition of model parameter sets ensemble

The model has a total of 27 adjustable parameters. We implemented a Monte Carlo routine based on the Metropolis algorithm (Hao et al., 2012; Metropolis et al., 1953) to directly and globally align the model and the data set. The goal of this exercise was not to identify a single parameter set that fits the data best (arguably, a fruitless task for models with even modest complexity). Rather, the algorithm collects a large ensemble of parameter sets that fit the data near-optimally. The algorithm was implemented in MATLAB (MathWorks), adapted from code described in detail previously (Cirit and Haugh, 2012). A summary of the method follows (see also figure 3.8).

1. An initial set of parameters $k_i$ is chosen. Using these initial values, the dimensionless model output is computed.
2. The model output is aligned to the means of the experimental data by scaling the model output by alignment factors for each readout $j$ (phospho-Akt, phospho-TSC2, phospho-PRAS40 and phospho-S6K1). For each parameter set $i$, the value of each alignment factor $j$ is chosen to minimize the sum of squared deviations, $SSD_{ij}$. This is achieved via a branch-and-bound subroutine. In calculating the $SSD$, we weighted the data for different PDGF
doses by the following factors, to offset differences in the magnitudes of the data values: 1 for 0.3 and 1 nM PDGF, 2 for 0.03 nM PDGF, and 5 for 0.01 nM PDGF.

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

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

4. Each parameter $k_i$ is updated according to the following equation.

$$k_{i+1} = k_i (1 + \Delta \mu \text{ randn})$$

where randn is a random number drawn form a standard normal distribution. For this study, the value of the parameter $\Delta \mu$ was 0.03. The step is redone if $k_{i+1}$ is chosen to be less than $10^{-4}$ or greater than $10^4$.

5. Steps 2 and 3 are repeated for the new parameter set.

6. If $cSSD_{i+1} < cSSD_i$, the new parameter set is accepted, and $i$ is incremented. Otherwise, it might still be accepted, with a probability given by the following formula.

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

$$T_i = T^0 cSSD_i$$

The value of $T_i$ is called the ‘temperature’, which determines how forgiving the algorithm is when the fit fails to improve. The parameter $T^0$ is the ‘standard’ temperature (corresponding to $cSSD_i = 1$), which was set to a value of 0.01 for this study i.e., a parameter set with much greater than 1% higher $cSSD$ than its predecessor is severely penalized. If the new parameter set is rejected, the procedure is repeated using the previous parameter set as the input.
7. The algorithm is run for a sufficient long time, until at least 50,000 parameter sets are accepted. From these, we selected the 10,000 parameter sets with the lowest $cSSD_i$ to comprise the parameter set ensemble used to generate modeling results.

For each parameter set in the ensemble, the model was recalculated, and the relevant outputs were scaled by their respective alignment factors for comparison to the experimental data. The scaled model output for each time course is reported as the ensemble mean ± standard deviation, allowing visual assessment of the accuracy and precision of the fit.

Figure 3.8: Identification of a large ensemble of parameter sets that fit the data near optimally. A) Block flow diagram of the Monte Carlo algorithm used to generate and choose the parameter sets. B) $cSSD$ values (orange) for each accepted parameter set, in the order accepted. Those with $cSSD$ values below the green line were the best 10,000 parameter sets, constituting the ensemble.
3.5 Model output

Figure 3.9 shows the ensemble averaged model output along with the means of the experimental data. Visual observations indicate that the aligned model outputs show clear agreement with the experimental data and captures all of the aforementioned features of the training data. Each Akt phosphorylation time course shows decay from the peak value at later time points, and the model successfully captures the sensitivity of these time courses with respect to PDGF doses. Compared to Akt phosphorylation kinetics, those of TSC2 and PRAS40 for the higher doses of PDGF are more sustained (i.e., slow decay). The interpretation of the model is that the phosphorylation of TSC2 and PRAS40 response is close to saturation. Our model clearly follows the TSC2 phosphorylation kinetics both for lower and higher doses of PDGF. Compared with TSC2 phosphorylation, S6K1 phosphorylation has a kinetic delay to reach the maximum phosphorylation (maximum TSC2 phosphorylation is at 15 minutes, whereas maximum S6K1 phosphorylation is at 30 minutes), and shows more, not less, sensitivity to the Akt phosphorylation level. These features are consistent with activation by deactivation of a negative regulator mechanism (discussed in Chapter-2). Our model captures all of these features. Hence, we assert that the data are indeed consistent with the biochemical mechanisms encoded by the model equations. Variations of the model, in which it is assumed that mTORC1 is regulated solely by TSC1/2 or by PRAS40, did not yield a better fit to the data (Appendix).
Figure 3.9: Ensemble averaged model output fit to the kinetic data. Each quadrant shows the fit for a different dose of PDGF as indicated; note that y-axes are scaled differently to allow the quality of fit to be more fully assessed. The ensemble averaged (mean) model output (green curves) may be compared to the experimental data (red circles). Black dashed lines indicate mean ± SD for the model output, indicating the level of variation in the fit.

Table 3.1 shows the statistics for the parameters set ensemble. The minimum, first quartile, median, third quartile and maximum are displayed for each set of the 27 adjustable parameters. Figure 3.10 shows the distribution of the parameter values across the ensemble. The statistics indicate that some parameters are tightly distributed, whereas others are far less constrained (e.g., arbitrarily high/low).
Figure 3.10: Distributions of parameter values across the ensemble. A) For each parameter of the model, the distribution given in Table 3.1 is plotted as a box (1st and third quartiles, with the red line marking the median value) and whiskers (minimum and maximum). All rate constants have units of min\(^{-1}\); all other parameters are dimensionless. B) Same as A, except the parameters are sorted according to the ratio of the third quartile to the first quartile (low to high). A smaller ratio indicates a more tightly constrained parameter.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Min</th>
<th>Q1</th>
<th>Median</th>
<th>Q3</th>
<th>Max</th>
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<tbody>
<tr>
<td>$P$ (0.01)</td>
<td>Signal plateau, 0.01nM PDGF</td>
<td>0.0001</td>
<td>0.00261</td>
<td>0.00347</td>
<td>0.00423</td>
<td>0.00915</td>
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<td>$A$ (0.01)</td>
<td>Signal amplitude, 0.01nM PDGF</td>
<td>0.000168</td>
<td>0.00653</td>
<td>0.00833</td>
<td>0.0104</td>
<td>0.0188</td>
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<td>$P$ (0.03)</td>
<td>Signal plateau, 0.03nM PDGF</td>
<td>0.695</td>
<td>1.51</td>
<td>1.86</td>
<td>2.86</td>
<td>6.96</td>
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<td>$A$ (0.03)</td>
<td>Signal amplitude, 0.03nM PDGF</td>
<td>0.108</td>
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<td>0.347</td>
<td>0.560</td>
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<td>$P$ (0.3)</td>
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<td>$P$ (1)</td>
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<td>7.38</td>
<td>24.9</td>
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<td>55.9</td>
<td>143</td>
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<td>$A$ (1)</td>
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<td>$k_1$</td>
<td>Rate constant, receptor activity decay</td>
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<td>0.137</td>
<td>0.155</td>
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<td>$k_2$</td>
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<td>0.0768</td>
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<td>0.238</td>
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<tr>
<td>$k_{SP}$</td>
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<td>0.00319</td>
<td>0.0146</td>
<td>0.0256</td>
<td>0.0969</td>
<td>0.209</td>
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<td>$k_a$</td>
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<td>$k_{g0}$</td>
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<td>0.0133</td>
<td>0.0225</td>
<td>0.0262</td>
<td>0.0314</td>
<td>0.0595</td>
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<td>$k_g$</td>
<td>Rate constant, TSC2 phosph. by Akt</td>
<td>3.44</td>
<td>16.8</td>
<td>32.3</td>
<td>52.1</td>
<td>143</td>
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<tr>
<td>$k_{g0}$</td>
<td>Rate constant, basal PRAS40 phosph.</td>
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<td>0.199</td>
<td>0.231</td>
<td>0.276</td>
<td>0.509</td>
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<td>$k_p$</td>
<td>Rate constant, PRAS40 phosph. by Akt</td>
<td>7.57</td>
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<td>162</td>
<td>395</td>
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<tr>
<td>$k_{p0}$</td>
<td>Rate constant, PRAS40 dephosph.</td>
<td>0.0350</td>
<td>0.104</td>
<td>0.137</td>
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<td>0.432</td>
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<tr>
<td>$k_r$</td>
<td>Rate constant, Rheb-GDP → -GTP</td>
<td>0.0007</td>
<td>0.00422</td>
<td>0.00604</td>
<td>0.00903</td>
<td>0.0401</td>
</tr>
<tr>
<td>$k_s$</td>
<td>Rate constant, Rheb-GTP hydrolysis</td>
<td>0.039</td>
<td>0.189</td>
<td>0.304</td>
<td>0.427</td>
<td>1.08</td>
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<tr>
<td>$K_r$</td>
<td>Equilibrium constant, Rheb/mTOR</td>
<td>1.51</td>
<td>12.6</td>
<td>16.8</td>
<td>38.4</td>
<td>99.2</td>
</tr>
<tr>
<td>$K_p$</td>
<td>Equilibrium constant, PRAS40/mTOR</td>
<td>108</td>
<td>520</td>
<td>781</td>
<td>2.78e3</td>
<td>5.27e3</td>
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<tr>
<td>$k_{r0}$</td>
<td>Rate constant, S6K1 phosph.</td>
<td>0.612</td>
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<td>4.79</td>
<td>6.37</td>
<td>14.6</td>
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<tr>
<td>$k_s$</td>
<td>Rate constant, pS6K1 dephosph.</td>
<td>0.0245</td>
<td>0.0473</td>
<td>0.0582</td>
<td>0.0706</td>
<td>0.143</td>
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<td>$\beta$</td>
<td>mTORC1 cooperativity parameter</td>
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<td>0.000197</td>
<td>0.000292</td>
<td>0.000818</td>
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<td>$\alpha$</td>
<td>mTORC1 activity parameter</td>
<td>0.235</td>
<td>0.784</td>
<td>0.888</td>
<td>0.951</td>
<td>0.999</td>
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</tbody>
</table>

Table 3.1: Statistics of the parameter set ensemble for the main model of the Akt/mTORC1 signaling axis. All parameters labeled as rate constants have units of min$^{-1}$; all others are dimensionless. For each parameter, the minimum (Min), first quartile (Q1), median, third quartile (Q3), and maximum (Max) of the 10,000 values in the ensemble are listed.
3.6 Discussion

The phosphorylation time course and dose response data show that Akt phosphorylation reached a peak value after roughly 15 minutes and decayed thereafter for all doses of PDGF. Compared to the Akt phosphorylation kinetics, those of TSC2 and PRAS40 phosphorylation were more sustained for higher doses of PDGF. The hypothesis explaining this observation is that phosphorylation of TSC2 and PRAS40 are close to saturation, which also explains the dramatic difference between the shapes of the time courses for 0.03 and 0.3 nM PDGF in the case of TSC2 phosphorylation. Note that both TSC2 and PRAS40 are direct substrates of Akt and negative regulators of mTORC1. Akt-mediated phosphorylation of these proteins neutralizes their inhibitory effect to mTORC1. As described under chapter-2, this indirect mechanism of activation of mTORC1 by deactivating negative regulators has distinct properties relative to a direct activation mechanism (i.e., activating a positive regulator). Compared to TSC2 phosphorylation, S6K1 phosphorylation has a kinetic delay to reach the maximum (maximum TSC2 phosphorylation is at 15 minutes, whereas maximum S6K1 phosphorylation is at 30 minutes), and shows more, not less, sensitivity to the magnitude of Akt phosphorylation. Overall, these data provide a basis for building and constraining a kinetic model of the PI3K/Akt/mTOR pathway and reveal a number of characteristic features of the Akt/mTORC1 signaling axis.

The algorithm for calculating the values of the parameters provided an ensemble of 10,000 parameter sets, that fit the data equally well, instead of one best fit. This approach allows one to analyze the distribution of parameter values as shown in figure 3.10. The distributions show that some parameters are tightly constrained compared to the others; i.e., the system will be more sensitive to changes in those parameters that are more tightly
distributed. The absolute values of the parameters individually might not reflect the true values i.e., they may be arbitrarily high or low, but the relative values of the parameters compared to one another gives a sense of slower or faster kinetics of a particular reaction step. The parameter set ensemble can be used to generate a corresponding ensemble of model predictions for various hypothetical scenarios. Before applying the model in this manner, we judged the quality of the fit and deemed that the ensemble average of the aligned model output captures all of the critical features of the training data.
References


CHAPTER 4

mTOR activation dynamics controlled by deactivation of negative regulator motif and Rheb- & PRAS40-mediated seesaw mechanism
Activation of mTORC1 mediated by Akt is characterized by neutralization of TSC2, leading to accumulation of the mTORC1 activator, Rheb-GTP, and of the mTORC1 inhibitor, PRAS40. A mechanistic mathematical model, trained on quantitative measurements, was used to predict the contributions of these two Akt-dependent processes on mTORC1 regulation. The simulations predict that both pathways are substantially important and constitute a feedforward loop to activate mTORC1. Protein depletion experiments using short-hairpin RNAs were performed to validate the model predictions, and these measurements showed a semi-quantitative match to the model predictions. Further analysis of the model revealed the regulatory motif called activation by deactivation of a negative regulator and a seesaw regulation mechanism that describes how activated and inhibited states of mTORC1 are controlled in concert to produce a nonlinear, ultrasensitive response.
4.1 Introduction

Growth factor stimulation elicits activation of the mTOR pathway, and PI3K/Akt signaling has proven to be the dominant mechanism of mTORC1 activation in fibroblasts (Chapter-3). Akt activates mTORC1 by phosphorylating TSC2 and thus inhibiting the GAP activity of TSC1/2 complex that maintains Rheb in its inactive, GDP-bound state. However, in recent studies, it has been shown that PRAS40 phosphorylation by Akt might also play an important role (Nascimento et al., 2010; Wang et al., 2012). PRAS40 is a component of the mTORC1 complex that negatively regulates catalytic activity. PRAS40 binds to the mTOR carboxy-terminal kinase domain, and this interaction prevails under conditions that inhibit mTOR signaling, such as nutrient or serum deprivation or mitochondrial metabolic inhibition. PRAS40 prevents constitutive activation of mTORC1 in TSC1/2-null cell lines (Laplante and Sabatini, 2009). mTORC1 activation causes PRAS40 phosphorylation at Ser183 that ultimately leads to PRAS40 dissociation and further activation of the complex. It has been reported that Akt also phosphorylates PRAS40. The direct phosphorylation of PRAS40 at Thr246 by Akt activates mTORC1 (Nascimento et al., 2010; Wang et al., 2008). Therefore, PI3K/Akt signaling apparently regulates mTORC1 activation in two ways: through TSC2 phosphorylation, leading to accumulation of Rheb-GTP, and through direct phosphorylation of PRAS40 (figure 4.1) (Vander Haar et al., 2007). Yet, the balance of these two mechanisms in their contributions to mTORC1 activation is presently unclear. If both mechanisms proved to be significant, we would recognize the collective network structure as a coherent feedforward loop. Here, we used a trained mathematical model (Chapter-3) to predict the contributions in terms of mTORC1 phosphorylation of S6K1. To test \textit{a priori} predictions of
the model, short hairpin RNAs (shRNAs) were used to generate cell lines with key regulatory proteins depleted, and another set of kinetic measurements were acquired.

Figure 4.1: mTORC1 regulation by an Akt-mediated feedforward loop with two arms: a) TSC2-Rheb and b) PRAS40

Lentiviral shRNAs targeting mouse TSC2, Rheb or PRAS40 were used to investigate whether TSC2-independent PRAS40 phosphorylation or TSC2-dependent Rheb activation by Akt is sufficient to stimulate mTORC1 activity. We note that there are no pharmacological inhibitors available that block the function of TSC2, Rheb, or PRAS40. A non-targeting shRNA served as a negative control. The endoribonuclease Dicer heterogeneously cleaves shRNAs into biologically active small interfering RNAs that have differing efficacies and off-target effects. To deal with the off-target issue, multiple individual shRNAs targeting the same gene were used. As each shRNA is likely to have a unique off-target spectrum, but the same intended target, observing the same phenotype with multiple individual shRNAs confirms that the phenotype results from silencing the intended target.
Finally, the validated model was analyzed to reveal important dynamical motifs in the Akt/mTORC1 signaling axis, namely deactivation of a negative regulator (described under Chapter-2) and seesaw regulation. The latter describes how the status of the mTORC1 complex is modulated in an ultrasensitive manner relative to the activity of Akt.

4.2 Materials and Methods

4.2.1 Plasmids and lentiviral infections

pLKO-puro vectors containing short hairpin sequences targeting *Mus musculus* TSC2, Rheb and PRAS40 were acquired from the UNC Lenti-shRNA Core Facility (UNC Chapel Hill, NC). Lentivirus was produced by lipofection of 293T cells with shRNA containing vector and the packaging plasmids pCMV-VSVG (8454; Addgene) and pCMV-DR8.91. The control plasmid containing a nontargeting shRNA sequence (5′-TTATCGCGCATATCA CGCG-3′), pLKO-shNEG-puro (Everett, 2010), and pCMV-DR8.91 were gifts from R. Everett (Medical Research Council-University of Glasgow Centre for Virus Research, Glasgow, Scotland, UK). Virus was harvested 24, 48 and 72 hours after transfection, and the pooled, conditioned medium was supplemented with 8 μg/ml polybrene before adding to target cells. After incubating for 24 hours, the infected cells were selected in growth medium supplemented with 2 μg/ml puromycin (Fisher Scientific).

*Short hairpin sequences:* (sequences with highest knockdown efficiencies are underlined)

TSC2:

5' - TAACAAAGGCACATAGGCTGC - 3'
5' - AATGAGGCTCTCATACACTCG - 3'
5' - AAACCCTTAGTGATATAAGGG - 3'
5' - ATATCAAGTTTAAGAGAGG - 3'
5' - ATTGGAGAACACATATCGGC - 3'
Rheb:
5’ - ACCAACATAATAGGTATCTGC - 3’
5’ - AAGACTTTTCTTTGTAAGCTG - 3’
5’ - AAATTGGCCTTCAACAAACTG - 3’
5’ - TTTATCTTCAAGGATGACGGG - 3’
5’ - AATATCTATGGAGTATGTCTG - 3’

PRAS40:
5’ - TTCTGGAAGTGCCTGGTATTG - 3’
5’ - TAATATTTCCGCTTCAGCTTC - 3’
5’ - ATTCTCCTCATCTGACGATCG - 3’
5’ - AACCGGGATACAAATTGTCAGG - 3’

4.2.2 Predictions based on shRNA-mediated depletion of regulatory proteins

To predict how system dynamics are affected by depletion of regulatory proteins, certain parameters were adjusted across the ensemble. For example, to represent 80% knockdown of Rheb, the parameter $K_r$ was reduced by 80%; i.e., the value of $K_r$ in each parameter set was multiplied by 0.2. Likewise, for TSC2 and PRAS40 knockdown, the parameters adjusted are $k_r$ and $K_p$, respectively. Then the estimated pS6K1 time course was computed for each parameter set (with the previously determined alignment factor applied), and the ensemble mean constitutes the model prediction. To allow the model prediction to be compared to the corresponding experiment, both were normalized so that the mean of the control (for the same time points) equals 1.
4.2.3 Steady-state analysis based on fixed Akt activity

In this analysis, the Akt activity fraction, \( a \), was fixed at various values from 0 to 1 and was considered as the input. The corresponding steady-state values of \( g, p, r, \) and \( m \) were derived by setting the right-hand sides of the differential equations equal to zero and simplifying the resulting algebraic equations. These equations were solved for each parameter set to generate ensemble predictions.

4.3 Results

4.3.1 Both Akt/TSC2/Rheb and Akt/PRAS40 substantially regulate mTORC1

To assess the contributions of the two arms of the putative feedforward loop, we designed a new set of perturbation experiments that could be directly compared to \emph{a priori} model predictions. Specifically, we chose to deplete each of the major regulatory proteins (TSC2, Rheb, and PRAS40) by shRNA-mediated knockdown to generate various cell lines. Initially, five shRNAs were tested for each of TSC2 and Rheb, and four shRNAs were tested for PRAS40. Thus, we made a total of 15 new cell lines (14 with targeting shRNAs and 1 for the non-targeting, control shRNA). For each targeting shRNA, the extent of protein depletion was quantified by immunoblotting. Finally, for each target we identified the two cell lines with the highest knockdown efficiencies. These cells were stimulated with PDGF, and time courses of S6K1 phosphorylation were compared to the control cell line to determine how the system was perturbed (figure 4.2A).
Figure 4.2: Perturbation of the mTORC1 regulation. A) Stable shRNA-expressing cell lines were established to perturb key regulatory proteins in the Akt/mTORC1 axis: TSC2, PRAS40, and Rheb. For each target, two hairpins targeting different sequences were selected, based on the extent of protein knockdown (% KD, as indicated), along with a non-targeting control line (shNEG). Representative immunoblots (left) and quantification (right) of target protein knockdown and S6K1 phosphorylation for the indicated PDGF stimulation conditions are shown. Phospho-S6K1 data are normalized by total S6K1 and then by the mean value of each shNEG time course. The normalized data are reported as mean ± SE (n = 3 independent experiments). B) The corresponding model predictions are the means of the model ensemble (n = 10,000) for each condition, normalized in the same manner as the data.

The phospho-S6K1 data show that depletion of either TSC2 or PRAS40 significantly enhanced S6K1 phosphorylation in the cells stimulated with a moderate dose of PDGF (0.03 nM), whereas depletion of Rheb diminished the response in cells with a saturating dose (1nM). In all cases, the effect was magnified according to the extent of protein depletion. These data already imply that both arms of the feedforward loop (the parallel deactivation of TSC2 and PRAS40) are significant for regulation of mTORC1.
Turning to the model, we accounted for the estimated degree of protein knockdown in each cell line as a corresponding fractional change in the associated kinetic parameter. Adjusted thusly, each parameter set in the ensemble was used to predict the phospho-S6K1 time course for each scenario relative to the unperturbed control (the latter time course being used to normalize both the data and model predictions) (figure 4.2B). The \textit{a priori} predictions stand in semi-quantitative agreement with the experimental data, validating the modeling approach. Apparently, the dynamic response of the pathway to changing Akt activity, affected by PDGF dose in the training data set, consistently reflects the sensitivities of mTORC1 to regulation by TSC1/2/Rheb and PRAS40, as probed by the shRNA depletion experiments.

4.3.2 Analysis of the model reveals neutralization of negative regulators and seesaw regulation as dynamical motifs of the Akt/mTORC1 signaling axis

To gain further insight into the model predictions, we performed a computational analysis of the steady state, with the variable representing Akt activity artificially held at various fixed values. As anticipated based on inspection of the kinetic data, phosphorylation of TSC2 and of PRAS40 are predicted to be readily saturated with increasing Akt activity, whereas the predicted responses of Rheb-GTP and mTORC1 activity are far less so (figure 4.3). This is attributed to the indirect mechanism of mTORC1 activation, whereby phosphorylation by Akt neutralizes the negative regulators TSC1/2 and PRAS40. We recently proposed deactivation/neutralization of a negative regulator as a common but as yet underappreciated motif in signaling networks (Chapter-2) (Rahman and Haugh, 2014); the mechanism yields a broader range of sensitivity to the input, beyond saturation of regulator phosphorylation, because it is the unphosphorylated state of the regulator that exerts influence on the pathway (figure 4.3). This property of the motif is manifest in the model predictions.
and, accordingly, explains the ability of the model to capture complex features of the kinetic data.

![Figure 4.3: Neutralization of negative regulators. Ensemble predictions of the model at steady state (mean ± SD, n = 10,000) show that phosphorylation of the negative regulators TSC2 and PRAS40 approach saturation at low stoichiometries of Akt activation. This offers maximal sensitivity of Rheb-GTP loading and mTORC1 activation, because TSC2 and PRAS40 regulate those responses in their unphosphorylated states.](image)

We further used the steady-state analysis to explore the status of the mTORC1 complex, revealing another characteristic pattern (figure 4.4A). The model predicts that almost all of the mTORC1 is either PRAS40-bound or in the Rheb-induced state, rather than the so-called free state (neither PRAS40-bound nor Rheb-induced). The implication is that Akt exerts feedforward control of mTORC1 activity via a “seesaw” regulation mechanism (figure 4.4B), simultaneously weakening the inhibitory effect of PRAS40 while boosting the catalytic
function of mTORC1 through Rheb. Accordingly, the interpretation of the model is that
depletion of TSC2, PRAS40, or Rheb perturbs that balance (figure 4.5).

Figure 4.4: Seesaw regulation of mTORC1. A) Steady-state analysis of the model predicts the
status of the mTORC1 complex (mean ± SD, n = 10,000), with a transition from largely PRAS-
inhibited and largely Rheb-induced states. As shown for 10 randomly selected parameter sets
(right), this mode of enzyme regulation encodes an ultrasensitive, nonlinear input-output
relationship over the relevant range of Akt activity (i.e., with Akt activity scaled as it was in
the alignment to phospho-Akt data, where a value of 1 corresponds to the mean of the 1 nM
PDGF time course). B) Depiction of the seesaw mechanism and mathematical expression of
mTOR sensitivity to Akt.
Figure 4.5: Predicted effects of regulatory protein depletion on the status of the mTORC1 complex. The results for the control (ensemble mean ± SD) are from figure 4.4A. In the other panels, results for the ensemble mean of the control are reprised (dashed curves) for comparison to that of 80% knockdown (KD) of TSC2, PRAS40, or Rheb (solid curves). As a measure of pathway potency, the bar plot at right compares the Akt activity required to achieve 50% of mTORC1 in the active, Rheb-induced state for the control and knockdown conditions.

For the predictions of protein depletion (each at 80% knockdown), figure 4.5 shows that the more direct interactions are predicted to have greater effects on the balance between mTORC1 activation vs. inhibition. Knockdown of Rheb, the activator of mTORC1, is predicted to have the greatest effect of the three regulator proteins, followed by knockdown of PRAS40, which inhibits mTORC1 via direct binding. The weakest effect is that of TSC2 knockdown, which influences mTORC1 indirectly via Rheb.

What quantitative feature of this regulation mechanism is encoded in the kinetic data? A signature of this mode of regulation is a nonlinear, ultrasensitive response of mTORC1 activity to Akt activation [figure 4.4A (right)]. As shown through a mathematical analysis (Appendix), the maximum sensitivity of the pathway is achieved when mTORC1 activity is
proportional to the Rheb-GTP level and inversely proportional to the concentration of unphosphorylated PRAS40; given that PRAS40 phosphorylation is close to saturation, mTORC1 activity is roughly proportional to the Akt activity squared (figure 4.4B), i.e., with a Hill coefficient approaching 2.

Figure 4.6. Detailed view of the ensemble fit to kinetic data, with the parameter constraints $\alpha = \beta = 1$. Each quadrant shows the fit for a different dose of PDGF as indicated; note that y-axes are scaled differently to allow the quality of fit to be more fully assessed. The ensemble averaged (mean) model output (green curves) may be compared to the experimental data (red circles). Black dashed lines indicate mean ± SD for the model output, indicating the level of variation in the fit. The quality of fit may be visually compared to that shown in figure 4.3, and numerical comparisons are given in Table 3A1.
Across the ensemble of parameter sets chosen by our fitting algorithm, the value of the parameter that determines competition vs. synergy between the PRAS40-bound and Rheb-induced states of mTORC1 ($\beta$) was consistently chosen such that the two states are mutually exclusive. According to the same mathematical analysis noted above, this is not a necessary condition for ultrasensitivity; the scenario in which Rheb-GTP and PRAS40 bind mTORC1 independently, but PRAS40 binding trumps Rheb induction, is also capable of ultrasensitivity and is therefore only subtly different from the situation favored by the fitting algorithm. Constraining the fitting algorithm to conform to this scenario ($\alpha = \beta = 1$) yielded a poorer fit, but the difference is quite modest and imperceptible by eye (Table 3A1, figure 4.6). However, this model variation yielded noticeably inferior predictions (figure 4.7) that only qualitatively match the experimental measurements shown in figure 4.2A.
Figure 4.7: Model predictions with the parameter constraints $\alpha = \beta = 1$. The experimental data are reprised from figure 4.2A. The corresponding model predictions, with the parameters $\alpha$ and $\beta$ set equal to 1, are the means of the model ensemble ($n = 10,000$) for each condition, normalized in the same manner as the data.

### 4.4 Discussion

After building a mathematical model that successfully fits available data, the logical next step is to devise experiments in which the system is perturbed in ways that can be predicted by the model. Here, model predictions related to protein knockdown experiments showed a semi-quantitative match. In contrast, the prediction made with an alternative model (with $\alpha = \beta = 1$) did not, even though the fit to the training data was not substantially poorer in that case. This might serve as a case study exemplifying how a model might fit the constraining data well, yet the prediction might be far off from the validating experiments; in such cases, further refinement or a reformulation of the model might be attempted.
The model predictions presented here shed light on the hypothesis that multiple modes of mTORC1 activation (via activation of Rheb and neutralization of PRAS40) act in concert in a feedforward loop mediated by Akt. Specifically, the model predicts that most of the mTOR is either PRAS40-bound or Rheb-activated, and the role of Akt is shift that balance in a coordinated way (seesaw regulation). To test this hypothesis, we performed preliminary co-immunoprecipitation experiments to pull down mTOR, PRAS40, or Rheb and thus interrogate the status of mTORC1 complex. The chemical crosslinking agent DSP was used to stabilize protein-protein interactions, and the results were compared to the condition without crosslinking. This pull-down strategy did not yield conclusive results, possibly because the antibodies used to capture the corresponding proteins might not be able to bind when the protein is in complex. We found no literature describing such a pull-down strategy for endogenous mTORC1 components; instead, overexpression of epitope-tagged proteins was employed (Foster et al., 2010; Jain et al., 2014; Ramirez-Rangel et al., 2011; Wang et al., 2007; Yip et al., 2010). There is also no reported evidence of a direct, physical interaction between Rheb and mTOR in cells.

Seesaw regulation, with competitive Rheb and PRAS40 influences on substrate interactions, would be consistent with the Rheb-induced state representing mTORC1 with substrate bound, implying that mTORC1 substrates also compete with each other for a limiting amount of enzyme. An analogous enzyme limitation for ERK was previously described (Ahmed et al., 2014; Kim et al., 2011b; Kim et al., 2011c). mTORC1 phosphorylates and activates/inactivates many downstream substrates, including ULK1, ATG13, 4E-BP1, and others along with S6K1. If mTORC1 availability were limiting, modulating the expression of
high affinity substrates would have an inverse effect on the phosphorylation status of other substrates. shRNA knockdown strategies could be employed to test this hypothesis.

The model analysis also reveals that the mTORC1 activation response is ultrasensitive with respect to Akt activity (figure 4.4A). As discussed earlier, ultrasensitivity arises due to the dependence of mTORC1 activation on two Akt-mediated mechanisms. Further mathematical analysis shows that mTORC1 activity responds to Akt activity with a Hill coefficient approaching 2 for the perfectly competitive case (appendix).
References


CHAPTER 5
Conclusions and future work
5.1 Conclusions

The mTOR protein kinase is a key hub mediating diverse cellular processes. The mTORC1 complex links signal transduction and cell metabolism, and its dysregulation has been linked to diabetes, aging, and cancer (Cargnello et al., 2015; Guertin and Sabatini, 2007). Activation of this pathway increases anabolism and decreases catabolism and thus increases biosynthesis and cell survival. Although significant work has been done to define the regulation of mTORC1, quantitative studies and formulation of kinetic models of the pathway were lacking. In this thesis, we identified PI3K-Akt signaling as the dominant pathway of mTORC1 activation in NIH3T3 fibroblasts. A mathematical model of this pathway were built, considering deactivation of negative regulators as a general motif, and a consistent fit between the most successful model and newly acquired kinetic data was achieved. Moving forward, we used the model to predict the outcomes of perturbations mimicking depletion of key regulatory proteins of a putative feedforward loop mediated by Akt. This approach quantified the substantial contributions of both TSC2-dependent and PRAS40-dependent regulation of mTORC1. Further analysis of the model implicates seesaw regulation of mTORC1 at the level of the complex, with ultrasensitivity of mTORC1 activity relative to Akt activity as an emergent property that is essential for a good fit to the available data. These insights advance our understanding of mTORC1 signaling, and the model and data set we established could serve as a framework for further analysis and hypothesis generation.
5.2 Future work

5.2.1 Analysis of mTORC1 binding states, guided by model predictions

The model predictions concerning the status of mTORC1 suggest that most of the complex is either Rheb-induced or PRAS40-bound; a sparing amount of mTORC1 is in the free state. Experiments should be done to test this hypothesis. We performed co-immunoprecipitation experiments, without and with chemical crosslinking, to pull down mTOR, Rheb, or PRAS40 and detect the presence of other, associated proteins. This approach proved unfruitful, possibly because the ability of the antibodies to capture the proteins while they are in complex might be compromised. As discussed under Chapter-4, the Rheb-induced state in the model might represent mTORC1 saturated with its many substrates. If depletion or overexpression of a prominent substrate increases or decreases phosphorylation of others and vice versa, it would offer evidence that free mTORC1 is limiting.

5.2.2 Elucidate mTORC1 signaling dynamics in dysfunctional cell context

A dysfunctional signaling network may be defined as a network in which a signaling protein is either silenced or autonomously activated (loss or gain of function, respectively). Spontaneous gene mutations are considered by many to be the primary basis of cancer progression (Hanahan and Weinberg, 2011), for example. Molecular interventions such as introduction of oncogenes and/or gene deletions can be done to mimic cancerous/precancerous cell states and study how a signaling network responds under extreme conditions. Cell lines with oncogenic variants or cell lines derived from genetically engineered mouse models (GEMMs) of melanoma can be used to study the important pathways in dysfunctional networks. GEMMs are emerging as better defined, genetically stable experimental models to study metastasis and responsiveness to small-molecule drugs (Sharpless and Depinho, 2006;
Winter et al., 2011). Dysfunctional systems can also be investigated theoretically if there is an already established mathematical model of the system.

5.2.3 Introducing Ras/Raf/MEK/ERK pathway and mTORC2 in the model system

Although the contribution of the Ras/Raf/MEK/ERK pathway in the activation of mTORC1 was found to be minimal in our experimental system, signaling through this pathway might be important in other contexts. The parallel contributions of both Ras/Raf/MEK/ERK and PI3K/Akt pathways to mTOR activation is established in the literature. Cell lines having active Ras or Raf mutant show amplified contribution of this pathway to downstream signaling (Ballif et al., 2005; Huang and Manning, 2008; Kawashima et al., 2015; Winter et al., 2011; Zheng et al., 2009). So, incorporating this pathway into our model could be a key refinement.

Full activation of Akt requires phosphorylation on two sites: T308 and S473. T308 phosphorylation is mediated by PtdIns(3,4,5)P3 and the serine-threonine kinase, PDK1, whereas S473 is phosphorylated by mTORC2 (and perhaps other kinases) (Laplante and Sabatini, 2009; Yang et al., 2015). The activity of mTORC2 thus feeds into the activation of mTORC1, and hence it will be important to understand how mTORC2 is regulated by external factors and how that affects the status of the Akt/mTORC1 pathway.

5.2.4 mTORC1 signaling to cell functional response

mTORC1 signaling affects numerous cellular functions. mTORC1 enhances protein synthesis through parallel phosphorylation of S6K1 and 4E-BP1, enhances lipid synthesis by phosphorylating SREBP1, and inhibits autophagy by phosphorylating ULK1/ATG13 (figure 5.1). We noted previously that certain pathways downstream of mTORC1 follow the deactivation of a negative regulator motif, such as 4E-BP1 regulation (Rahman and Haugh, 2014) (Chapter-2).
Figure 5.1: mTORC1 control of protein synthesis, lipid synthesis and autophagy via S6K1, 4E-BP1, SREBP1 and ULK1/ATG13

The rate of overall protein synthesis can be measured using a commercial kit (e.g., Click-iT HPG from ThermoFisher). Along with the quantification of protein synthesis, the phosphorylation levels of S6K1 and 4E-BP1 can be quantified using phospho-specific antibodies, and a correlation between S6K1 and 4E-BP1 phosphorylation and protein synthesis rate can be made. Likewise, autophagy can be quantified using an autophagy detection kit (Abcam), which can be correlated to mTORC1-mediated ULK1/ATG13 phosphorylation. These measurements would allow us to develop a simple, data-driven model of cell physiology controlled by mTORC1. Perturbed mTORC1 signaling can also be related to cellular functions. Depletion of either TSC2 or PRAS40 yields a dysregulated signaling network that apparently extends the range of maximal mTORC1 signaling in our system, whereas Rheb depletion perturbs the network in the opposite way. Protein synthesis can be quantified along with mTORC1-dependent phosphorylation of S6K1 and 4E-BP1 for the 7 shRNA-expressing cell lines described under Chapter-4, and the hypothesis that protein synthesis is related to mTORC1 signaling can be tested further in a quantitatively consistent way.
5.2.5 Role of S6K1 negative feedback to IRS-1

mTORC1 is a critical signaling hub in the regulation of metabolism governed by insulin or insulin-like growth factors (IGFs). Whereas PDGF receptors directly activate type IA PI3Ks, leading to activation of Akt, activation of PI3Ks by insulin and IGF receptors require the adaptor protein, insulin receptor substrate (IRS)-1 (Dann et al., 2007; Manning, 2004). As depicted in figure 1.5, mTORC1 activates S6K1, which phosphorylates IRS-1 on S270, S636 and S639, which inhibits PI3K activation (Shah and Hunter, 2006; Zhang et al., 2008). Inhibition of mTORC1 by rapamycin suppresses the regulation of insulin-stimulated PI3K signaling, and over-activation of compensatory pathways enhances cell survival in an mTORC1-independent manner (Rozengurt et al., 2014; Veilleux et al., 2010). Our mathematical model could be extended to the systems in which IRS-1 plays an important role by incorporating IRS-1 mediated PI3K activation and S6K1-mediated negative feedback.

5.2.6 Evaluate the generality of activation by deactivation of negative regulator motif amongst other Akt substrates

As described in Chapter-1, Akt is a general mediator of growth factor-induced survival and has been shown to suppress apoptotic death induced by a variety of stressors, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion, and DNA damage (Ahmed et al., 1997). Akt promotes cell growth, proliferation, and survival by phosphorylating protein substrates such as BAD, FoxO and GSK3 (Engelman, 2009). Akt-mediated phosphorylation inhibits the activities of these proteins, and we hypothesize that the deactivation of a negative regulator motif applies generally to those Akt substrates. Experiments can be directed towards identifying the phosphorylation kinetics of those proteins under defined stimulation and/or stress conditions. If the deactivation of a negative regulator
motif is present, our work on TSC2 and PRAS40 has shown that phosphorylation of the negative regulator near saturation is a hallmark.
References


APPENDICES
Appendix A: Development of the models for two mechanisms (chapter-2)

We consider two distinct mechanisms by which a protein kinase might promote signal transduction through substrate phosphorylation: (I) increasing the activity of a positive regulator (activator) or (II) decreasing that of a negative regulator (deactivator). In both cases, the common step is phosphorylation of the regulatory molecule by the kinase. To describe this in the simplest manner possible, we consider that the relative activity of the upstream kinase may be represented by a time-dependent rate constant, \( k_k(t) \). Neglecting concentration gradients and saturation of the upstream kinase or of the opposing (constitutively active) phosphatase(s), we express the conservation of phosphorylated activator (mechanism I) as follows.

\[
\frac{dA_p}{dt} = k_k(t)A_k \cdot k_p; \quad A_p(0) = 0 \quad (S1)
\]

In the equation above, \( A_p \) is the concentration of phosphorylated activator, \( A \) is the concentration of unphosphorylated activator, and \( k_p \) is the pseudo-first-order rate constant of protein dephosphorylation. With the assumption that the sum of the phosphorylated and unphosphorylated regulator is conserved during the time scale of interest, we define the fraction of phosphorylated regulator as the dimensionless variable, \( \phi \), and we define the dimensionless signal function, \( s(t) \), to scale \( k_k(t) \) by \( k_p \).

\[
A + A_p = A_{Tot} = \text{constant}
\]

\[
\phi = \frac{A_p}{A_{Tot}}; \quad s(t) = \frac{k_k(t)}{k_p} \quad (S2)
\]

Substituting these definitions into Eq. S1 yields Eq. 1 in the main text, reprised here.

\[
\frac{d\phi}{dt} = k_p[s(1 - \phi) - \phi]; \quad \phi(0) = 0
\]

Up to this point, mechanism II is developed identically (i.e., Eq. 1 in the main text applies to
both mechanisms), except for the conceptual distinction that the substrate of the kinase is a deactivator; therefore, $D$ and $D_p$ take the place of $A$ and $A_p$, respectively.

Next we consider the downstream response element, which is found in either an inactive (off) or active (on) state, with concentrations $R$ and $R^*$, respectively. Following analogous assumptions as above, we write conservation equations for two mechanisms — activation (mechanism I) and relief of deactivation (mechanism II) — as follows.

$$\frac{dR^*}{dt} = \begin{cases} (k_{a,0}A + k_{a,max}A_p)A_{Tot}^{-1}R - k_{d,0}R^* & (I) \\ k_{a,0}R - (k_{d,0}D + k_{d,min}D_p)D_{Tot}^{-1}R^* & (II) \end{cases}$$

(S3)

In each of these equations, the first term describes activation, and the second, deactivation. In mechanism I, the activation term contains contributions from both the unphosphorylated and phosphorylated activator, with rate constants $k_{a,0}$ and $k_{a,max}$, respectively (dividing the activation term by the constant $A_{Tot}$ makes these rate constants pseudo-first order); the deactivation rate constant is fixed at $k_{d,0}$. Conversely, in mechanism II, the deactivation term contains contributions from both the unphosphorylated and phosphorylated deactivator, with rate constants $k_{d,0}$ and $k_{d,min}$, respectively (dividing the deactivation term by the constant $D_{Tot}$ makes these rate constants pseudo-first order); in this mechanism, the activation rate constant is fixed at $k_{a,0}$. With the assumption that the total concentration of the response element ($R_{Tot} = R + R^*$) is constant, and with the definition $\rho = R^*/R_{Tot}$ (along with the definition of $\phi$ for each mechanism), Eq. S3 is modified to obtain Eq. 2 in the main text, reprised here.

$$\frac{d\rho}{dt} = \begin{cases} [k_{a,0} + (k_{a,max} - k_{a,0})\phi](1 - \rho) - k_{d,0}\rho & (I) \\ k_{a,0}(1 - \rho) - [k_{d,0} - (k_{d,min})\phi]\rho & (II) \end{cases}$$

The initial conditions are assigned as follows, so that $\rho$ is stationary when $\phi = 0$ for either mechanism.
\[ \rho(0) = \frac{k_{a,0}}{k_{a,0} + k_{d,0}} \]  

(S4)

To set the models for mechanisms I and II on a common basis for comparison, we enforce that both mechanisms yield the same maximum steady-state value of \( \rho \) (i.e., with \( \phi_{ss} = 1 \)), which is achieved if we define a common, dimensionless gain parameter, \( g \), as follows.

\[ g = \frac{k_{a,\text{max}}}{k_{a,0}} = \frac{k_{d,0}}{k_{d,\text{min}}} \]  

(S5)

Defining \( K = k_{a,0}/k_{d,0} \), each conservation equation is reduced to a dimensionless form with only two adjustable constants (\( g \) and \( K \)) as follows. Thus, main text Eq. 2 was reduced to main text Eq. 4, reprised here.

\[ \frac{1}{k_{d,0}} \frac{d\rho}{dt} = \left\{ K \left[ 1 + (g - 1)\phi_{ss} \right] (1 - \rho) - \rho \right\} K(1 - \rho) - [1 - (1 - g^{-1})\phi_{ss}] \rho \]  

(I)

\[ \rho(0) = \frac{K}{1 + K} \]

(II)

\[ \rho(0) = \frac{K}{1 + K} \]

**Analysis of the steady state**

For constant \( s \), the steady-state solution of main text Eq. 1, \( \phi_{ss} \), is as follows.

\[ \phi_{ss} = \frac{s}{1 + s} \]  

(S6)

For each of the two mechanisms, the steady-state response \( \rho_{ss} \) is derived in terms of \( \phi_{ss} \), and hence in terms of \( s \), as follows.

\[ \rho_{ss} = \left\{ \begin{array}{ll}
K [1 + (g - 1)\phi_{ss}] & 1 + K [1 + (g - 1)\phi_{ss}] \\
K & 1 - (1 - g^{-1})\phi_{ss} + K
\end{array} \right\} = \frac{K(1 + gs)}{1 + K + (1 + gK)s} \]  

(I)

(S7)

\[ \rho_{ss} = \left\{ \begin{array}{ll}
K [1 + (g - 1)\phi_{ss}] & 1 + K [1 + (g - 1)\phi_{ss}] \\
K & 1 - (1 - g^{-1})\phi_{ss} + K
\end{array} \right\} = \frac{K(1 + gs)}{1 + K + g^{-1}(1 + gK)s} \]  

(II)

To lend additional insight, the steady-state response may be expressed as a fold change relative
to the basal value.

$$\frac{\rho_{ss}(s) - \rho(0)}{\rho(0)} = \begin{cases} \frac{(g - 1)s}{1 + K + (1 + gK)s} & (I) \\ \frac{(g - 1)s}{g(1 + K) + (1 + gK)s} & (II) \end{cases} \quad (S8)$$

As one might expect, each of these fold-change expressions can be rearranged to give the familiar hyperbolic form. Adopting the vernacular of a pharmacological dose-response relationship, we define the $EC_{50}$ here as the value of $s$ that yields the half-maximal value of the fold change. By rearrangement of Eq. S8, we obtain

$$EC_{50} = \begin{cases} \frac{1 + K}{1 + gK} & (S9) \end{cases}$$

By inspection of Eq. S9, one concludes the following.

1) The $EC_{50}$ value of mechanism I is less than 1, the value of $s$ for which $\phi_{ss} = 0.5$, provided that $g > 1$ (phosphorylation activates the regulator). Therefore, mechanism I generally saturates at a lower value of $s$ relative to phosphorylation of the positive regulator. The substrate of the activator (the response element in the ‘off’ state) is progressively depleted as the input increases, and so there is sub-linear sensitivity of the response with respect to the increasing activity of the activator. This is the nature of a sequential activation pathway with potential for saturation at each step.

2) The $EC_{50}$ value of mechanism II is greater than 1, the value of $s$ for which $\phi_{ss} = 0.5$, provided that $g > 1$ (phosphorylation deactivates the regulator). Therefore, II generally saturates at a higher value of $s$ relative to phosphorylation of the negative regulator. In this case, the substrate of the deactivator (the response element in the ‘on’ state) becomes more abundant as the input increases, and so there is supra-linear sensitivity of the response with respect to
the decreasing activity of the deactivator. This offsets the decreasing sensitivity of $\phi$ with respect to $s$.

3) Mechanism II has an $EC_{50}$ value that is greater, by a factor of $g$, than that of I. Thus, as $g$ is increased to enhance the maximum fold-change of the response, the dynamic range of $s$ (over which the response shows near-linear sensitivity) shrinks for I, whereas it is expanded for II.

Since $g$ is defined so that the maximum and minimum values of $\rho_{ss}(s)$ are the same for both mechanisms, the higher $EC_{50}$ for mechanism II implies a lower sensitivity in the limit $s, \phi_{ss} \ll 1$, as shown in figure 2.2 d&e. For mechanism II, greater sensitivity near saturation implies low sensitivity when the system is far from saturation, whereas the opposite is well appreciated to be true for the ‘canonical’ mechanism I.

**Analysis of time scales**

Transient behaviors of the two mechanisms are shown in figure 2.4 of the paper. The results were obtained by numerical integration of the differential equations, using a stiff implicit solver in MATLAB. Though this is simple enough, we find that approximations of the ‘exact’ solutions are instructive. Such analyses are outlined below.

In the examples shown in figure 2.4 a&b, a step change in the input function $s(t)$ is assumed, i.e., constant $s$ for $t > 0$. The transient solution of main text Eq. 1 for these conditions is as follows, with $\phi_{ss}$ taken from Eq. S6.

$$\phi(t) = \phi_{ss}\{1 - \exp[-(1 + s)k_p t]\} \quad (S10)$$
Therefore, the kinetics of $\phi(t)$ approaching the steady-state value become progressively faster as $s$ increases, with a time scale of $[(1 + s)k_p]^{-1}$. Based on the parameter values chosen for the calculations shown in figure 2.4, we reasoned that the kinetics of $\phi(t)$ were relatively fast. With this conjecture, we substitute the approximation $\phi(t) \approx \phi_{ss}$ in main text Eq. 2 and simplify as follows.

$$\frac{d\rho}{dt} \approx \begin{cases} \frac{k_{d,0}}{1 - \rho_{ss}}(\rho_{ss} - \rho) & (I) \\ \frac{k_{d,0}}{\rho_{ss}}(\rho_{ss} - \rho) & (II) \end{cases}$$

(S11)

For the calculated examples, $k_{d,0} = 0.1k_p$, $k_{a,0} = 0.005k_p$, and $\rho_{ss}$ varies from 0.047 ($s = 0$) and 0.33 ($s >> 1$). Therefore, we confirm that the time scale of $\phi(t)$ is never rate limiting under the conditions tested.

This analysis also shows how the kinetics of $\rho(t)$ for mechanism I generally become faster, and how the kinetics for II become slower, as $s$ increases. By inspection of Eq. S11 above, we identify the characteristic time scale $\tau$ as the inverse of the effective rate constant, i.e., with $d\rho/dt \approx \tau^{-1}(\rho_{ss} - \rho)$. The time constant thusly identified for mechanism I is $(1 - \rho_{ss})/k_{d,0}$, which decreases (faster kinetics) as $\rho_{ss}$ increases. As explained in the main text, this is intuitive because signaling increases the frequency of activation. The time constant for mechanism II is $\rho_{ss}/k_{a,0}$, which increases (slower kinetics) as $\rho_{ss}$ increases; here, signaling decreases the frequency of deactivation, while that of activation is constant.

In the examples shown in Fig. 2c&d, a time-decaying input was considered.

$$s(t) = s(0) \exp\left(-k_{\text{decay}}t\right)$$

(S12)

The rate constant of decay was $k_{\text{decay}} = 0.03k_p$, i.e., slow enough for $\phi(t)$ to respond according to the following quasi-steady state approximation.
\[ \phi(t) \approx \frac{s(t)}{1 + s(t)} \quad S(13) \]

It is readily shown that this function decays, on a relative basis, slower than does \( s(t) \). This is intuitive when \( s(t) \gg 1 \), because \( \phi(t) \) is pegged close to 1. This insight along with the steady-state sensitivity results presented in figure 2.2 d&e provides at least a qualitative explanation of the kinetics shown in figure 2.4 c&d. For mechanism I, the slow decay of \( \phi(t) \) for saturating \( s(0) \) is compounded by the modest sensitivity of \( \rho \) to \( \phi(t) \) near saturation (figure 2.2d); thus, the response peaks rapidly but decays slowly under such conditions (figure 2.4c). In contrast, mechanism II shows ultrasensitivity to \( \phi(t) \) in that regime (figure 2.2e); thus, after a prolonged equilibration period (reflected in the time at which the response achieves its peak, consistent with the slow kinetics shown in figure 2.4b), the time scale associated with the decay of the response is much closer to that of \( s(t) \) (figure 2.4d).
Appendix B: Variations of the model tested (chapter-3)

Along with the main model described in the previous sections, two variations were tested; we refer to these ‘control’ models as Rheb only and PRAS40 only. In these variations, the equation describing the regulation of mTOR (species $m$) is replaced by a differential equation as follows.

\[
\frac{dm}{dt} = k_m r (1 - m) - k_m m; \quad m(0) = \frac{k_m r_0}{k_m r_0 + k_m} \quad \text{(Rheb only)}
\]

\[
\frac{dm}{dt} = k_m (1 - m) - k_m (1 - p) m; \quad m(0) = \frac{k_m}{k_m + k_m (1 - p_0)} \quad \text{(PRAS40 only)}
\]

Thus, in the Rheb only model it is assumed that mTOR is simply activated by Rheb; modulation of PRAS40 is neglected. Conversely, in the PRAS40 only model, mTOR is simply activated through neutralization of PRAS40. Table 3A1 shows a comparison of how well the main model and these two model variations fit the data. Cumulative SSD values of each readout (Akt, TSC2, PRAS40 and S6K1), reported as mean ± s.d. for the ensemble, are reported; a lower cSSD indicates a closer overall fit. The main model consistently outperforms the two variations in terms of the cSSD values for each readout. Finally, a comparison index that penalizes models having more adjustable parameters, the Akaike Information Criterion (AIC) (Akaike, 1974), was calculated for each model (lower AIC is better). The main model and PRAS40 only model have approximately the same mean AIC values, despite the main model having 4 more adjustable parameters. That said, the subsequent measurements in Rheb- and TSC2-depleted cells show that the underlying assumption of the PRAS40 only model is false.

Also shown in Table 3A1 are the results of an alternative fit of the main model, with the parameter constraints $\alpha = 1$, $\beta = 1$ ($\alpha\beta1$ model). This corresponds to the scenario where Rheb-GTP and PRAS40 engage mTORC1 independently, with PRAS40 canceling the Rheb-induced
activation of mTORC1. In terms of cSSD and AIC values, the αβ1 model does not do well compared to main model.

<table>
<thead>
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<th></th>
<th>Main model</th>
<th>Rheb only</th>
<th>PRAS40 only</th>
<th>α = 1, β = 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>cSSD_{Akt}</td>
<td>2.93 ± 0.40</td>
<td>4.34 ± 0.39</td>
<td>3.23 ± 0.40</td>
<td>3.93 ± 0.37</td>
</tr>
<tr>
<td>cSSD_{TSC2}</td>
<td>2.48 ± 0.26</td>
<td>3.74 ± 0.44</td>
<td>2.46 ± 0.25</td>
<td>2.34 ± 0.23</td>
</tr>
<tr>
<td>cSSD_{PRAS40}</td>
<td>1.17 ± 0.15</td>
<td>1.01 ± 0.15</td>
<td>1.52 ± 0.17</td>
<td>1.32 ± 0.21</td>
</tr>
<tr>
<td>cSSD_{S6K1}</td>
<td>2.56 ± 0.24</td>
<td>3.84 ± 0.41</td>
<td>2.77 ± 0.27</td>
<td>3.57 ± 0.36</td>
</tr>
<tr>
<td>AIC (N=84)</td>
<td>106.2</td>
<td>131.1</td>
<td>105.5</td>
<td>118.9</td>
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<tr>
<td># of parameters (k)</td>
<td>27</td>
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<td>25</td>
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</tbody>
</table>

Table 3A1: Model comparisons. Cumulative SSD values for each readout (ensemble mean ± SD) and the Akaike Information Criterion (AIC) value is listed for each model. The formula for AIC is $AIC = N \left[ 1 + \ln(2\pi) + \ln(cSSD/N) \right] + 2k$, where $N$ is the number of observations and $k$ is the number of parameters.

References

Appendix C: Mathematical analysis of mTORC1 regulation by PRAS40 and Rheb-GTP: Ultrasensitivity (chapter-4)

The model considers two distinct ways for PRAS40 to antagonize Rheb-induced mTORC1 activity: through binding competition ($\beta < 1$) or through modulation of catalytic activity ($\alpha < 0.5$). Although combinations thereof are allowed in the model, we find it instructive to examine the limiting cases, $\beta = 0$ (perfectly competitive) vs. $\beta = 1$, $\alpha = 1$ (noncompetitive).

Considering the perfectly competitive case, with $\beta = 0$ and $\alpha$ arbitrarily set to 1, the mTORC1 activity is given by

$$m = \frac{K_r r}{1 + K_r r + K_p (1 - p)}$$

At steady state, it is readily shown that $r$ and $p$ depend on Akt activity $a$ as follows.

$$r = \frac{r_0 a_r^* + a}{a_r^* + a}; \quad a_r^* = \frac{k_{g0} + k_{-g}(1 + k_{-r}/k_r)}{k_g}$$

$$p = \frac{p_0 a_p^* + a}{a_p^* + a}; \quad a_p^* = \frac{k_{p0} + k_{-p}}{k_p}$$

Substituting,

$$m = \frac{K_r [r_0 a_r^* a_p^* + (r_0 a_r^* + a_p^*)a + a^2]}{[1 + K_r r_0 + K_p (1 - p_0)] a_r^* a_p^* + [(1 + K_r r_0) a_r^* + (1 + K_r + K_p (1 - p_0)] a_p^*} a + (1 + K_r) a^2$$

This equation shows that mTORC1 activity will respond to that of Akt with substantial ultrasensitivity (with a Hill coefficient approaching 2), provided that certain conditions are met. Evaluating the numerator in the equation above, one condition for ultrasensitivity is

$$r_0 a_r^* + a_p^* \ll 1$$
This inequality is readily achieved, requiring only that the basal Rheb-GTP level, $r_0$, is low and that PRAS40 phosphorylation is close to saturation; the latter is required for the sensitivity of the neutralization of a negative regulator motif (Rahman and Haugh, 2014). Another condition for ultrasensitivity is based on the denominator of the $m(a)$ function above:

$$[1 + K_r r_0 + K_p (1 - p_0)]{a_r}^*{a_p}^* \gg \{(1 + K_r r_0){a_r}^* + [1 + K_r + K_p (1 - p_0)]{a_p}^*\}(r_0{a_r}^* + {a_p}^*)$$

In the limit of $r_0$ and $p_0 \approx 0$, this inequality reduces to

$$K_p {a_r}^* \gg (1 + K_r + K_p){a_p}^*$$

This condition is readily met if $K_p \gg 1$ (high affinity of unphosphorylated PRAS40 binding to mTORC1) and ${a_r}^* \gg {a_p}^*$ (again, if PRAS40 phosphorylation is highly saturable).

Turning to the noncompetitive case, the mTORC1 activity is given by

$$m = \frac{K_r r}{(1 + K_r r)(1 + K_p (1 - p))}$$

Substituting the steady-state expressions for $r$ and $p$, and simplifying,

$$m = \frac{K_r [r_0{a_r}^* + (r_0{a_r}^* + {a_p}^*)a + a^2]}{(1 + K_r r_0)[1 + K_p (1 - p_0)]{a_r}^* + [(1 + K_r r_0){a_r}^* + (1 + K_r)(1 + K_p (1 - p_0)]{a_p}^*a + (1 + K_r)a^2}$$

In this case, the numerator inequality is the same, but the denominator inequality (again, in the limit of $r_0$ and $p_0 \approx 0$) is different:

$$K_p {a_r}^* \gg (1 + K_r)(1 + K_p){a_p}^*$$

This imposes a constraint on the magnitude of the parameter $K_r$ that is more stringent than the competitive case, but this scenario is nonetheless capable of an ultrasensitive response.