Stochastic Regulation of Signaling in Lymphocytes

by

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Abstract

Humans are exposed to a variety of infectious pathogens, such as virus, bacteria, etc. But we survive thanks to our immune systems. One of the important immune systems is the adaptive immune system, which mounts pathogen specific immune responses. The orchestrators are lymphocytes, including B cells and T cells. When lymphocytes are stimulated strongly enough by infected or antigen-presenting cells, the signal will be transmitted through a complex network of biochemical reactions underlying the cellular functions. Such a complex network is fundamentally regulated by stochastic principles with intriguing behaviors. This thesis aims to understand the signaling network in lymphocytes and the stochastic regulation behind.

In lymphocytes, mitogen-activated protein (MAP) kinases can be triggered by various stimuli (growth factors, cytokines, etc.) with significant cellular responses (proliferation, inflammation, etc.). We first investigate the roles of upstream proteins, Son of Sevenless (SOS) and Ras guanyl-releasing protein (RasGRP), in MAP kinases, Erk and P38, activation. The signaling pathways of P38 activation are still elusive. In Chapter 3, we study two signaling pathways for P38 activation, the classical pathway through the linker for activation of T cells (LAT) complex and the alternative pathway directly via ZAP-70 molecules. In both of studies, we bring together computational tools, stochastic theories and cell biology by collaborating with biologists, Professor Jeroen Roose and Dr. Jesse Jun, in University of California, San Francisco.

In biochemical systems, like lymphocytes, fluctuations are ubiquitous. Such noise may drive biochemical systems from one stable state to the other with biological significance. In this part of the work, we investigate fluctuation-driven transitions in two biochemical models: genetic toggle switches (GTS) and self/non-self peptide-major histocompatibility complex (MHC) discrimination models. In the GTS model, we introduce dynamical disorder in rate coefficients and study its influences in optimal transition paths, transition rates, and the stationary probability distribution of the system. In peptide-MHC discrimination part, we investigate the effects of a conformational change step of ZAP-70 molecules on the sensitivity and robustness of the discrimination.
To those whom I love, those who love me, and especially my parents and sister:

陈和平 (Chen, Heping) & 王春梅 (Wang, Chunmei), 陈沛 (Chen, Pei)
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This doctoral thesis has been examined by a Committee of the Department of Chemistry as follows:

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Chapter 1

Introduction

On exposure to infectious pathogens, humans survive thanks to the immune system with innate and adaptive immunity. [50, 56] As one type of white blood cells, lymphocytes play a key role in the adaptive immune system. Lymphocytes originate in the bone marrow by the process of hematopoiesis. Then lymphocytes migrate to lymphatic systems and reside in various lymphoid organs, such as lymph nodes, spleen, and thymus. There are two major types of lymphocytes, T lymphocytes (T cells) and B lymphocytes (B cells). On their surfaces, T cells and B cells express immunoglobulin proteins, T cell receptors (TCRs) and B cell receptors (BCRs). The distinct receptors can identify molecular signatures of specific infectious pathogens with responses. For example, TCRs can recognize antigen-derived peptides bound to proteins of the major histocompatibility complexes (MHCs) expressed on the surfaces of antigen-presenting cells (APC). [12, 63, 117, 36, 116] And such TCR-induced signals, transmitted along a complex intracellular network of proteins, may lead to a variety of cell decisions, including proliferation and differentiation. The lymphocytes proliferate and differentiate in a balanced way to maintain a self-renewing immune system. Otherwise, dysfunction of cellular regulation will result in diseases, such as cancer, immunodeficiency or autoimmune diseases [22, 42].

To avoid or conquer these immune disorders, we first need to depict the relationships among interacting proteins as components of the signaling networks in lymphocytes. The development of advanced experimental techniques in biology [5, 47],
enabling us to detect the evolution of specific proteins in the networks and collect more biological data, has shed light on the network modeling and the analysis of signaling mechanisms. In such biochemical networks, biological features (e.g. digital signaling [19], rare transitions among stable states[38], etc.), underlying pathological cellular functions, are fundamentally regulated by stochastic theories[118, 14, 24]. In this thesis, we seek to investigate the stochastic regulations of the signaling in lymphocytes by bringing together experimental techniques in cell biology (e.g., single-cell phospho-flow cytometry[99]), computational tools (e.g., Gillespie algorithm[33, 34, 35]), and theoretical theories in stochastic dynamics (e.g., large deviation theory[28, 113, 44]). Before we go into the details of each chapter, we begin with a brief description of the cellular networks we will focus on and the background of the fluctuation-driven transitions we will study, followed by an outline of the thesis.

1.1 Mitogen-activated protein kinases signaling

The intracellular biochemical reactions form a complex signaling network. As evolutionarily conserved enzymes, mitogen-activated protein kinases (MAPKs) construct a downstream signaling pathway, activated by various extracellular stimuli and in the control of gene expression, cell proliferation, differentiation, apoptosis and other cellular processes. [15, 21, 94] In mammalian cells, there are three main groups of MAP kinases: the extracellular signal-regulated protein kinases (ERK), the P38 protein kinases, and the c-Jun NH2-terminal kinases (JNK). The MAPK signaling cascade is a three-tiered network, composed of MAP kinases, MAPK kinases, and MAPKK kinases or MEK kinases. In terms of phosphorylation processes, these three protein kinases get activated in succession from MAPKKK proteins to MAPKK proteins, and eventually to MAPK proteins. To trigger such a series of activations, a bunch of upstream proteins are involved and some positive or negative feedback loops are formed with biological significance. The functional roles of these upstream proteins involved in the downstream MAP kinase activation are elusive. In this thesis, we focus on the study of ERK and P38 MAP kinases signaling pathways. We, Chakraborty lab, col-
laborate with Professor Jeroen Roose's lab at University of California, San Francisco, to investigate the MAPK signaling by combining in silico computer modeling and simulation with biology-based experiments. The work on requirements and functions of upstream proteins, e.g. Sos of Sevenless (SOS) and Ras guanyl-releasing protein (RasGRP), for ERK and P38 activation is described in Chapter 2. For P38 activation, two distinct pathways, classical and alternative pathways, from TCR signal are proposed by biologists based on experimental observations\[4, 95\]. But the precise functions and contributions of each pathway still remain open questions. In Chapter 3, we explore these two pathways of TCR-mediated P38 activation.

1.2 Fluctuation-driven transitions in biochemical networks

In living cells, the cellular machinery is composed of biochemical reactions underlying biological functions. Biochemical systems sometimes exhibit distinct or even counter-intuitive features due to the inherent stochasticity of the reaction networks with small numbers of molecules involved.\[55, 85, 86\] Recently, more studies have been focused on the stochastic regulation of lymphocyte signaling dynamics with some significant impact. \[49, 3\] For the systems with multistable states, an interesting stochastic behavior is that noise may drive the system from one stable state to another, resulting in functional responses. And the fluctuation of parameters of reaction rates and protein concentrations will also affect such transitions. To obtain such mechanistic pictures, we calculate how small changes in rate parameters and protein concentrations affect the rate of a stochastic transition with biological significance. It is very computationally complex to determine these sensitivities. Because it is nearly too time consuming to sample a fluctuation-driven transition which is very rare. Also, there are a large amount of reaction rates and concentrations in a realistic biochemical network, and each of parameters must be perturbed to determine if it affects the transition significantly. In our Chakraborty group, a semianalytical technique
has been developed that aims to overcome these challenges by exploiting ideas from large deviation theory. We also exploit an efficient geometric minimal action method (gMAM), developed in Vanden-Eijnden group at New York University, to find the optimal paths of the fluctuation-driven transitions. With such a powerful framework, we investigate two biological systems. In Chapter 4, in collaboration with Professor Jian-shu Cao and my colleague, Peter Thill, we investigate the transitions in genetic toggle switches and how it is affected by dynamical disorder in rate coefficients. Chapter 5 is concerned with the fluctuation effects in self/foreign peptide-MHC discrimination.
Chapter 2

ERK but not P38 requires allosteric SOS activation

Thymocytes convert graded TCR signals into positive selection or deletion, and activation of ERK, P38, and JNK MAPKs have been postulated to play a discriminatory role. Two families of Ras Guanine nucleotide Exchange Factors (RasGEFs), SOS and RasGRP, activate Ras and the downstream RAF-MEK-ERK pathway. The pathways leading to lymphocyte P38 and JNK activation are less well defined. We previously described how RasGRP alone induces analog Ras-ERK activation, while SOS and RasGRP cooperate to establish bimodal ERK activation. Here we employed computational modeling and biochemical experiments with model cell lines and thymocytes to show that TCR-induced ERK activation grows exponentially in thymocytes, and that a W729E allosteric pocket mutant SOS1 can only reconstitute analog ERK signaling. In agreement with RasGRP allosterically priming SOS, exponential ERK activation is severely decreased by pharmacological or genetic perturbation of the PLC-γ-diacylglycerol-Rasgrpl pathway. By contrast, P38 activation is not sharply thresholded and requires high level TCR signal input. Rac and P38 activation depends on SOS1 expression but not allosteric activation. Based on computational predictions and experiments exploring whether SOS functions as a RacGEF or adaptor in Rac-P38 activation, we established that the presence of SOS1, but not its enzymatic activity is critical for P38 activation.

1The content of this chapter has been published on Molecular and Cellular Biology[53]
2.1 Introduction

MAP kinase (MAPK) signaling cascades are conserved pathways that can be activated by a wide variety of stimuli and play a role in diverse cellular processes [15]. Early on it was recognized that activation of the ERK MAPK can be graded or switch-like, and this impacts biological outcome [69]. Specifically, stimulation of rat adrenal pheochromocytoma (PC-12) cells with neuronal growth factor (NGF) results in sustained activation of the MAPK ERK and differentiation, whereas stimulation with epidermal growth factor (EGF) elicits transient ERK activation and cell proliferation [69]. Synthetic engineering to rewire the feedback loops in the NGF- and EGF-ERK networks alters cell fate, further demonstrating a causative link between mode of ERK activation and cell biological effect [96]. Individual Xenopus oocytes demonstrate a very large Hill coefficient for ERK activation, and ERK functions as an ultrasensitive switch to convert graded progesterone stimuli into all-or-none biological responses [24]. Various mechanisms have been described to account for the switch-like ERK activation, including high cooperativity intrinsic to the ERK module [68, 78], signal amplification towards digital patterns in Ras nano-clusters [112], dual negative feedback control by phosphatase SHP1 [1], as well as sub-cellular location of cascade activity [40].

T cells can also convert analog receptor input into digital or bimodal ERK activation [1, 19, 62, 81]. Developing thymocytes must undergo all-or-none selection processes to retain functional T cells but delete auto-reactive T cells [104]. The small GTPase Ras in the Ras-RAF-MEK-ERK pathway [31, 18]plays a critical role during thymocyte selection [107]. At least two families of Ras guanine nucleotide exchange factors (RasGEFs) establish T cell receptor (TCR)-induced GTP-loading of Ras in thymocytes, namely RasGRP and SOS [105]. RasGRP1 plays a non-redundant role in Ras-ERK pathway activation; positive selection of thymocytes and TCR-induced ERK phosphorylation is impaired in Rasgrp1−/− mice [22]. Similarly, deletion of RasGRP1 and RasGRP3 in the chicken DT40 model B cell line greatly impairs B cell receptor (BCR)-induced ERK activation [77, 93]. SOS RasGEFs are recruited to the
plasma membrane via the small adapter molecule Grb2 [9, 65]. In addition to other forms of regulation, SOS1’s catalytic activity critically relies on an allosteric binding pocket for Ras previously identified in SOS1’s crystal structure [66]. Efficient binding of RasGTP to this pocket induces a conformational switch to enhance SOS1’s GEF activity [27]. We previously demonstrated that RasGRP1 alone sends analog ERK signals [13, 19, 81]. We also proposed that positive feedback regulation of SOS enables bimodal, TCR-/BCR-triggered ERK activation through a mechanism that involves allosteric activation of SOS by RasGTP [13, 19, 81].

Results from several studies including our own have culminated in a model for discriminatory thymocyte selection signals that is based on modest, analog TCR-ERK signals via RasGRP driving positive selection and strong, digital SOS-ERK signals via SOS establishing negative selection (reviewed in [79, 104]). In agreement with this model, RasGRP1 without SOS sends analog ERK signals [13, 19, 81], ERK phosphorylation and positive selection is impaired in Rasgrp1−/− mice [22], ERK1 and ERK2 doubly deficient thymocytes demonstrate impaired positive selection [25], and Grb2 haploinsufficiency [37] or conditional inactivation of Sos1 [57] in thymocytes leaves positive selection intact. However, recent studies have demonstrated that this model is incomplete; negative selection is intact in ERK1−/−ERK2−/− thymocytes [70] as well as in thymocytes with conditional inactivation of Sos1 [57], even in the context of Sos2 deletion [57]. Thus TCR-induced digital ERK activation alone cannot be sufficient for the negative selection signal. A modified model explaining the TCR-induced opposing fates of developing thymocytes, life or death, has been put forth on the basis of differential activation of the ERK-, P38-, and JNK-MAPK pathways [79, 104]. In support of this model, pharmacological inhibition of P38 MAPK blocks negative selection of thymocytes in fetal thymic organ cultures [106], and P38 and JNK activation and negative selection are impaired in Grb2 heterozygous mice [37]. However, the molecular mechanisms of P38 and JNK activation pathways in lymphocytes are largely unknown [90, 127].

The above-mentioned studies motivated us to explore how RasGEFs might coordinate activation of different MAPK pathways. We took an approach that combined
synergistic computer simulations and biochemical assays using model cell lines and primary thymocytes to examine the role of SOS in regulating the quantity and quality of Ras and MAPK signal output. Here we demonstrate that highly sensitive digital ERK activation requires allosteric activation of SOS in lymphocytes, and that RasGRP is an important catalyst for ERK activation. Antigen receptor-stimulated activation of the Rac-P38 pathway depends on SOS1 more than it does on RasGRP. In contrast to ERK activation, optimal P38 activation does not critically depend on positive feedback regulation via SOS1’s allosteric pocket. In addition, P38 activation was not affected by enzymatic crippling of SOS1’s RasGEF or Dbl domains, implying that SOS1 functions as an adapter in the P38 pathway. These results show a stark contrast between the mechanisms of ERK and P38 activation, and propose new hypotheses for the role of SOS in MAPK activation in selecting thymocytes. We also anticipate that our studies will be applicable to SOS function in other cell types, downstream of other receptors systems like the epidermal growth factor receptor (EGFR).

2.2 Results

2.2.1 Robust ERK activation occurs in thymocytes and requires both RasGRP and SOS

It has been suggested that thymocytes require low levels of TCR signal input to activate ERK but higher levels of TCR stimulation to also activate p38 and JNK [104, 127, 21]. To test this, we stimulated total thymocytes from mice with a low or high dose of anti-CD3ε cross-linking antibody. ERK phosphorylation was robustly induced by low levels of TCR stimulation, whereas phosphorylation of p38 was more modest and critically depended on a high dose of stimulating anti-CD3ε antibody. Phosphorylation of JNK was efficiently induced by sorbitol but was not triggered by CD3ε cross-linking (Fig. 2-1A). Similarly, wild-type chicken DT40 B cells activate ERK in a very sensitive manner, following anti-BCR stimulating antibody (M4). The presence of both RasGRP and SOS RasGEFs is required for triggering high levels
of ERK activation at the population level, measured by Western blotting (Fig. 2-1B). These results in DT40 cells are in agreement with the reported ERK activation impairment in Rasgrp1−/− [23], Sos1−/− [57], and Sos1−/− Sos2−/− [58] thymocytes. We previously reported that bimodal ERK activation disappears in the absence of SOS1/SOS2 [19]. We expanded on these findings by performing extensive time courses and dose responses. As before [19], mean fluorescence values of ERK phosphorylation data points were used as input for Hartigan’s analyses. Here we further increased the stringency of these Hartigan’s analyses so that ERK phosphorylation was termed bimodal (B) only when a default null hypothesis of unimodal phospho-ERK (pERK) patterns is rejected. As such, we established that BCR stimulation-induced ERK activation is not bimodal for any dose or at any time point when SOS RasGEFs are missing (Fig. 2-1C). We hypothesized that the lack of bimodal ERK activation in SOS-deficient cells is caused by the absence of the allosteric feedback to SOS.

2.2.2 In silico modeling: allosteric activation of SOS1 is required for bimodal ERK activation.

To test our hypothesis, we extended our previous computational models for Ras activation to include ERK activation downstream of Ras and carried out stochastic computer simulations of the Ras-ERK signaling cascade [19, 89, 64]. Thus, we explored the effect of SOS, in particular, the SOS allosteric pocket, in ERK activation. By altering the computational parameters depicted in Fig. 2-11 in the supplemental material, we modeled ERK activation in cells with wild-type SOS, without SOS, or with allosteric mutant SOS as a function of time and stimulus dosage. We predicted ERK activation profiles at different time points with three different stimulus levels, namely, (i) weak, (ii) intermediate, and (iii) strong stimulation. These stochastic simulations predict that bimodal ERK activation will occur in cells with wild-type SOS but that bimodality will disappear when SOS is absent or when SOS cannot be allosterically activated, regardless of dosage level or stimulation time (Fig. 2-2A to C).
Figure 2-1: (A) Activation of MAPK ERK and P38 in total mouse thymocytes induced with 1x (2 µg/ml) and 5x (10 µg/ml) αCD3e (2C11) stimulation. SB indicates 30 min stimulation with 0.4 M sorbitol as a positive control for phospho-P38 (pP38) and phospho-JNK1/2 (pJNK1/2, black arrow heads). Asterisk (*) indicates heavy chain of stimulatory antibody. (B) Deficiency in either RasGRP or SOS leads to impaired ERK activation in DT40 cells. Wild-type, RasGRP1-3- and SOS1-2- DT40 cells were activated with intermediate dosage of M4 αBCR stimulation for indicated time. (C) SOS1/2-deficient DT40 cells do not demonstrate a bimodal pERK pattern after 3 min BCR stimulation, regardless of strength of BCR stimulus (range is 1:32,000 to 1:1,000 dilution). Bimodality is statistically tested by Hartigan’s analysis. A case with lower than threshold dip value in Hartigan’s unimodality test (p <0.05) is classified as bimodal (indicated as 'B'), while cases with p>0.05 are considered unimodal. Numbers represent the percentage ratio to maximum phospho-protein level normalized to total levels of that specific protein. All data are representative presentations of at least three independent experiments.

The output through the SOS-Ras-RAF-MEK-ERK pathway is influenced by feedback regulation with both negative and positive feedback loops connecting to ERK and SOS. The exact nature of negative feedback loops is not understood, and there appear to be differences between lymphoid and nonlymphoid (epithelial) cell lineages [124, 80, 17, 130]. To explore the negative feedback to SOS, we modified our model to include these parameters [54], the phosphorylation rate of SOS1 by pERK, kp = 0.02 s⁻¹µM⁻¹, and the dephosphorylation rate of pSOS1, kd. The process of de-
In silico prediction of ERK activation pattern under weak, intermediate or strong stimulations

Figure 2-2: ERK activation in cells with wild-type SOS, without SOS, or with allostERIC mutant W729E SOS expression, is projected at increasing time points. Each plot is a histogram of 8000 realizations from stochastic simulations. (A-C) In the simulations, there is no negative feedback loop from ERK to SOS and the positive feedback loop in the RAF-MEK-ERK module. (D-F) The negative feedback loop from ERK to SOS is added. (G-I) The simulations include both the negative feedback loop from ERK to SOS and the modest positive feedback loop in the RAF-MEK-ERK module.

Inclusion of these feedback loops does not change the overall qualitative behavior of our stochastic models that explore the wild-type, no-SOS1, and lack-of-allostERIC-activation-of-SOS situations. The main new effect is that the activated ERK levels begin to decline at longer times (Fig. 2-2D to F). We next expanded our model to include a positive feedback loop in the RAF-MEK-ERK module [6]. The exact rate, or strength, of positive feedback regulation is not known, so we varied these parameters. For weak positive feedback regulation, there is no effect, whereas for very strong positive feedback, all trajectories shifted to

phosphorylation of pSOS1 is extremely slow; we therefore take kd as 0 s^{-1}\mu M^{-1} here (see Fig. 2-12 in the supplemental material). Inclusion of these feedback loops does not change the overall qualitative behavior of our stochastic models that explore the wild-type, no-SOS1, and lack-of-allostERIC-activation-of-SOS situations. The main new effect is that the activated ERK levels begin to decline at longer times (Fig. 2-2D to F). We next expanded our model to include a positive feedback loop in the RAF-MEK-ERK module [6]. The exact rate, or strength, of positive feedback regulation is not known, so we varied these parameters. For weak positive feedback regulation, there is no effect, whereas for very strong positive feedback, all trajectories shifted to
maximal ERK phosphorylation very rapidly (data not shown). For moderate positive feedback regulation, which seems physiologically realistic, we still find bimodal ERK responses only for the wild-type SOS. But the bimodality is less sharply defined, with more simulation trajectories "caught" between the on and off states at any given time point (Fig. 2-2G to I). Notice that these simulation results resemble the experimental results more closely than if feedback regulation is not included.

2.2.3 Disruption of SOS1's allosteric activation negates bimodal Ras-ERK activation

Based on the structure of SOS1, tryptophan residue 729 (W729) was identified as a key residue coordinating Ras binding within the allosteric pocket [66, 103]. A single-amino-acid alteration of tryptophan into glutamic acid (W729E) cripples allosteric activation of nucleotide exchange activity of the isolated catalytic domain of SOS1 in vitro and in transfected cells [19, 103]. To directly test our predictions from the simulations in Fig. 2-2, we stably reconstituted SOS1- SOS2- DT40 cells with either WT or W729E (allosteric pocket mutant) human SOS1 (hSOS1). Selected stable clones, WT DT40 and DT40 mutants, expressed comparable levels of surface BCR (sBCR) (Fig. 2-3A), and WT and W729E hSOS1 proteins were expressed at comparable levels as well (Fig. 2-3C). We first analyzed Ras and ERK activation at the population levels and established that WT hSOS1 reconstitution rescued the BCR-induced Ras and ERK activation defect in SOS1- SOS2- DT40 cells. In contrast, W729E hSOS1-reconstituted cells demonstrated significantly lower levels of activated Ras and ERK than the WT hSOS1 stable clone (Fig. 2-3C). WT hSOS1-reconstituted SOS1- SOS2-DT40 cells demonstrated slightly enhanced responses compared to parental DT40 cells. This may be a reflection of differences between chicken and human SOS1. Alternatively, hSOS1 may be expressed at higher levels than chicken SOS1, but lack of a cross-reactive SOS1-specific antibody prevented us from testing this. WT and W729E hSOS1-reconstituted cells were therefore directly compared in the following experiments. Significantly, BCR stimulation of WT hSOS1-reconstituted SOS1-
SOS2- cells resulted in a rescue of the bimodal ERK activation pattern, revealed by dose responses and time courses of BCR-induced phospho-ERK flow cytometry and Hartigan’s analyses of the resulting phospho-ERK histograms (Fig. 2-3D and E). In contrast, bimodal ERK activation was never observed in W729E hSOS1-reconstituted SOS1- SOS2- DT40 cells regardless of stimulation dose or time (Fig. 2-3D and E). Expression of W729E hSOS1 did not lead to a general impairment of ERK activation, as strong stimulation of cells with phorbol myristate acetate (PMA) (a synthetic analog of diacylglycerol [DAG]) resulted in very similar ERK activation in WT DT40, SOS1-SOS2- WT hSOS1, and SOS1- SOS2- W729E hSOS1 cells (Fig. 2-3B). Likewise, the observed results did not reflect uniquely selected stable clones; additional WT and W729E hSOS1 stable clones were tested in parallel and exhibited similar dynamics and patterns of BCR-induced pERK (data not shown).

2.2.4 Optimal p38 activation preferentially requires SOS1.

Pharmacological inhibition of p38 MAPK blocks negative selection of thymocytes in fetal thymic organ cultures [106]. In Grb2+/- thymocytes, TCR-induced p38 activation is impaired [37], suggestive of a possible role for SOS RasGEFs that are recruited by Grb2, but TCR-induced p38 activation has not been examined in Sos1−/− [57] or Sos1−/− Sos2−/− thymocytes [58] to date or in Rasgrp1−/− thymocytes [23]. We previously reported how RasGRP1 plays a more dominant role in ERK activation in TCR-stimulated Jurkat T cells or human CD4+ T cells than SOS1 [93] (Fig. 2-4A and B). To address how these RasGEFs might affect the p38 MAPK pathway and how this compares to their roles on the ERK pathway, we determined TCR-induced p38 activation in the exact same samples in which RasGRP1 or SOS1 expression was targeted by siRNA duplexes. We found that the role of RasGRP1 in p38 activation is more modest than that for ERK activation. However, TCR-induced activation of p38 was consistently more impaired by reduced SOS1 expression than by a similar level of reduced RasGRP1 expression in both Jurkat T cells and human CD4+ T cells (Fig. 2-4C and D). These siRNA approaches yield only partial knockdown of RasGRP1 or SOS1. To determine the effects under complete absence of RasGRP-1
Efficient and bimodal Ras-ERK signaling requires an intact allosteric pocket in SOS1(W729)

Figure 2-3: (A) Surface BCR levels of various DT40 cell lines. SOS1/2-deficient DT40 cells stably reconstituted with wild-type (WT; clone 1-9) or allosteric mutant W729E hSOS1 (clone 2-9) express comparable surface BCR level to wild-type or other mutant DT40 cells. (B) Various DT40 B cell lines were stimulated with 25 ng/ml PMA for 10 min. MAPK ERK activation is measured by phospho-flow cytometry. Two dashed lines indicate unstimulated and PMA-induced levels of phospho-ERK in wild type DT40 cells. (C) BCR-induced Ras and ERK activation depends on SOS and on SOS' allosteric pocket. Various DT40 cells were stimulated with low dose of M4 αBCR antibody and analyzed as in Fig 1B. Part of lysates were used to assay BCR-induced Ras-GTP accumulation. Percentage relative to wild-type maximum activation is shown. (D and E) Reconstitution of SOS1-2- DT40 cells with the W729E hSOS1 does not lead to rescue of the bimodal pERK pattern, regardless of dose or time course of stimulation. Bimodality statistically supported by Hartigan’s analysis is marked as ‘B’ as in Fig. 2-1C. Presented data are representative of at least three independent experiments.

and -3 or of SOS-1 and -2, we turned to our RasGRP1- RasGRP3- and SOS1- SOS2-DT40 cell lines, which substantiated the siRNA findings; the SOS RasGEFs made a larger contribution toward BCR-induced p38 activation than the RasGRP RasGEFs, whereas the opposite holds true for activation of ERK (Fig. 2-4E and F). Given the differential contribution of RasGRP and SOS to ERK versus p38 activation and that p38 activation is not sharply thresholded as in the digital ERK activation, we hypothesized that activation of ERK and p38 in thymocytes occurs in a mechanistically
different way downstream of these RasGEFs.

Optimal P38 activation preferentially requires SOS1

Figure 2-4: (A-D) siRNA-driven knockdown of SOS1 in Jurkat T cells (A and C) or in human peripheral blood CD4+ T cells (B and D) result in a reduction of TCR-triggered P38 activation that is more pronounced than when RasGRP1 expression levels are reduced by siRNA-driven knockdown. Panels A and B are partial reproduction of figure 2B&C in MCB.01882-06 and experimental details are described there. Mouse Rasgrp1-specific siRNA duplex was used for off-target effect control in both experiments. Residual expression levels of SOS1 and RasGRP1 are represented as the percentage of control level. Representative of three independent experiments. (E and F) Impaired BCR-induced P38 activation in the absence of SOS. Wild-type and mutant DT40 cells were stimulated with high dosage of M4 αBCR antibody for indicated time. Percentage relative to wild type maximum response of pERK and pP38 normalized to each total protein level is plotted in (F). Data represent mean ± SD from four independent experiments.
2.2.5 Modest contribution from the PLCγ-RasGRP1 axis to thymocyte p38 activation

We first utilized a pharmacological approach to inhibit phospholipase C (PLC) with U73122, the enzyme that produces the second messengers IP3 and DAG. The latter is essential for RasGRP1 membrane recruitment and activation in thymocytes and T cell lines (17). As expected based on the established role of the DAG-RasGRP1-Ras pathway for ERK activation, exposure of total mouse thymocytes and Jurkat cells to U73122 reduced the maximal induction of ERK activation by 65% and 47%, respectively, following TCR stimulation compared to that of cells treated with inert analog U73343 as a control (Fig. 2-5A and B). In contrast, U73122 treatment in thymocytes had a more modest effect (reduction by 33%) on the TCR-induced activation of p38 (Fig. 2-5A and B). U73122 had a small effect on proximal TCR signals, evidenced by the small decrease in phosphorylation of ZAP70 (Fig. 2-5A). Jurkat cells show delayed kinetics of TCR-induced p38 activation compared to mouse and human primary T cells (Fig. 2-4C and D) (data not shown). U73122 treatment of Jurkat cells only minutely affected TCR-induced p38 activation, especially at 5 min, while the impact at the later time point was greater (reduction by 31% at 15 min) (Fig. 2-5B). Interestingly, ZAP70 activation was not reduced in U73122-treated Jurkat cells, while the upstream MKK3/6 activation was completely abolished (Fig. 2-5B). Activation of p38 downstream of the TCR has been described to occur via LAT-dependent classical and LAT-independent alternative pathways [4]. It is possible that the difference between thymocytes and Jurkat cells comes from different contributions of classical and alternative p38 pathways. To examine the contribution of the LAT-independent pathway in our own hands, we compared wild-type Jurkat and LAT-deficient J.Cam2 cells (Fig. 2-5C). In contrast to what has been reported [95], we did not see any significant TCR-induced p38 activation in J.Cam2 cells, whereas we confirmed the earlier documented [95] elevated baseline p38 phosphorylation in this cell line. It should be noted that we examined dual T180/Y182 p38 phosphorylation in our study presented here. There is some evidence that the alternative p38 pathway preferentially
involves monophosphorylation of T180 [73], although T180 p38 monophosphorylation following extensive time courses and dose responses has not been explored to date.

**PLCγ-RasGRP1 axis contributes only minimally to P38 activation in thymocytes.**

![Figure 2-5](image)

**Figure 2-5:** (A&B) Pharmacological inhibition of DAG-producing PLCγ by U73122 results in a modest reduction of P38 activation compared to the reduction in ERK activation in TCR-stimulated total thymocytes and Jurkat cells. U73343, inert analog is used as a treatment control. Percentage relative to control maximum activation is shown. Representative of two experiments. (C) TCR-stimulated MKK3/6 and P38 activation is compared between wild-type and LAT-deficient J.Cam2 Jurkat cells. (D and E) Minute reduction in TCR-induced P38 activation in Rasgrp1-deficient thymocytes. Control Rasgrp1+/+ thymocytes were obtained from MHCI/II-double deficient mice. Data represent mean ± SD from at least three mice of each genotype (Student t test vale, p < 0.05 for *, p<0.01 for **).

We next took a genetic approach by comparing thymocytes from Rasgrp1−/− mice with those from β2m/MHC class II doubly deficient mice. In both cases, thymocyte development is arrested, and thymi consist of a rather uniform cell population of CD4+ CD8+ thymocytes [92]. In Rasgrp1−/− mice, the defect is intrinsic to the thymocytes, whereas in the β2m/MHC class II doubly deficient mice, the defect lies in the epithelial cells and not in the thymocytes. ERK activation was severely impaired in TCR-stimulated CD4+ CD8+ thymocytes that are deficient for RasGRP1 (Fig. 2-5D and E). In contrast, the consistently modest TCR-induced p38 activation was
not significantly affected by the genetic deletion of RasGRP1 (Fig. 2-5D and E) ($P = 0.07$). In summary, the mechanism of p38 activation appears to be very different from that of ERK activation, is not sharply thresholded, and is mostly independent of RasGRP1.

### 2.2.6 Allosteric activation of SOS1 is dispensable for BCR-stimulated p38 activation.

Results from Fig. 2-4 and 2-5 demonstrated defective p38 activation in SOS1- SOS2-DT40 cells but only a minimal role for RasGRP1 in activation of the p38 MAPK. We therefore focused on the feedback loop to the allosteric pocket in SOS1 next and examined how uncoupling this loop via the W729E point mutation affects the activation of p38. Under strong BCR stimulation, the p38 activation defect in SOS1- SOS2- DT40 cells was restored by reconstitution with WT hSOS1 (Fig. 2-6A). A very similar level of restoration was obtained in SOS1- SOS2- W729E hSOS1 cells (Fig. 2-6A). Extensive time courses and dose responses revealed levels of p38 activation in W729E hSOS1-reconstituted cells that were consistently similar to those in WT hSOS1-reconstituted cells, demonstrating that the allosteric pocket in SOS1 is dispensable for optimal BCR-triggered p38 activation (Fig. 2-6B, C, and D). These results were further validated with transient reconstitution of SOS1- SOS2- cells with empty vector, WT hSOS1, or W729E hSOS1. W729E hSOS1 rescued the p38 activation defect with the same efficiency as WT hSOS1 (Fig. 2-6E). As a control for the impairment of allosteric activation of SOS, ERK activation was verified in all these assays. W729E hSOS1 cells always demonstrated ERK activation defects compared to WT hSOS1-expressing cells, and these defects were most pronounced under low-level stimulation conditions, reiterating the importance of allosteric activation of SOS1 for optimal ERK activation (Fig. 2-6A to E).
SOS1's allosteric pocket is dispensable for optimal P38 activation

Figure 2-6: (A, B&C) W729E hSOS1 and WT hSOS1 similarly rescue the SOS1-2-DT40 in terms of P38 activation regardless of stimuli dosage. Quantitation of pERK and pP38 signal relative to maximum WT control level is plotted in (C). Note that the ERK activation defect in W729E hSOS1 reconstituted cells is most noticeable with low αBCR stimulation. (D) P38 activation at 10 min stimulation is measured in response to two-fold serial dilution of λsBCR antibody at a range of intermediate dosage. (E) SOS1-2-DT40 cells were transiently transfected with empty vector, WT hSOS1 or W729E hSOS1 and stimulated as indicated. Transfected cells were magnetically purified by positive selection of co-transfected human CD16/7 fusion protein. Numbers for phospho-ERK and âÅP38 are generated by taking the ratio of pERK/ERK and pP38/P38 in the individual samples and setting the maximum phospho-protein level in the wildtype control to 100%. Representative blots of at least three experiments.

2.2.7 SOS1, Rac, and p38 activation.

SOS has potential dual GEF activity for both Ras and Rac, in which SOS’s atypical Dbl homology/Pleckstrin homology (DH-PH) domain may perform the exchange reaction of GDP for GTP on Rac [75, 76, 100, 72]. Rac is typically positioned upstream of the MKK3/6-p38 MAPK pathway, although most research has been done
in nonlymphoid cell types [90, 21]. As an initial start to map a pathway from SOS1 toward p38 in lymphoid cells, we examined activation of the small GTPase Rac. GTP loading of Rac induced by BCR stimulation was drastically reduced in SOS1- SOS2-DT40 cells. RasGRPs are not predicted to have Rac GEF activity [105]. Surprisingly, Rac activation was also impaired in RasGRP1- RasGRP3- cells, albeit more modestly (Fig. 2-7A). The differences in RasGRP and SOS requirements for Rac activation somewhat paralleled their degree of requirement for MKK3/6 and p38 activation but completely contrasted the need for RasGRP or SOS in terms of ERK activation (Fig. 2-7A); i.e., compared to SOS, RasGRP deficiency results in more-modest Rac and p38 defects but more-severe ERK activation impairment. Supporting a potential SOS-Rac-MKK3/6-p38 pathway, stable reconstitution of SOS1- SOS2- cells with W729E hSOS1 resulted in an effective rescue of BCR-induced Rac activation, mirroring the MKK3/6 and p38 activation rescue, approximating the levels obtained in WT hSOS1-reconstituted cells (Fig. 2-7B).

**BCR-induced RAC activation requires SOS but not allosteric activation of SOS1**

![Figure 2-7](image)

Figure 2-7: (A) RAC activation in response to high αBCR dosage is measured by RAC pull-down assay. (B) RAC activation in stably hSOS1-reconstituted DT40s is measured by RAC pull-down assay. Low αBCR dosage is used for stimulation. Percentage relative to wild-type maximum activation is shown. Data are representative of two experiments.
2.2.8 Computational explorations of SOS’s role in Rac-p38 activation.

We built a coarse-grained computational model to formulate new hypotheses that might be explored to provide mechanistic underpinnings to our discovery of SOS’s role in Rac-p38 activation and that (unlike ERK activation) the allosteric pocket of SOS is not critical for p38. First, we focused on the differences in activation profiles of Rac and p38 (Fig. 2-7A): RasGRP1- RasGRP3- cells displayed a significant defect in Rac activation but fairly robust p38 activation; in contrast, SOS1- SOS2- cells had even lower Rac activation and failed to activate p38 effectively. We considered two possible mechanisms to explain this: (i) Rac activated by RasGRP-mediated pathways may be localized in a different compartment than that activated by SOS-mediated pathways, and SOS-activated Rac is more efficient in activating p38 (this is not our focus here but may be tested in future imaging studies), and (ii) a threshold amount of active Rac might be needed to effectively activate p38, and RasGRP1- RasGRP3- cells can generate enough active Rac to cross that threshold but SOS1-SOS2- cells cannot. To test the second hypothesis, we introduced a thresholding effect for Rac-p38 activation. In biochemical systems, this is most conveniently modeled by using a Hill coefficient greater than 1, as this result is in a nonlinear dependence. We determined that a Hill coefficient of 2 for p38 activation would be sufficient to produce the thresholding effect observed in Fig. 2-7A.

Next, we investigated whether SOS may function as a RacGEF, an adaptor, or both. Published work indicated that SOS may possess RacGEF activity through its DH-PH domain [75] and/or functions as an adaptor affecting LAT oligomerization which may help recruit and stabilize RacGEFs at the LAT signalosome [46]. In our coarse-grained computational model, we assumed that RasGRP also facilitates the stable formation of the LAT signalosome by a cooperative mechanism [81] and hence may regulate RacGEFs as well. We then predicted the Rac-p38 activation profiles for wild-type, RasGRP-deficient, and SOS-deficient cells and SOS cells with disrupted RacGEF activity (DH-PHmut) under three scenarios (Fig. 2-8): SOS is both a
RacGEF and an adaptor, SOS is only an adaptor, and SOS is only a RacGEF. For panels A and D, DH-PHmut leaves SOS’s adaptor functionality intact, and hence, the Rac activation profile for DH-PHmut falls between that for the wild type and that for SOS deficiency, crossing the threshold to fully activate P38. For panels B and E, DH-PHmut does not alter SOS’s adapter function and has the same Rac-p38 activation profile as the wild type; for panels C and F, DH-PHmut completely impairs SOS’s RasGEF functions and the DH-PH mutant is as deleterious as SOS deficiency. These simulations suggest that the observed Rac and p38 activation patterns may be a result of either SOS’s RacGEF or adaptor function or a combination of the two, which we experimentally tested next.

**Computational predictions for SOS activating the Rac-P38 pathway**

![Figure 2-8: When SOS functions as (A&D) both a RacGEF and an adaptor, (B&E) an adaptor only, and (C&F) a RacGEF only, the activation profile of Rac-P38 for cells with DH-PH domain mutant SOS is (A&D) between that for wild-type SOS and that for SOS1-2-, (B&E) the same as wild-type SOS, and (C&F) the same as SOS1-2-.](image)

2.2.9 BCR-induced p38 activation does not require enzymatic activities of SOS1.

To test computer-based predictions, we sought to cripple SOS1’s enzymatic functions in a manner that would keep a potential adapter function intact. We have previously demonstrated that an F929A substitution in SOS1 abrogates RasGEF activity (Fig.
S26 in reference [19]). SOS contains a DH-PH domain that potentially may function as a Rac GEF in addition to a Ras GEF [75]. No evidence for direct GTP loading of Rac by SOS is available. Instead, a distinct SOS1 complex in association with EPS8 and E3b1/Abi-1 cofactors was shown to have in vitro RacGEF activity [100]. SOS1’s DH domain is atypical [102], making it challenging to design function-impairing point mutations. However, composite deletions in the Dbl oncogene resulted in reduced in vitro activation of the small GTPase Cdc42 [41] and inspired studies with a similarly mutated DH domain of SOS1 in fibroblast focal transformation assays [84]. A similar 7-amino-acid mutation in the Rac exchange factor Vav (LLLQELV to IIIQDAI) results in severely impaired TCR-induced Rac activation in Jurkat T cells [59].

We first generated SOS1-DBLmut, an expression construct of human SOS1 with the LHYFELL-to-IIIRDII substitution [84]. Repeated attempts to stably reconstitute SOS1- SOS2-DT40 cells with SOS1 F929A and SOS1 DBLmut were unsuccessful (data not shown). We therefore resorted to a transient-transfection approach. To this end, we first developed an intracellular FACS approach for p38 phosphorylation (Fig. 2-9A) so that p38 activation could be assessed without purifying and manipulating transfected cells prior to stimulation and analysis. Next, we transfected SOS1- SOS2-DT40 cells with WT, F929A, or DBLmut hSOS1 cells, stimulated these with anti-BCR antibodies, and performed simultaneous intracellular FACS stainings for phospho-p38 and hSOS1. Comparisons of cells expressing equal amounts of SOS1 via the electronic gating strategy depicted in Fig. 2-9B demonstrated that SOS1 F929A and SOS1 DBLmut restored BCR-induced p38 activation in SOS1- SOS2-DT40 cells with a similar efficiency as WT SOS1 (Fig. 2-9E and F). Even a lower level of BCR stimulation did not reveal significant defects in p38 activation in cells reconstituted with SOS1-DBLmut (data not shown). In contrast, the same perturbations in the RasGEF and DH domains of SOS1 did impact the magnitude of BCR-induced ERK activation (Fig. 2-9C and D). Thus, SOS1 appears to play an adapter role in the pathway that leads to p38 activation, whereas allosteric regulation of its catalytic function is critical for ERK activation (Fig. 2-10).
Enzymatic activity of SOS1 is dispensable for BCR-induced P38 activation

Figure 2-9: (A) Flow cytometric assay of pP38 induced by BCR-crosslinking in wild-type DT40s. (B) hSOS1 expression profile of transiently transfected SOS1-2- DT40 cells. Two gates show hSOS1 positive (hSOS+) and negative (hSOS-) populations. (C) BCR-induced ERK and P38 activation in transiently transfected SOS1-2-DT40 cells. hSOS1- (open histogram) of each transfection is compared with hSOS1+ (gray histogram) subset as gated in (B). (C) Two arbitrarily chosen reference lines in the histogram plot represent basal (left) and maximal (right) pERK intensity of wild-type hSOS1 transfection. (E) Reference line represents basal pP38 intensity. (D&F) Graphical representation of pERK and pP38 mean fluorescence intensity (MFI) shown in (C) and (E). SOS1 negative represents the average pP38 MFI value of hSOS- subset in three samples. Data are representative of two experiments.

2.3 Discussion

In the present study, we combined computer simulations and biological experiments in cell lines and primary cells to interrogate the requirements for antigen receptor-induced activation of the ERK and p38 MAPK pathways. We find that sensitive and bimodal ERK activation occurs in thymocytes and cell lines (Fig. 2-1). Bimodal ERK activation critically relies on allosteric activation of SOS1, and an allosteric pocket W729E mutant SOS1 signals to ERK in an analog manner (Fig. 2-3). In agreement
Graphic representation of the contrasting roles of SOS in activating the ERK versus P38 pathway

Figure 2-10: ERK activation is bimodal and sharply thresholded. These characteristics critically rely on allosteric activation of SOS. By contrast, P38 activation is not sharply thresholded. Whereas optimal P38 activation does require the presence of SOS1, it does not depend on allosteric activation of SOS1, on an intact RasGEF domain, or on an intact DH domain. These results suggest that SOS1 has an adaptor function in the P38 pathway. Graphic design by Anna Hupalowska.

with initial facilitation of allosteric activation of SOS by RasGRP1-derived RasGTP, pharmacological or genetic perturbations of the PLCγ-RasGRP1-Ras pathway in thymocytes and cell lines drastically impair the ERK output (Fig. 2-5). In contrast to ERK, antigen receptor-induced phosphorylation of p38 is modest and depends on SOS more than on RasGRP but is not affected by mutation of its allosteric pocket in cell lines (Fig. 2-5 and 2-6). Moreover, p38 activation does not appear to be affected by crippling mutations in either SOS1’s RasGEF domain or its DH domain, which argues that SOS1 functions as an adapter in the p38 pathway (Fig. 2-8 and 2-9). These findings provide mechanistic insights into the differential activation of ERK and p38 MAPK, which are proposed to play opposing roles during thymocyte
selection [104, 79]. SOS also plays a critical role downstream of the EGF receptor and other receptor tyrosine kinases [9, 65], and allosteric activation of SOS plays a critical role in EGF-stimulated Ras activation [8]. Therefore, our results and hypotheses not only provide new insights into thymocyte MAPK selection signals but also provide a framework for future studies on the impact of distinct MAPK pathway activation in other cellular systems.

Pharmacological inhibition of p38 blocks in vitro negative selection of thymocytes [106], but a clear understanding of TCR-induced p38 activation is lacking to date [4]. Here we focused on a potential role for the small GTPase Rac, since studies in nonlymphoid cells have positioned Rac upstream of the MKK3/6-p38 MAPK pathway [90, 21] and also postulated that SOS may contain RacGEF activity [76, 100, 72]. We find that BCR-induced Rac activation is severely impaired in SOS1- SOS2- DT40 cells but is efficiently rescued by reconstitution with both WT and allosteric pocket mutant SOS1, paralleling the requirements of SOS for p38 activation (Fig. 2-3). In support of this pathway, inhibition of RacGTP accumulation correlates with impaired BCR-induced p38 activity in DT40 cells [43]. RasGRP also contributes to RacGTP generation, albeit more modestly than SOS, but does not appear to contribute much to p38 activation (Fig. 2-5 and 2-7).

In T cells but not in B cells, it has been reported that antigen receptor stimulation can directly link to p38 activation via lck-ZAP70 activation, via an alternative pathway that bypasses conventional MAPK cascade components, such as LAT and MKK3/6 [4]. We explored the LAT-independent pathway but were not able to reproduce the normal p38 activation that had been reported in the LAT-deficient J.CaM2 cell line [95]. Instead, we find a defect in p38 activation in J.CaM2 cells (Fig. 2-5C). It is, however, difficult to rule out the existence of an alternative p38 pathway in J.CaM2 cells because these cells express reduced levels of surface TCR [67], which leads to downstream impairments, such as the suppressed TCR-induced ZAP70 activation in J.Cam2 cells (Fig. 2-5C), and the alternative pathway is known to be dependent on ZAP70. It is worth noting that blocking DAG production completely abolished TCR-induced MKK3/6 activation, while downstream p38 activation is only
partially impaired in U73122-treated Jurkat cells (Fig. 2-5B). The phospho-p38 reduction is especially more pronounced at the later time point. The residual p38 activation in the near absence of phospho-MKK3/6 might come from an alternative p38 activation pathway (Fig. 2-5A and B). We postulate that TCR-induced p38 activation may be the sum of two temporally separated pathways: an alternative pathway mediating the early time point, while the later p38 activation is through a conventional MKK3/6-dependent p38 activation pathway. In addition, it is very possible that there are temporal or qualitative differences in mono- and dual phosphorylation. These hypotheses, as well as the differences between p38 activation in T cells and B cells that appear to lack the alternative pathway, are important areas for future research, requiring careful analyses of pT180-p38 and pT180/Y182-p38 following different stimuli, ideally also including the mouse models, such as the recently generated p38 knock-in mice [51].

Since the allosteric pocket of SOS is not involved in Rac activation, the defect in Rac activation in RasGRP-deficient cells cannot be explained by impaired allosteric activation of SOS acting as a RacGEF. Instead, we postulate that RasGRP-dependent Rac activation involves a different RacGEF and may couple to downstream pathways other than p38 activation. How RasGRP contributes to Rac activation, what RacGEF is involved, and at what cellular localization Rac activation occurs are important problems for future research. Potential candidates include Tiam1 and Vav [115, 126]. Whereas Tiam1 has not been connected to antigen receptor stimulation [32], its RacGEF activity is stimulated by binding to RasGTP [60] and may therefore be RasGRP dependent. Vav family proteins are the best-studied RacGEFs downstream of lymphocyte antigen receptor [114]. Vav is not directly responsive to RasGTP, but Vav’s GEF activity is regulated indirectly by RasGTP, coupling to its effector phosphatidylinositol 3-kinase [126, 39]. Additionally, several studies indicate cross talk between Vav and RasGRP1 [10, 88, 131].

Exactly through what mechanism SOS1 functions as an adapter in a Rac-p38 pathway in lymphocytes remains to be resolved through future studies. Complexes of Grb2 and SOS1 at a 2:1 ratio were shown to mediate clustering of multiple LATs
and associated proteins in T cells [46, 101], and it is feasible that the function of RacGEFs like Vav is impacted by LAT clustering. Conversely, Vav has recently been reported to stabilize T cell microclusters that contain the adaptors LAT and SLP76 [109]; thus, lymphocyte Rac-p38 activation is likely impacted by many factors.

We anticipate that our studies here will stimulate future investigations of SOS and its role in lymphocyte MAPK activation and thymocyte selection. We hypothesize that strong TCR signal input, as would occur in thymocytes that carry potentially self-reactive TCR and need to be deleted, results in digital ERK activation that depends on allosteric activation of SOS. However, given the data with ERK1 and ERK2 doubly deficient [70] and SOS1 and SOS2 doubly deficient [58] thymocytes, we postulate that the digital ERK activation may be merely a passenger effect of the strong TCR signal and not a driver of negative selection. As such, SOS-dependent strong digital ERK activation, while important for mature T cell activation, may be irrelevant for negative selection. It may also be a mechanism to rapidly downregulate ERK activation by strongly engaging negative feedback loops, and not having sustained ERK activation may be required for negative selection. It has to be noted that some residual bimodal ERK activation was detected in SOS1 and SOS2 doubly deficient thymocytes whereas deficiency for only SOS1 does appear to result in a loss of distinct bimodal ERK activation patterns [58]. One possible explanation for this phenomenon is that complete deficiency for both SOS1 and SOS2 likely results in increased access to Grb2 for other proteins, such as Themis1. Themis1 is a relatively recently identified protein that is expressed predominantly in thymocytes and not in B cells, interacts with Grb2, and positively regulates ERK activation [29, 52, 61]. We have also described digital ERK activation patterns for TCR-stimulated peripheral T cells in the past [19]. Recently, SOS1 has been suggested to be of more limited importance in TCR-induced ERK activation in peripheral T cells [122], although FACS patterns of ERK activation were not examined in this study. Future studies should address the question of whether differences in SOS-mediated ERK activation with the various experimental models are a reflection of compensatory mechanisms or due to the developmental stage of the cell population in the analysis.
We hypothesize that strong TCR input leading to relatively modest p38 activation helps drive negative selection and propose that SOS plays an important role here whereas RasGRP's contribution is more minor. However, Rasgrpl's contribution toward p38 may become more critical when SOS function is limiting, which is supported by a recent study demonstrating compensatory mechanisms between RasGRPI and SOS1 during negative selection of thymocytes [58]. Given the many functions that SOS may fulfill and our results that point to an adaptor function for SOS1 in Rac and p38 activation and a feedback-regulated catalytic function for ERK activation, we anticipate that future studies of specific point mutant versions of SOS will be most informative and will reveal unanticipated compensation mechanisms.

2.4 Materials and methods

2.4.1 Cell lines, mice, stimulations, and inhibitor treatment.

Cultures of human Jurkat leukemic T cells, chicken DT40 cell lines, and DT40-derived lines were maintained as described previously [19, 77, 93]. All experimental mice were used at the age of 6 to 7 weeks. Age- and sex-matched major histocompatibility complex type I and II (MHCI/II)-double-deficient (Abb/β2m) mice were purchased from Taconic (Hudson, NY). For cell stimulation, harvested cells were rested for 30 min in phosphate-buffered saline (PBS) or plain RPMI at 37°C. For phospholipase Cγ (PLCγ) inhibition, cells were preloaded for 20 min with U73122 inhibitor (Calbiochem) or its inactive analog U73343 at 5 μM (Calbiochem). Stimulations of DT40 cells were carried out in PBS or plain RPMI at 37°C with the indicated doses of M4 anti-BCR antibody hybridoma ascites fluid preparation.

2.4.2 Hartigan’s analysis.

We previously employed Hartigan’s statistical analyses of phospho-ERK (pERK) histograms to label them as unimodal or bimodal [19]. Here we used the average of cell counts in the neighboring five gates to smooth the cell count data in each gate in
the pERK flow cytometry experiment. We repopulated the pERK levels uniformly within each gate to generate a continuous distribution of pERK expression patterns and applied Hartigan's test to the generated distribution.

2.4.3 Computer modeling.

For Fig. 2-2, we used an established computational model of Ras activation via receptor input, as described in Fig. 2-1A of Riese et al. [89], with the extension of the Ras-Raf-MEK-ERK activation cascade [64], as depicted in Fig. 2-11 in the supplemental material. In this model, SOS1 and SOS2 are represented by SOS, i.e., we do not make a distinction between SOS1 and SOS2 in the model. To further explore the effects of downstream regulation of Ras activation, we included negative and positive feedback loops, as depicted in Fig. 2-12 in the supplemental material. We used our implementation of the standard Gillespie algorithm [34], the stochastic simulation compiler (SSC), to simulate the described signaling network stochastically. In all stochastic simulations, we used a spatially homogeneous simulation box with size $V$ of area ($4 \text{ mm}^2$) times height (0.02 mm). This choice of the system size ensures that the system is well mixed. The initial concentrations and the rate constants are those in Tables S1 to S4 of Das et al. [19], Tables 1 and 2 of Riese et al. [89], and the supplemental material of Locasale et al. [64], except we used 36 molecules/$\mu$m$^2$ for PIP2 to match published results [89]. More details of the choice of parameters can be found in the supplemental materials of Das et al. [19] and Riese et al. [89].

For the results shown in Fig. 2-8, we also performed stochastic simulations to investigate Rac activation when SOS functions directly as a RacGEF and/or functions as an adaptor recruiting and stabilizing RacGEF at the LAT signalosome. We assumed that a threshold level of active Rac would be needed for p38 activation. To mimic such a thresholding behavior, we modeled p38 activation kinetics as second order in the amount of active Rac, which is equivalent to a Hill coefficient of two for p38 activation. The detailed model specification and parameter choice are indicated here. We made the following assumptions in the coarse-grained models: (i) the LAT signalosome, possibly through Gads, activates RacGEF, and (ii) RasGRP, possibly
Table 2.1: Number of molecules in the system

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAT</td>
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</tr>
<tr>
<td>PLCγ-Rasgrp</td>
<td>100</td>
</tr>
<tr>
<td>Grb2-SOS</td>
<td>100</td>
</tr>
<tr>
<td>Gads</td>
<td>100</td>
</tr>
<tr>
<td>Rac</td>
<td>1000</td>
</tr>
<tr>
<td>P38</td>
<td>1000</td>
</tr>
</tbody>
</table>

recruited by PLCγ, stabilizes the LAT signalosome and hence regulates RacGEF. In all Gillespie simulations, we used a spatially homogeneous simulation box with a size V of area (4 mm²) times height (0.02 mm). All the rate parameters reported below have the unit of s⁻¹, which is directly used in the simulations; all the species concentrations reported below have the unit of number of molecules per simulation box. For SOS-deficient, RasGRP-deficient, and SOS DH-PH mutant cases, the SOS concentration, RasGRP concentration, and rate of Rac activation by SOS (if it exists), respectively, are set to zero (Table 2.1).

Case 1: SOS functions as both a RacGEF and an adaptor. When both Grb2-SOS and PLCγ-RasGRP bind to LAT, the LAT signalosome complex is more stable and more effective in activating Rac. Grb2-SOS, when bound to LAT, activates Rac directly (Table 2.2).

Case 2: SOS functions as an adaptor only. Grb2-SOS binding to LAT stabilizes the LAT signalosome complex and increases its RacGEF activity; PLCγ-RasGRP’s binding further enhances such cooperativity. Grb2-SOS does not activate Rac directly (Table 2.3).

Case 3: SOS functions as a RacGEF. Grb2-SOS, when bound to LAT, activates Rac directly. RasGRP, when bound to the LAT signalosome, cooperatively enhances SOS’s RacGEF. Rac can also be activated via a Gads-mediated pathway, but SOS’s binding to LAT does not contribute to it (Table 2.4).
Table 2.2: Case 1: SOS functions as both a RacGEF and an adaptor. When both Grb2-SOS and PLC\(\gamma\) -Rasgrp bind to LAT, LAT signalosome complex is more stable and more effective in activating Rac. Grb2-SOS, when bound to LAT, activates Rac directly.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>(k_{on}(1/s))</th>
<th>(k_{off}(1/s))</th>
<th>(k_{cat}(1/s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gads binding to, unbinding from LAT without cooperativity</td>
<td>0.1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Grb2-SOS binding, unbinding to LAT</td>
<td>0.12</td>
<td>0.8</td>
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<tr>
<td>PLC(\gamma) -Rasgrp binding, unbinding to LAT</td>
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<tr>
<td>Gads unbinding from LAT bound with SOS and Rasgrp</td>
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<td>SOS activating Rac</td>
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<td>Gads activating Rac without co-operativity</td>
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<tr>
<td>Gads activating Rac with co-operativity</td>
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<tr>
<td>Rac self-decaying to inactive form</td>
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<td>80</td>
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<tr>
<td>Rac activating P38</td>
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</tr>
<tr>
<td>P38 self-decaying to inactive form</td>
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<td></td>
<td>35</td>
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Table 2.3: Case 2: SOS functions as an adaptor only. Grb2-SOS' binding to LAT stabilizes LAT signalosome complex and increases its RacGEF activity; PLC\(\gamma\) -Rasgrp’s binding further enhances such cooperativity. Grb2-SOS does not activate Rac directly.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>(k_{on}(1/s))</th>
<th>(k_{off}(1/s))</th>
<th>(k_{cat}(1/s))</th>
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<td>5</td>
<td></td>
</tr>
<tr>
<td>Grb2-SOS binding, unbinding to LAT</td>
<td>0.12</td>
<td>0.8</td>
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</tr>
<tr>
<td>PLC(\gamma) -Rasgrp binding, unbinding to LAT</td>
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<tr>
<td>Gads unbinding from LAT bound with Rasgrp only</td>
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<td>5</td>
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<tr>
<td>Gads unbinding from LAT bound with SOS only</td>
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<td>0.05</td>
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<tr>
<td>Gads unbinding from LAT bound with SOS and Rasgrp</td>
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<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Gads activating Rac without co-operativity</td>
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<td>0.005</td>
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<tr>
<td>Gads activating Rac when LAT bound with Rasgrp only</td>
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<td></td>
<td>0.005</td>
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<tr>
<td>Rac self-decaying to inactive form</td>
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<td>80</td>
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<tr>
<td>Rac activating P38</td>
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<td>P38 self-decaying to inactive form</td>
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Table 2.4: Case 3: SOS functions as a RacGEF. Grb2-SOS, when bound to LAT, activates Rac directly. Rasgrp, when bound to LAT signalosome, cooperatively enhances SOS’ RacGEF. Rac can also be activated via a Gads mediated pathway, but SOS’ binding to LAT does not contribute to it.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>kon(1/s)</th>
<th>koff(1/s)</th>
<th>kcat(1/s)</th>
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</thead>
<tbody>
<tr>
<td>Gads binding to, unbinding from LAT without cooperativity</td>
<td>0.1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Grb2-SOS binding, unbinding to LAT without cooperativity</td>
<td>0.12</td>
<td>0.8</td>
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</tr>
<tr>
<td>PLCγ-Rasgrp binding, unbinding to LAT</td>
<td>0.16</td>
<td>0.5</td>
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</tr>
<tr>
<td>Grb2-SOS unbinding from LAT bound with Rasgrp</td>
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<tr>
<td>Gads activating Rac</td>
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<td>SOS activating Rac when LAT bound with Rasgrp</td>
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<td>SOS activating Rac when LAT unbound with Rasgrp</td>
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<td>Rac activating P38</td>
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</tr>
<tr>
<td>P38 self-decaying to inactive form</td>
<td></td>
<td></td>
<td>20</td>
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</tbody>
</table>

2.4.4 Plasmids and stable and transient transfection

Plasmid expressing full-length hSOS1 with a DBL domain mutation (pEF6-hSOS1 DBLmut) was constructed by modifying pEF6 hSOS1 wild type (WT) with 7 amino acid substitutions (LHYFELL to IIIRDII) [84]. Other plasmids and the small interfering RNA (siRNA) duplex used in this study were described previously [19, 93, 81]. For transient cotransfection of hSOS1 with the hCD16/7 fusion construct, 300 μl of SOS1- SOS2- DT40 cell suspension at 66 × 106 cells/ml in plain RPMI was electroporated with 10 μg of each plasmid by using a Bio-Rad electroporator (Bio-Rad) set at square wave, 300 V, with a 10-ms single pulse. Transfected cells were cultured for 16 to 20 h prior to phosho-flow assay or to anti-hCD16-biotin magnetically activated cell sorting (MACS) purifications by following the manufacturer’s guidelines (Miltenyi Biotec). For stable reconstitution of human SOS1, SOS1- SOS2- DT40 cells were transfected with 10 μg hSOS1 plasmid by electroporation set at exponential decay, 250 V, 950 μF. Transfected cells were selected by 10 μg/ml final blasticidin S (Fischer Biotech) from 24 h posttransfection. Single cell-derived clones were isolated from a set of serially diluted cultures on 96-well plates after 1 to 2 weeks of seeding. Isolated clones were screened for hSOS1 and surface BCR (sBCR) expression by Western blotting and fluorescence-activated cell sorting (FACS), respectively.
2.4.5 Western blot analysis of cell lysates

Levels of various proteins were measured by Western blotting as previously described [19, 93]. The following antibodies were purchased from Cell Signaling: phospho-p44/42 MAPK ERK1/2 (pT204/pY204), total ERK1/2, phospho-p38 (pT180/pY182, clone 3D7), total p38, phospho-PLCγ1 (pY783), and phospho-ZAP70 (pY319). Other antibodies were human SOS1 (BD Transduction Lab), mRasGRP1 (clone m199), Grb2 (SC-255; Santa Cruz Biotech), α-tubulin (Sigma), Rac1 (clone 23A8; Millipore), and pan-Ras (clone Ab-3; Calbiochem) for detection of chicken Ras. Proteins were visualized using enhanced chemiluminescence (ECL) Western blot substrate (Pierce) and the LAS-4000 image system (Fujifilm Life Science). Densitometric analysis was performed using Multi Gauge V3.0 (Fujifilm Life Science). Shown results are representative of two or more independent experiments.

2.4.6 Intracellular FACS staining for pERK, phospho-p38, and hSOS1

BCR-induced MAPK activation and hSOS1 protein levels were measured by intracellular FACS staining as previously described [19]. In brief, DT40 cells were resuspended/rested in PBS or plain RPMI at 2.0 × 10^6 cells/75 µl (per well of a 96-well plate). Cells were stimulated for the desired amount of time by adding 75 µl of 2× stimulation mix and subsequently fixed for 20 min at room temperature by mixing with 150 µl fixation buffer (Cytofix/Cytoperm; BD Biosciences). Cells were washed and permeabilized for at least 30 min with 90% methanol at -20°C. Fixed/permeabilized cells were stained with anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (number 9101; Cell Signaling), phospho-p38 (pT180/pY182, clone 3D7), and anti-hSOS1 (BD Transduction Lab) at room temperature (RT) in the presence of 2% (vol/vol) normal goat serum (Jackson ImmunoResearch Laboratories). Subsequently, cells were washed twice and stained with phycoerythrin (PE)- or allophycocyanin (APC)-conjugated AffiniPure F(ab')2 fragment donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) with Alexa Fluor 488-conjugated don-
key anti-mouse IgG antibody (Invitrogen). Stained cells were washed 3 times and directly analyzed by FACS.

2.4.7 Ras and Rac activation assays.

BCR-induced Ras and Rac activation were analyzed by Ras/RacGTP pulldown assays according to the manufacturer’s instructions (Upstate).
2.5 Supplementary material

Coarse-grained model of ERK activation [19, 64, 89]

Figure 2-11: Ras can be activated via both DAG-Rasgrp1-mediated and SOS-mediated pathways. The catalytic rate of SOS depends on the state of its allosteric pocket: empty, bound to RasGDP, or bound to RasGTP, with increasing level of catalytic activities. In particular, the catalytic rate of SOS with RasGTP bound to its allosteric pocket is much larger than that with RasGDP bound to its allosteric pocket. This constitutes a SOS-mediated positive feedback for Ras activation. Ras can be deactivated by RasGAP, while DAG can be metabolized by DGK. Activated Ras can trigger the activation cascade of Ras-Raf-MEK-ERK.
Coarse-grained model of ERK activation with negative and positive feedback loops [54, 6]

Figure 2-12: Based on the coarse-grained model in Fig. 2-11, a negative feedback loop and a positive feedback loop are added. The negative feedback loop is generated by the phosphorylation of SOS1 by activated ERK. Ras can also be activated via phospho-ERK, which leads to the positive feedback.
Chapter 3

Regulatory T cell differentiation requires P38 signal pathway through TCR-SOS1/2-dependent classical MAPK cascade

P38 is a key MAPK involved in various aspects of inflammatory responses, including T-cell mediated inflammation. TCR stimulation can connect to P38 activation at least by two distinct pathways: one dependent on LAT signalosome and MAPK cascade (classical) and the other via ZAP70-mediated P38 auto-activation (alternate) pathway. Here we utilized single-cell level phosphor-flow cytometry and in-silico computer modeling to investigate TCR-induced P38 activation. We show that both pathways are active downstream of TCR. Two pathways contribute differently to P38 signal output: ZAP70-mediated alternate pathway allows rapid P38 activation by lowering the activation threshold, while classical pathway determines the magnitude of response, contributing to the amplification of P38 activation. Classical P38 pathway depends on LAT and SOS1/2 signalosome and is important for differentiation of induced regulatory T (iTreg) cells in vitro and in vivo. These results highlight that two P38 pathways differently contribute to TCR-induced response, and the balance between
two pathways dictate the physiological outcome during inflammatory responses.

3.1 Introduction

P38 protein is a key mitogen-activated protein kinase (MAPK) involved in a variety of aspects of inflammatory responses, including T-cell mediated inflammation. Based on previous studies [81, 53], P38 is activated through a classical signaling pathway: activated ZAP70 phosphorylates the tyrosines on an adaptor molecule called linker for activation of T cells (LAT). Several proteins related to the downstream P38 activation bind to phosphotyrosines of LAT to assemble a signalosome. Gads binds to phosphorylated LAT, and subsequently recruits SLP-76, which binds to PLCγ1. SOS is also recruited to LAT through the binding of its C-terminal proline-rich regions to an adaptor protein Grb2’s SH3 domains. It is shown that knockdown or deletion of SOS1 attenuates TCR/BCR-induced P38 activation[53]. The mutual interactions among these proteins cooperatively stabilize the signalosome. The LAT signalosome, possibly through Gads, activates RacGEF. The activated Rac leads to the mitogen-activated protein kinase (MAPK) signaling pathway to activate P38.

The Ashwell group also proposes a LAT-independent alternative pathway by which activated ZAP70 phosphorylates P38 directly on Tyr323. In turn, the phosphorylation of Tyr323 induces the autophosphorylation of P38 on Thr180 and Tyr182. [95, 4]

To study P38 activation, we build up a minimal model including both the classical pathway and the alternative pathway. For the classical pathway, both the identities and the exact functions of each protein bound to LAT is unclear. But we can classify them as one kind of proteins, called GPS, representing Gads, PLCγ1, SOS, and other proteins, which are involved in the formation of LAT signalosome. Also, from the experimental results, we find there exists a phase change with a time delay from an analog P38 activation to a digital signal. To explain the phase change with the time delay in our mathematical model, we introduce a positive feedback loop from phosphorylated P38 (pP38) to the formation of GPS-LAT complex. For the alternative pathway, activated ZAP70 activates P38 directly.
Roncagalli's study shows that there exists a temporal delay in the assembly of LAT complex when T cell receptor is stimulated. To represent the delay effect, we study two models with the delayed signaling happening at the different steps. In Model I, we assume that there is a delay in the process of the assembly of the LAT-GPS complex. In Model II, we assume that there is a delay because a certain threshold in P38 activation needs to be overcome first before the feedback occurs.

Prior to T cell stimulation, there exists some basal pY323 already in resting T cells. So, in the last section, we add a basal signaling in the model and investigate its effect.

3.2 Results

3.2.1 Classical P38 pathway is operating downstream of TCR

We have previously shown that LAT-deficient Jurkat cells (J.Cam2) do not activate significant P38 upon TCR crosslinking [53]. This observation is consistent even when activated P38 is measured in a single-cell level by phosphor-flow assay (Figure 3-1). We noted J.Cam2 cells express reduced amount of surface TCR and exhibit reduced ZAP70 activation in response to TCR crosslinking [53, 67]. To fairly compare TCR-activated P38, wild-type and J.Cam2 cells were co-stained with anti-pTpY P38 and pZAP70 antisera. Cells activating comparable degree of pZAP70 to basal resting level were sub-gated and compared between wild-type and J.Cam2 cells. Even in the absence of LAT, there was noticeable activation P38 upon TCR stimulation, indicating TCR is connected to P38 independent of LAT signalosome and classical pathway. However, the magnitude of response was reduced compared to wild-type level, more markedly at the later time point (Figure 3-2).

We examined time kinetics of activated signal transducers in murine CD4 T cells by Western blotting (Figure 3-3). Upon CD3 crosslinking, pZAP70 and pY323 shows acute and transient kinetics of phosphorylation, reaching the peak level at 30 sec time point. However, activation of MAPK ERK1/2 and p38 (pTpY) occur at delayed time
Figure 3-1: Only a small increase of pTpY P38 is induced in the absence of LAT. Wild-type Jurkat or LAT-deficient Jurkat (JCam2) cells were stimulated with TCR crosslinking antibody. TCR-induced pTpY P38 were measure by pFLOW. Number in each histogram represents mean fluorescence intensity (MFI) of pTpY P38 signal.

Figure 3-2: LAT is required for optimal P38 activation, especially for later time point, reaching the max level at 1.5–2 min. Importantly, molecular events between pY323 and pTpY P38 are temporally separated.

3.2.2 Full P38 activation is delayed and requires SOS as well as feedback from P38 activity

Knowing TCR is connected to P38 by at least two pathways, we investigated to see if there are any quantitative or qualitative differences between two different P38 pathways by using pFLOW assay. Since alternate pathway relies on ZAP70, B cells provide an ideal system to examine P38 activation kinetics independent of alternate
Figure 3-3: Phosphorylation of Y323 P38 temporally precedes the dual phosphorylation of activation motif (pT180pY182). CD4+ T cell blast from wild-type C57BL6 mouse were stimulated with αCD3 antibody for indicated time. Specific signal (arrow heads); Non-specific signal (Asterisk).

pathway. We used Jurkat and DT40 cells as representative T and B cell lines for this study. Both cells have been widely used for the study of MAPK signaling. For acute induction of intracellular signals, antigen receptors can be stimulated by single-step exposure to mouse IgM asciate specific for TCR (clone C305) and BCR (clone M4), making the physical forms of stimulating antibodies comparable.

Jurkat T cells achieve the max pTpY P38 level, while signal to P38 is delayed in DT40 B cells (Figure 3-4). Time taken for DT40 cells to reach the max pTpY P38 level is delayed regardless of anti-BCR dosage (Figure 3-4). Interestingly, the pattern of P38 activation also differs between Jurkat T and DT40 B cells.

We previously reported SOS1, independent of its enzymatic activity, is required for optimal activation of P38 upon TCR/BCR crosslinking [53]. When BCR-induced P38 signal is measured by pFLOW, both phases of P38 activation is decreased. More significantly, the delayed exponential phase is almost abolished in the absence of SOS1/2 expression (Figure 3-5). The activation of P38 through classical pathway is induced time delayed and SOS1/2-dependent.

Since SOS1/2 are required for optimal activation of MAPK ERK and P38, we investigated whether high-level P38 activation also depends on feedback from these kinases activity. BCR-induced activation of ERK and P38 was blocked by specific-inhibitors U0126 and SB203580 respectively. Treated DT40 cells were stimulated
Figure 3-4: Different patterns of P38 activation (pTpY) in DT40 B cells and Jurkat T cells. Two different strength of BCR- or TCR-crosslinking antibodies were used representing high (Hi) or low (Lo) level strength. To note, TCR-induced P38 activation is rapid regardless of stimuli strength. While, P38 activation in DT40 B cells shows early analog phase and later exponential phase. Plots show the percentage to maximum pTpY P38 MFI level in each cell type for high and low level stimuli.

Figure 3-5: SOS1/2 are required for late, exponential phase P38 activation. BCR-induced pERK and pP38 were measured in wild-type (open histogram) and SOS1-2-(filled histogram) DT40 B cells.

with BCR crosslinking and induced pMAPK were measured by pFLOW in parallel with DMSO-treated control cells.
Specific inhibitors blocked the activation of corresponding MAPK as expected. Inhibition of P38 did not impact on ERK activation (Sup Fig XX) but blocked the late exponential phase of P38 activation (Figure 3-6). Inhibition of ERK activation did not affect P38 activation, indicating full activation of P38 requires intact P38 activity.

![Graph showing P38 activation over time with treatments](image)

Figure 3-6: Thresholded exponential phase of P38 activation requires SOS and P38 activity, but not ERK activation. Wild-type DT40 cells were treated with DMSO, P38 inhibitor (SB203580) or MEK1/2 inhibitor (U0126). To note, the last exponential P38 activation requires P38 activity. Number in each histogram represents mean fluorescence intensity of pTpY P38 signal.

### 3.3 In silico modeling of two distinct pathways driving P38 activation

#### 3.3.1 Minimal Model I

In Model I, we assume that there is a delay in the assembly process of the LAT-GPS complex that results in the activation of P38 through the classical pathway lagging
behind the activation through the alternative pathway. To represent the time delay, we take the rate constant of the P38 activation by LAT-GPS as a step function. At an earlier time, the activation rate of P38 by GPS-LAT is zero. After a certain time, \( \tau \), the classical pathway is triggered with a non-zero activation rate of P38 by GPS-LAT.

Mathematically, the rate constant of the phosphorylation process of P38 by GPS-LAT can be described as a Heaviside step function, \( k_p = k_p \theta(t - \tau) \), where \( \tau \) is the critical time point. This model is illustrated in Figure 3-7 below.

![Figure 3-7: Model I: there exists a delay in the assembly process of the LAT-GPS complex. The classical pathway is triggered after a critical time point.](image)

### 3.3.2 Minimal Model II

In Model II, we assume that a certain threshold in phosphorylated P38 needs to be overcome first, before the positive feedback regulation from pP38 to LAT-GPS complex kicks in. To represent the delay of the feedback loop from pP38 to GPS-LAT complex, we take the rate constant of the feedback reaction as a step function. When the amount of phosphorylated P38 is small, the rate constant of the feedback loop from pP38 to GPS-LAT is zero. After phosphorylated P38 is accumulated to a certain threshold, \( pP38^0 \), the feedback loop is triggered with a non-zero rate constant.
Mathematically, \( k_f = k_f \theta (pP38 - pP38^0) \). See the illustration of Model II in Figure 3-8.

![Model II Diagram](image)

Figure 3-8: Model II: a certain threshold in phosphorylated P38 needs to be overcome before the positive feedback regulation from pP38 to LAT-GPS complex kicks in.

### 3.3.3 Simulations

The Gillespie algorithm is implemented to simulate the described models, Model I and Model II. In all stochastic simulations, we used a spatially homogeneous simulation box of size \( V = Area(4mm^2) \times Height(0.02mm^2) \). This choice of the system size ensures that the system is well-mixed. The initial numbers of species and the rate constants are listed in Table 3.1 and Table 3.2 [53].

<table>
<thead>
<tr>
<th>Species</th>
<th>Initial number of molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZAP70</td>
<td>100</td>
</tr>
<tr>
<td>GPS</td>
<td>100</td>
</tr>
<tr>
<td>LAT</td>
<td>200</td>
</tr>
<tr>
<td>P38</td>
<td>1000</td>
</tr>
</tbody>
</table>

Table 3.1: Initial Numbers of Species
Table 3.2: Rate Constants

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Rate constant k (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated ZAP70 phosphorylates LAT, $k_{zd}$</td>
<td>0.05</td>
</tr>
<tr>
<td>Activated ZAP70 phosphorylates P38, $k_{zp}$</td>
<td>0.005</td>
</tr>
<tr>
<td>pLAT self-decays to the inactive form, $k_{df}$</td>
<td>1</td>
</tr>
<tr>
<td>GPS binds to pLAT, $k_{bg}$</td>
<td>0.0001</td>
</tr>
<tr>
<td>GPS unbinds to pLAT, $k_{bg}$</td>
<td>5</td>
</tr>
<tr>
<td>GPS-LAT complex phosphorylates P38, $ka$</td>
<td>0.1</td>
</tr>
<tr>
<td>pP38 self-decays to the inactive form, $k_{dp}$</td>
<td>40</td>
</tr>
<tr>
<td>The positive feedback of pP38 to GPS-LAT complex, $k_f$</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Simulations of Model I

We carried out simulations by setting different critical time points. Before the critical time point, $\tau$, there is only the alternative pathway. After the critical time point, the classical pathway is triggered.

When the critical time, $\tau = 1 \text{ sec}$, the simulation results are plotted in the following histograms at four different time points. Each histogram represents the distribution of in silico "cells" with various levels of phosphorylated P38.

![Histograms of P38 activation](image)

Figure 3-9: Set the critical time at $\tau = 1s$. The histograms of P38 activation at different time points.

When $\tau$ is decreased to 0.02 sec, the simulation results are plotted in Figure 3-10.

From the simulations, Model I does not recapture the characteristics of the full
Figure 3-10: Set the critical time at $\tau = 0.02s$. The histograms of P38 activation at different time points.

P38 activation no matter which critical time point we choose. That is because, after the classical pathway is triggered (after the critical time point), the peak of pP38 will move directly to the final stable state in an analog way. In other words, the delay of the assembly of the LAT-GPS complex cannot explain the behavior of the full P38 activation.

Simulations of Model II

We carried out the stochastic simulations based on Model II.

Classical Pathway vs pP38 Threshold  The simulation results are shown in Figure 3-11 with different strengths of classical pathways and different thresholds of amount of $pP38^0$. They show that the threshold of amount of $pP38^0$ and the strength of the classical pathway affect the behavior of P38 activation in a coordinated manner.

For a certain strength of the classical pathway (e.g., intermediate, when $k_a = 0.1$ in Figure 3-11), the threshold of amount of $pP38^0$ cannot be too high, otherwise, the positive feedback loop cannot be triggered which means that the full activated P38 cannot be seen. Interestingly, at a certain threshold (e.g., intermediate level, when
As the strength of the classical pathway is increased, the magnitude of the end point of phosphorylated P38 is augmented.

![Figure 3-11: Simulation of Model II with different strengths of the classical pathway and different levels of the threshold of amount of pP380. As the strength of the classical pathway is increased, the magnitude of the end point of phosphorylated P38 is augmented.](image)

**Alternative Pathway vs pP38 Threshold**  The simulations with different strengths of the alternative pathway versus the thresholds of amount of pP380 are carried out and the results are shown in Figure 3-12.

Similar to the analysis of varying classical pathway, for a certain strength of the alternative pathway (e.g., weak, when \( k_s = 0.1 \) in Figure 3-12), the threshold of amount of pP380 cannot be too high, otherwise, the threshold will never be reached.
On the contrary, the alternative pathway does not affect the magnitude of the end point of the pP38 that much, but it accelerates the onset of the full P38 activation.

![Diagram](image)

Figure 3-12: Simulation of Model II with different strengths of the alternative pathway and different levels of the threshold of amount of pP38. Stronger alternative pathway accelerates the onset of the full P38 activation.

3.3.4 Only the alternative pathway

To test the alternative pathway-only P38 activation, we deleted LAT. The result is shown in Figure 3-13. We cannot see the full activation of P38. But there is still an analog pP38 signal which is in good agreement with experimental results.
3.4 The Model with Basal pZAP70

Prior to T cell stimulation, there exists some basal level of activated ZAP70. In this section, we include a basal signaling in the model and study the corresponding influence.

From Figure 3-15, the basal pZAP70 signal makes TCR-induced P38 activation reaches the threshold level faster.
3.5 Variations

To clearly see the different effects of the classical pathway and the alternative pathway, here, we ignore stochastic fluctuations in the number of molecules. By solving rate equations of the network, we obtain the temporal evolution of the average amount of phosphorylated P38.

Figure 3-16a and Figure 3-16b show greater classical pathway but not alternative pathway increases the magnitude of the end point of P38 activation. However, strengthening alternative pathway accelerates the onset of P38 activation.
3.6 Mathematical Analysis

To understand the principles underlying the minimal model, we study the steady states and their stabilities based on the classical pathway network shown in Figure 3-16.

Denote

[P] as the number of unphosphorylated P38
[PP] as the number of phosphorylated P38
[L] as the number of LAT
[G] as the number of GPS
[GL] as the number of GPS-LAT complex
$k_{bg}$ as the rate constant of binding process of GPS and LAT

$k_{ug}$ as the rate constant of unbinding process of GPS-LAT complex

$k_a$ as the rate constant of the activation of P38 by GPS-LAT complex

$k_d$ as the rate constant of the deactivation of pP38

$k_f$ as the rate constant of the feedback from pP38 to GPS-LAT

$\alpha$ as the total amount of initial unphosphorylated P38

$\beta$ as the total amount of initial GPS

$\gamma$ as the total amount of initial LAT

We write down the rate equations for this minimal model.

\[
\frac{d[PP]}{dt} = -k_d[PP] + k_a[P][GL]
\]

\[
\frac{d[GL]}{dt} = -k_{ug}[GL] + k_{bg}[L][G] + k_f[L][G][PP]
\]

For steady states, we have

\[
0 = -k_d[PP] + k_a[P][GL]
\]

\[
0 = -k_{ug}[GL] + k_{bg}[L][G] + k_f[L][G][PP]
\]

From the conservation of species, we have

\[
[P] + [PP] = \alpha
\]

\[
[G] + [GL] = \beta
\]

\[
[L] + [GL] = \gamma
\]

Then, from equations 3.1 and 3.2, we obtain

\[
k_f(k_d + \gamma k_a)(k_d + \beta k_a)[PP]^3
\]

\[
+ (k_a k_d (\beta + \gamma)(k_{bg} - \alpha k_f) + k_a^2 \beta \gamma (k_{bg} - 2\alpha k_f) + k_{bg} k_d^2 + k_a k_d k_{ug})[PP]^2
\]

\[
+ (\alpha \beta \gamma k_a^2 (\alpha k_f - 2k_{bg}) - \alpha k_a k_d (k_{bg} (\beta + \gamma) + k_{ug}))[PP]
\]

\[
+ k_{bg} k_d^2 \alpha^2 \beta \gamma = 0
\]
Let $x = [PP]$, 
Then, $a_3x^3 + a_2x^2 + a_1x + a_0 = 0$

where, $a_0 = k_{bg}k_a^2\alpha^2\beta\gamma$

\[
a_1 = \alpha\beta\gamma k_a^2(\alpha k_f - 2k_{bg}) - \alpha k_a k_{d}(k_{bg}(\beta + \gamma) + k_{ug}),
\]
\[
a_2 = k_a k_{d}(\beta + \gamma)(k_{bg} - \alpha k_f) + k_a^2\beta\gamma(k_{bg} - 2\alpha k_f) + k_{bg}k_d^2 + k_{a}k_{d}k_{ug},
\]
and $a_3 = k_f(k_{d} + \gamma k_{a})(k_{d} + \beta k_{a})$

Because $a_0 = k_{bg}k_a^2\alpha^2\beta\gamma > 0$ and $a_3 = k_f(k_{d} + \gamma k_{a})(k_{d} + \beta k_{a}) > 0$, according to the Decartes rule, there are at most two real positive solutions. By approximation, we can estimate the three solutions of $x = [PP]$.

\[
x_1 \approx -\frac{k_{bg}}{k_f} \quad \text{Negative solution} \quad \text{With the effect of stochastic fluctuation, this is close to zero. By linear stability analysis, this is an unstable state.}
\]
\[
x_2 \approx \frac{\beta k_a \alpha}{\beta k_a + k_d} \quad \text{Positive solution} \quad \text{Stable State}
\]
\[
x_3 \approx \frac{\gamma k_a \alpha}{\gamma k_a + k_d} \quad \text{Positive solution} \quad \text{If it is true, the number of GPS-LAT complexes is larger than } \gamma, \text{ the total amount of LAT, which can NOT be biologically true.}
\]

### 3.6.1 Linear Stability Analysis

We then analyze the stability of the steady states obtained from solving ODEs.

Let $x = [PP]$ and $y = [GL]$, 

\[
\frac{dx}{dt} = -k_d x + k_a (\alpha - x) y
\]
\[
\frac{dy}{dt} = -k_{ug} y + (k_{bg} + k_f x)(\gamma - y)(\beta - y)
\]
Thus,
\[
\frac{d\delta x}{dt} = -(k_d + k_a\bar{y})\delta x + k_a(\alpha - \bar{x})\delta y \\
\frac{d\delta y}{dt} = k_f(\gamma - \bar{y})(\beta - \bar{y})\delta x - (k_{ug} + (k_{bg} + k_f\bar{x})(\beta + \gamma - 2\bar{y}))\delta y
\]

where, \(\bar{x}\) and \(\bar{y}\) are the concentrations of phosphorylated P38 and the GPS-LAT complex at steady states.

Jacobian Matrix is
\[
J = \begin{pmatrix}
-(k_d + k_a\bar{y}) & k_a(\alpha - \bar{x}) \\
k_f(\gamma - \bar{y})(\beta - \bar{y}) & -(k_{ug} + (k_{bg} + k_f\bar{x})(\beta + \gamma - 2\bar{y}))
\end{pmatrix}
\]

For the parameters, \(\alpha = 1000, \beta = 100, \gamma = 200, k_{bg} = 0.001, k_{ug} = 5, k_a = 0.1, k_d = 40, k_f = 0.01\), we obtain

\[
[PP] = -0.1 \quad \text{Unstable} \\
[PP] = 196 \quad \text{Stable} \\
[PP] = 337 \quad \text{Unstable}
\]

Thus, the system has two physical steady states. One is table and the other is unstable. The unstable one is close to zero and it is able to be observed in experiments due to stochastic fluctuation. The system will jump from the unstable state to the higher stable state. We can call it apparent bimodality.

### 3.7 Correlation Functions

To identify the existence of the positive feedback loop from phosphorylated P38 to the formation of LAT complex, we explore features of correlation functions of molecular numbers.
The reaction scheme of P38 activation can be simplified as

\[
\begin{align*}
\text{ZAP70} + \text{P38} & \xrightarrow{k_1} \text{ZAP70} + \text{pP38} \\
\text{ZAP70} + \text{LAT} & \xrightarrow{k_2} \text{ZAP70} + \text{pLAT} \\
\text{pLAT} + \text{P38} & \xrightarrow{k_3} \text{pLAT} + \text{pP38} \\
\text{pP38} + \text{LAT} & \xrightarrow{k_4} \text{pP38} + \text{pLAT} \\
\text{pLAT} & \xrightarrow{k_5} \text{LAT} \\
\text{pP38} & \xrightarrow{k_6} \text{P38}
\end{align*}
\]

From the conservation law, we have

\[
\begin{align*}
[P] + [PP] &= [P]_T = \alpha \\
[L] + [LL] &= [L]_T = \beta \\
[ZAP70] &= [Z]_T = \gamma
\end{align*}
\]

Thus,

\[
\begin{align*}
[P] &= \alpha - x \\
[L] &= \beta - y \\
[ZAP70] &= \gamma
\end{align*}
\]

The ordinary differential equations (ODEs) are

\[
\begin{align*}
\frac{d[pP38]}{dt} &= k_1[ZAP70][P38] + k_3[pLAT][P38] - k_6[pP38] \\
\frac{d[pLAT]}{dt} &= k_2[ZAP70][LAT] + k_4[pP38][LAT] - k_5[pLAT]
\end{align*}
\]

Substitute the notations to the ODEs

\[
\begin{align*}
\frac{dx}{dt} &= k_1\gamma(\alpha - x) + k_3y(\alpha - x) - k_6x \\
\frac{dy}{dt} &= k_2\gamma(\beta - y) + k_4x(\beta - y) - k_5y
\end{align*}
\] (3.3)
Then, we convert the concentrations to the number of species

\[
\begin{align*}
\frac{dX}{dt} &= \Omega^{-1}k_1NZ(N_P - X) + \Omega^{-1}k_3Y(N_P - X) - k_6X \\
\frac{dY}{dt} &= \Omega^{-1}k_2NZ(N_L - Y) + \Omega^{-1}k_4X(N_L - Y) - k_5Y
\end{align*}
\] (3.4)

The corresponding master equations are

\[
\begin{align*}
\frac{dP(X,Y,t)}{dt} &= \Omega^{-1}k_1(\mathbb{E}_X^{-1} - 1)NZ(N_P - X)P + \Omega^{-1}k_3(\mathbb{E}_X^{-1} - 1)Y(N_P - X)P \\
&\quad + k_6(\mathbb{E}_X^{-1} - 1)XP + \Omega^{-1}k_2(\mathbb{E}_Y^{-1} - 1)NZ(N_L - Y)P \\
&\quad + \Omega^{-1}k_4(\mathbb{E}_Y^{-1} - 1)X(N_L - Y)P + k_5(\mathbb{E}_Y^{-1} - 1)YP
\end{align*}
\] (3.5)

We assume that the number of species has the mean scaled with \(\Omega\) and the standard deviation scaled with \(\Omega^{1/2}\). With this assumption, we have the number of pP38 \(X = \Omega \phi(t) + \Omega^{1/2} \zeta(t)\) and the number of pLAT \(Y = \Omega \psi(t) + \Omega^{1/2} \eta(t)\)

Thus, we have

\[
\begin{align*}
\frac{d\zeta(t)}{dt} &= -\Omega^{1/2} \frac{d\phi(t)}{dt} \\
\frac{d\eta(t)}{dt} &= -\Omega^{1/2} \frac{d\psi(t)}{dt}
\end{align*}
\]

Now the probability can be written as a function of \(\zeta\) and \(\eta\)

\[
P(X,Y,t) = \Pi(\zeta, \eta, t)
\]

The step operator \(\mathbb{E}\) can be approximated by the Taylor expansion

\[
\begin{align*}
\mathbb{E}^{-1}_X &= 1 + \Omega^{-1/2} \frac{\partial}{\partial \zeta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \zeta^2} + \ldots \\
\mathbb{E}^{-1}_X &= 1 - \Omega^{-1/2} \frac{\partial}{\partial \zeta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \zeta^2} + \ldots \\
\mathbb{E}^{-1}_Y &= 1 + \Omega^{-1/2} \frac{\partial}{\partial \eta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \eta^2} + \ldots \\
\mathbb{E}^{-1}_Y &= 1 - \Omega^{-1/2} \frac{\partial}{\partial \eta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \eta^2} + \ldots
\end{align*}
\]
Thus, the master equation 3.5 in the new variables becomes

\[
\frac{d\Pi(\zeta, \eta, t)}{dt} = \frac{\partial \Pi}{\partial t} + \frac{\partial \Pi}{\partial \zeta} \frac{d\zeta}{dt} + \frac{\partial \Pi}{\partial \eta} \frac{d\eta}{dt}
\]

\[
= \gamma k_1(-\Omega^{-\frac{1}{2}} \frac{\partial}{\partial \zeta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \zeta^2})(\Omega \alpha - \Omega \phi - \Omega^{\frac{1}{2}} \zeta)\Pi 
+ k_3(-\Omega^{-\frac{1}{2}} \frac{\partial}{\partial \zeta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \zeta^2})(\Omega \psi + \Omega^{\frac{1}{2}} \eta)(\alpha - \phi - \Omega^{-\frac{1}{2}} \zeta)\Pi 
+ k_6(\Omega^{-\frac{1}{2}} \frac{\partial}{\partial \zeta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \zeta^2})(\Omega \phi + \Omega^{\frac{1}{2}} \zeta)\Pi 
+ k_2 \gamma(-\Omega^{-\frac{1}{2}} \frac{\partial}{\partial \eta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \eta^2})(\Omega \beta - \Omega \psi - \Omega^{\frac{1}{2}} \eta)\Pi 
+ k_4(-\Omega^{-\frac{1}{2}} \frac{\partial}{\partial \eta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \eta^2})(\Omega \phi + \Omega^{\frac{1}{2}} \zeta)(\beta - \psi - \Omega^{-\frac{1}{2}} \eta)\Pi 
+ k_5(\Omega^{-\frac{1}{2}} \frac{\partial}{\partial \eta} + \frac{1}{2} \Omega^{-1} \frac{\partial^2}{\partial \eta^2})(\Omega \psi + \Omega^{\frac{1}{2}} \eta)\Pi
\]

Collecting the terms of order \(O(\Omega^{\frac{1}{2}})\), we obtain

\[
\frac{d\phi}{dt} = \gamma k_1 (\alpha - \phi) + k_3 \psi (\alpha - \phi) - k_6 \phi
\]

\[
\frac{d\psi}{dt} = \gamma k_2 (\beta - \psi) + k_4 \phi (\beta - \psi) - k_5 \psi
\]

(3.6)

Not surprisingly, equations 3.6 have the same form of equations 3.4.

Collecting the terms of order \(O(\Omega^0)\), we obtain

\[
\frac{\partial \Pi}{\partial t} = (\gamma k_1 + k_3 \psi + k_6) \frac{\partial}{\partial \zeta} (\zeta \Pi) + (k_2 \gamma + k_4 \phi + k_5) \frac{\partial}{\partial \eta} (\eta \Pi)
- k_3 (\alpha - \phi) \frac{\partial}{\partial \zeta} (\eta \Pi) - k_4 (\beta - \psi) \frac{\partial}{\partial \eta} (\zeta \Pi)
+ \frac{1}{2} \gamma k_1 (\alpha - \phi) + \frac{1}{2} k_3 \psi (\alpha - \phi) + \frac{1}{2} k_6 \phi \frac{\partial^2}{\partial \zeta^2} \Pi
+ \frac{1}{2} \gamma k_2 (\beta - \psi) + \frac{1}{2} k_4 \phi (\beta - \psi) + \frac{1}{2} k_5 \psi \frac{\partial^2}{\partial \eta^2} \Pi
\]

(3.7)

The first moments of the fluctuations are

\[
\partial_t \langle \zeta \rangle = -(\gamma k_1 + k_3 \psi + k_6) \langle \zeta \rangle + k_3 (\alpha - \phi) \langle \eta \rangle
\]

\[
\partial_t \langle \eta \rangle = k_4 (\beta - \psi) \langle \zeta \rangle - (\gamma k_2 + k_4 \phi + k_5) \langle \eta \rangle
\]

(3.8)
Stoichiometric matrix $\nu$ is
\[
\nu = \begin{bmatrix}
1 & 0 \\
0 & 1 \\
1 & 0 \\
0 & 1 \\
0 & -1 \\
-1 & 0
\end{bmatrix}
\]

Define the Jacobian matrix $A$, where its entry $A_{ij} = \sum_{k=1}^{R} \nu_{ki} \left( \frac{\partial a_k}{\partial c_j} \right)$, in which $\nu$ represents the stoichiometric matrix, $c_j$ the concentration of species $j$, and $a_k$ the propensity of reaction $k$.

\[
A = \begin{bmatrix}
-(k_1 \gamma + k_3 \psi + k_6) & k_3(\alpha - \phi) \\
k_4(\beta - \psi) & -(k_2 \gamma + k_4 \phi + k_5)
\end{bmatrix}
\]

The eigenvalues of $A$ are
\[
\lambda_{1,2} = \frac{(A_{11} + A_{22}) \pm \sqrt{(A_{11} + A_{22})^2 - 4(A_{11}A_{22} - A_{12}A_{21})}}{2}
\]

The solution of equations 3.8 is
\[
\langle \zeta \rangle = \left( \frac{\lambda_2 - A_{11}}{\lambda_2 - \lambda_1} \right) \langle \zeta \rangle_0 - \left( \frac{\lambda_1 - A_{11}}{\lambda_2 - \lambda_1} \right) \langle \eta \rangle_0 e^{\lambda_1 t} - \left( \frac{\lambda_1 - A_{11}}{\lambda_2 - \lambda_1} \right) \langle \zeta \rangle_0 - \left( \frac{\lambda_2 - A_{11}}{\lambda_2 - \lambda_1} \right) \langle \eta \rangle_0 e^{\lambda_2 t}
\]
\[
\langle \eta \rangle = \frac{\lambda_1 - A_{11}}{A_{12}} \left( \frac{\lambda_2 - A_{11}}{\lambda_2 - \lambda_1} \right) \langle \zeta \rangle_0 - \frac{\lambda_2 - A_{11}}{A_{12}} \left( \frac{\lambda_1 - A_{11}}{\lambda_2 - \lambda_1} \right) \langle \eta \rangle_0 e^{\lambda_1 t} - \frac{\lambda_2 - A_{11}}{A_{12}} \left( \frac{\lambda_1 - A_{11}}{\lambda_2 - \lambda_1} \right) \langle \zeta \rangle_0 - \frac{\lambda_2 - A_{11}}{\lambda_2 - \lambda_1} \langle \eta \rangle_0 e^{\lambda_2 t}
\]

Define the diffusivity matrix $B$, where $B_{jk} = \sum_{i=1}^{R} a_i \nu_{ij} \nu_{ik}$, in which $a_i$ is the propensity of reaction $i$, and $\nu_{ij}$ is the stoichiometric matrix.

\[
B = \begin{bmatrix}
k_1 \gamma(\alpha - \phi) + k_3 \psi(\alpha - \phi) + k_6 \phi & 0 \\
0 & k_2 \gamma(\beta - \psi) + k_4 \phi(\beta - \psi) + k_5 \psi
\end{bmatrix}
\]
For the second moments of the fluctuations, we have

\[
\partial_t \langle \zeta^2 \rangle = 2A_{11} \langle \zeta^2 \rangle + 2A_{12} \langle \eta \zeta \rangle + B_{11} \\
\partial_t \langle \eta^2 \rangle = 2A_{22} \langle \eta^2 \rangle + 2A_{21} \langle \eta \zeta \rangle + B_{22} \\
\partial_t \langle \zeta \eta \rangle = A_{11} \langle \zeta \eta \rangle + A_{12} \langle \eta^2 \rangle + A_{21} \langle \zeta^2 \rangle + A_{22} \langle \zeta \eta \rangle
\]

We obtain the variance-covariance at the stationary state

\[
\langle \zeta \eta \rangle^s = \frac{A_{11}A_{12}B_{22} + A_{22}A_{21}B_{11}}{2(A_{11} + A_{22})(A_{11}A_{22} - A_{12}A_{21})}
\]

Then, we calculate the correlation function matrix

\[
\kappa_{12}(t) = \langle \zeta_0 \eta_t \rangle^s = \mathbb{E}[\zeta_0 \eta_t] = \mathbb{E}[\zeta_0 \eta_t | \zeta_0 \eta_0]
\]

\[
= \int \int \mathbb{E}[\zeta_0 \eta_t | \zeta_0 = \zeta, \eta_0 = \eta] P(\zeta_0 = \zeta, \eta_0 = \eta) d\zeta d\eta
\]

\[
= \int \int \zeta \mathbb{E}[\eta_t | \zeta_0 = \zeta, \eta_0 = \eta] P(\zeta_0 = \zeta, \eta_0 = \eta) d\zeta d\eta
\]

\[
= \int \int (\frac{\lambda_1 - A_{11}}{A_{12}}(\frac{\lambda_2 - A_{11} \zeta^2 - A_{12} \eta \zeta}{\lambda_2 - \lambda_1})e^{\lambda_1 t} - \frac{\lambda_2 + A_{11}}{A_{12}}(\frac{(\lambda_1 - A_{11}) \zeta^2 - A_{12} \eta \zeta}{\lambda_2 - \lambda_1})e^{\lambda_2 t})
\]

\[
P(\zeta_0 = \zeta, \eta_0 = \eta)d\zeta d\eta
\]

\[
= \frac{\lambda_1 - A_{11}}{A_{12}}(\frac{\lambda_2 - A_{11}}{\lambda_2 - \lambda_1})e^{\lambda_1 t} - \frac{\lambda_2 + A_{11}}{A_{12}}(\frac{(\lambda_1 - A_{11}) \zeta^2 - A_{12} \eta \zeta}{\lambda_2 - \lambda_1})e^{\lambda_2 t}
\]

When \( k_4 = 0, \ A_{21} = 0 \), which means the positive feedback loop is blocked, thus

\[
\partial_t \langle \zeta \rangle = -(\gamma k_1 + k_3 \psi + k_6) \langle \zeta \rangle + k_3 (\alpha - \phi) \langle \eta \rangle
\]

\[
\partial_t \langle \eta \rangle = -(\gamma k_2 + k_4 \phi + k_5) \langle \eta \rangle
\]

The solutions are

\[
\langle \zeta \rangle = (\langle \zeta \rangle_0 - \frac{A_{12} \langle \eta \rangle_0}{A_{22} - A_{11}})e^{A_{11} t} + \frac{A_{12} \langle \eta \rangle_0}{A_{22} - A_{11}}e^{A_{22} t}
\]

\[
\langle \eta \rangle = \langle \eta \rangle_0 e^{A_{22} t}
\]
Therefore

\[ \langle \eta_0 \eta_t \rangle^s = \langle \eta^2 \rangle^s e^{A_{22}t} \]
\[ \langle \zeta_0 \eta_t \rangle^s = \langle \zeta \eta \rangle^s e^{A_{22}t} \]  

(3.13)

Thus, the correlation functions have different forms (equations 3.10 and 3.13) for the P38 activation model with or without the positive feedback loop. And we can identify the existence of the positive feedback by examining the correlation functions.
Chapter 4

Transitions in genetic toggle switches driven by dynamic disorder in rate coefficients\textsuperscript{1}

In biochemical systems, intrinsic noise may drive the system switch from one stable state to another. We investigate how kinetic switching between stable states in a bistable network is influenced by dynamic disorder, i.e., fluctuations in the rate coefficients. Using the geometric minimum action method, we first investigate the optimal transition paths and the corresponding minimum actions based on a genetic toggle switch model in which reaction coefficients draw from a discrete probability distribution. For the continuous probability distribution of the rate coefficient, we then consider two models of dynamic disorder in which reaction coefficients undergo different stochastic processes with the same stationary distribution. In one, the kinetic parameters follow a discrete Markov process and in the other they follow continuous Langevin dynamics. We find that regulation of the parameters modulating the dynamic disorder, as has been demonstrated to occur through allosteric control in bistable networks in the immune system, can be crucial in shaping the statistics of optimal transition paths, transition probabilities, and the stationary probability distribution of the network.

\textsuperscript{1}The content of this chapter has been published on the Journal of Chemical Physics[16]
4.1 Introduction

Single molecule studies have demonstrated that enzymatic behavior is different from the static picture gleaned from ensemble averaged experiments [74, 125, 128], and a growing body of theoretical work has examined the consequences of fluctuating chemical kinetics in protein interaction networks [11, 129]. Enzymatic rates of catalysis can fluctuate over several orders of magnitude [120, 26]. Recent single molecule studies from Iversen et al. have shown that this type of behavior is even exhibited in bistable networks that are involved in the earliest stages of T-cell signaling in the immune system [49]. The particular behavior outlined in that work shows that enzymatic rate constants can remain at a fixed value for an interval of time before jumping and resampling from a heavy tailed distribution [97, 49]. Throughout this chapter, we will model such a behavior by a stochastic process in which the time intervals between switching events are exponentially distributed with a parameter \( \lambda [s^{-1}] \) that we call the switching rate. The single molecule studies find that, interestingly, allosteric regulation can affect both the switching rate \( \lambda \), and the shape of the probability distribution \( p(k) \) that the rate parameters draw from. It was shown through computational studies that the presence of dynamic disorder [132] can make the network more stable than it would be if all kinetic parameters acted at a single average value and did not fluctuate, in the sense that a stronger signal input would be required for the network to switch from one basin to the other.

In the present work, we investigate the effects of dynamic disorder in a general toggle switch model with bistability. [82, 30, 2, 123, 98] We first investigate a relatively simple model where the rate parameter \( k \) draws from a discrete probability distribution. This discrete distribution model is simple enough for us to examine the optimal transition paths between stable states and the corresponding actions by using the geometric minimum action method (gMAM) [87, 44]. The results of the optimal transition paths illustrate a deviation between the optimal path and the average transition path obtained from chemical rate equations. And, as expected, the optimal paths converge to the average path when we increase the switching rate. The
increasing switching rate also dramatically reduces the minimum action, which means the dynamic disorder makes the system more stable.

We then consider a more complex genetic toggle switch model in which the rate parameter $k$ draws from a continuous heavy tailed distribution. We see that by tuning the shape of the distribution $p(k)$ from which a particular rate constant samples, or by tuning the switching rate $\lambda$, the equilibrium probability mass function (PMF) is significantly changed and therefore one stable basin can be greatly stabilized relative to the other. This means that dynamic disorder (and specifically allosteric control of the parameters of the model) can dramatically influence the mean first passage time for transition from one stable basin to the other. We investigate this behavior both in the small system limit by looking at Gillespie simulations of the toggle switch network, and also in the large volume, fixed concentration limit by using numerical procedures to investigate how the minimum action path is influenced by the presence of dynamic disorder. We find that the results are qualitatively unchanged when the stochastic rate parameter follows a Markov jump process as opposed to stochastic Langevin dynamics.

4.2 Genetic toggle switches with three isomers

In genetic toggle switches, a pair of genes mutually represses each other [82, 30, 2]. A pair of genes encodes proteins $A$ and $B$ respectively, called transcription factors. Protein $A$ and $B$ in turn form homodimers $A_2$ and $B_2$ which bind to regulatory regions of DNA, called operators ($O_a$ and $O_b$), of the respective other gene. The binding of $A_2$ to the operator $O_b$ represses the production of protein $B$ and vice versa. We count the total copy numbers of the transcription factors $A$ ($N_A$) and $B$ ($N_B$) which include those in homodimers and those bound to the operators. Within a region in parameter space, the genetic toggle switches have two possible stable states: a state with a large concentration of $c_A$ and a small concentration of $c_B$, or the other state with small $c_A$ and large $c_B$. [123]

To incorporate dynamic disorder into the model, we investigate a model in which,
due to fluctuating conformational changes, the unbinding rate of $A_2$ from the operator $O_b$ is not fixed but instead fluctuates. We study an asymmetric model in which only polymer $A_2$ has a fluctuating unbinding rate. To examine the optimal transition paths and find the corresponding minimum actions, we begin with a relatively simple model where the unbinding rate of $O_b A_2$ samples from a discrete probability distribution, $P_1 = 0.1, P_2 = 0.8$ and $P_3 = 0.1$. In other words, there are three conformations of polymer $A_2$, $A_2^1$, $A_2^2$ and $A_2^3$ with different unbinding rates, and these homodimers switch among each other governed by a rule of transition probability matrix:

$$
R = \begin{pmatrix}
A^1_2 & A^2_2 & A^3_2 \\
A^1_2 & R_1 & R_2 & R_3 \\
A^2_2 & R_1 & R_2 & R_3 \\
A^3_2 & R_1 & R_2 & R_3
\end{pmatrix}
$$

where, $R_1 = 0.1, R_2 = 0.8, R_3 = 0.1$.

We model the genetic toggle switches as a discrete Markov jump process with the reaction scheme in Figure 5-1 and Table 4.1. The values of the rate constants are chosen based on Warren’s paper.[123] We assume that there is only one copy of the genome in the genetic toggle switch.
Figure 4-1: The model of the genetic toggle switches. Genes $a$ and $b$ encode proteins $A$ and $B$ respectively. Protein $A$ and $B$ in turn form homodimers $A_2$, with three conformations, and $B_2$ which bind to regulatory regions of DNA of the respective other gene. The binding of $A_2$ to the operator $O_b$ represses the production of protein $B$ and vice versa.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>$k_f$</th>
<th>$k_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production of proteins</td>
<td>$O_a \rightarrow O_a + A$</td>
<td>$O_b \rightarrow O_b + B$</td>
</tr>
<tr>
<td>Degradation of proteins</td>
<td>$A \rightarrow \emptyset$</td>
<td>$B \rightarrow \emptyset$</td>
</tr>
<tr>
<td>Formation of dimers</td>
<td>$A + A \rightleftharpoons A_2^1$</td>
<td>$B + B \rightleftharpoons B_2$</td>
</tr>
<tr>
<td></td>
<td>$A + A \rightleftharpoons A_2^2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$A + A \rightleftharpoons A_2^3$</td>
<td></td>
</tr>
<tr>
<td>Binding/unbinding to operators</td>
<td>$O_a + A_2^1 \rightleftharpoons O_b A_2^1$</td>
<td>$O_a + B_2 \rightleftharpoons O_b B_2$</td>
</tr>
<tr>
<td></td>
<td>$O_b + A_2^1 \rightleftharpoons O_b A_2^2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$O_b + A_2^2 \rightleftharpoons O_b A_2^3$</td>
<td></td>
</tr>
<tr>
<td>Switching among dimers</td>
<td>$A_2^1 \rightleftharpoons A_2^2$</td>
<td>$\lambda R_2$</td>
</tr>
<tr>
<td></td>
<td>$A_2^1 \rightleftharpoons A_2^3$</td>
<td>$\lambda R_3$</td>
</tr>
<tr>
<td></td>
<td>$A_2^2 \rightleftharpoons A_2^3$</td>
<td>$\lambda R_3$</td>
</tr>
<tr>
<td></td>
<td>$O_b A_2^1 \rightleftharpoons O_b A_2^2$</td>
<td>$\lambda R_2$</td>
</tr>
<tr>
<td></td>
<td>$O_b A_2^2 \rightleftharpoons O_b A_2^3$</td>
<td>$\lambda R_3$</td>
</tr>
<tr>
<td></td>
<td>$O_b A_2^3 \rightleftharpoons O_b A_2^1$</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Reaction Scheme of Genetic Toggle Switches with Three Isomers of $A_2$

1. $k_f$ and $k_b$ are the forward and backward rate constants of the corresponding reactions.
2. $P_{1,2,3}$ are the distribution probabilities of three polymers $A_2$.
3. $R_{1,2,3}$ are the switching probabilities of three polymers $A_2$.
4. $\lambda$ is the switching rate.

### 4.2.1 Optimal transition paths

As described in the last section, genetic switches normally have bimodality and the intrinsic noise can drive the genetic switches from one stable state to the other. From
the large deviation theory, the probability of a rare transition between stable states is dominated by the probability of the optimal transition path with the minimum action. [28, 113] In this section, we investigate the most probable transition paths for the genetic switches with different switching rates (λ) by implementing the geometric minimum action method (gMAM) [44].

We first obtain the stable states by solving ordinary differential equations (ODE) with different switching rates based on the reaction scheme in Table 4.1. We plot these stable states in Figure 4-2. The x and y axes represent the concentrations of species $A$ ($C_A = c_A + 2(c_{A_1} + c_{A_2} + c_{A_3} + c_{O_bA_1} + c_{O_bA_2} + c_{O_bA_3})$) and $B$ ($C_B = c_B + 2(c_{B_1} + c_{O_bB_2})$) respectively. Note that stable states, for both high $A$ (labeled as a in Figure 4-2) and low $A$ (labeled as b), of the system with different switching rates are distinct from each other. As the switching rate increases, the stable states shift in the direction of less $A$ and more $B$ (top left in Figure 4-2). The reason is that polymers $A_2$ switch among each other on operators and the overall behavior will be closer to the situation with mean value of the unbinding rate, $\bar{k}_u = 5$, which is larger than the most probable value of the unbinding rate, $k_u = 4.625$. And the larger unbinding rate of $A_2$ leads to more production of $B$.

![Figure 4-2: The optimal paths with the minimum actions. The solid lines and dashed lines are forward (from a to b) and backward (from b to a) transition paths respectively. The x and y axes are the concentrations of species A and B respectively. Different colored lines represent systems with different switching rates.](image)
By using the gMAM method, we then find the forward (from a to b) and backward (from b to a) optimal paths between these stable states, which are drawn in solid lines and dashed lines respectively in Figure 4-2. The forward and backward optimal paths do not coincide, which implies that the system does not obey detailed balance. More interestingly, when we increase the switching rate, both forward and backward paths converge to the middle green lines. The reason for the convergence is probably because the large switching rate diminishes the stochastic effect of the system with fluctuating conformational changes. To validate this argument, we then investigate the mean paths of the transitions. We choose two different state points, c and d, which are close to a and b respectively. Because they are unstable states, they will move to the stable states a and b respectively.

![Figure 4-3](image.png)

Figure 4-3: a). The optimal path with the minimum action is different from the average paths. The forward and backward optimal transition paths are indicated by dashed black and green lines respectively. The average paths are indicated by cyan cross lines. b). The deviation between the optimal paths and the average path as a function of the concentration of A.

We obtain the average transition paths by solving ODE where the system evolves with deterministic characteristics. The results in Figure 4-3a show that, from c to b, the average transition path is very different from the optimal paths when the switching rate is relatively small. However, when the switching rate is large, the optimal path coincides with the average path obtained by solving ODE. This observation demonstrates the decline of the stochastic characteristics due to the enhanced switching...
behavior among isomers.

To clearly see the difference between the optimal paths and the average path, we plot the deviation between them as a function of the concentration of A in Figure 4-3b. We pick three switching rates: 0.5, 1, and 10 and plot the deviations correspondingly. When the concentration of A is smaller, the deviation is larger due to the fluctuation. Also, a smaller switching rate results in a larger deviation.

4.2.2 The minimum action

According to the large deviation theory, the transition rate of a rare event is a negative exponential function of the action of the optimal path, \( K \approx A \exp(-VS^*) \), where \( S^* \) is the minimum action and \( V \) is the volume of the system[38]. If the minimum action is smaller, the probability of the transition will be larger. Thus, we use the minimum action to describe the probability of the transition between stable states.

![Graph of minimum action vs. switching rate](image)

Figure 4-4: The minimum action as a function of switching rate. The data points are marked by crosses. The action decreases dramatically as the switching rate increases, and then plateaus for large switching rate.

In Figure 4-4, we plot the minimum action as a function of the switching rate among isomers \( A_2 \). The action decreases dramatically as the switching rate increases, and then plateaus for large switching rate. The decrease of the action indicates that
a larger switching rate makes this system easier to shift from high A state to low A state. The reason for the plateau is that, at the large switching rate, the isomers $A_2$ are well mixed and the system behaves as the average, where the action reaches the lower-bound limit. The results demonstrate that the fluctuations of conformational polymers slow down the rare transition between stable states of the system.

4.3 Continuous space model

4.3.1 Discrete jumps

We then extend the simple genetic toggle switch model to a more complex one where rate coefficient samples from a continuous heavy tailed distribution instead of a discrete distribution. We begin by investigating the behavior of this toggle switch network in the small copy number limit using the Gillespie algorithm [33, 34, 35]. We first carry out the simulations for a model of dynamic disorder in which individual rate parameters remain at one value for a random, exponentially distributed amount of time (parameterized by a switching rate $\lambda[s^{-1}]$), before redrawing a value from a prescribed distribution. Following experimental evidence which shows fluctuating rate constants draw from heavy tailed distribution [128, 26, 49], we allow the rate constants to sample from a log normal distribution. We carry out these simulations with only one copy of genome in the simulation box like the discrete distribution case in the last section. For this model, the state space is continuous as an individual rate constant samples from a continuous probability distribution, but the jumps are discrete.

Because of the fluctuating conformational changes of polymer $A_2$, the unbinding rate of $A_2$ to the operator $O_b$ samples from a lognormal distribution. We fix the mean value of the lognormal distribution at a value known to yield a bistable network, and look at the effect of changing the variance/skewness of the distribution. For the lognormal distribution, changing the variance while keeping the mean fixed requires us to change both the $\mu$ and $\sigma$ parameters of the exponentiated normal. We first
Figure 4-5: a). Here we plot the mean first passage time to transition from a state of high A to a state of low A. The stable basins are found by Monte Carlo simulation, and we use $A = 0, B \geq 7$ for low A state and $B = 0, A \geq 7$ for high A state. The x axis is the standard deviation in the lognormal distribution for k, not the parameter $\sigma$ of the lognormal. b). Here we plot the mean first passage time to transition from a state of low A to a state of high A as a function of increasing the standard deviation in the distribution of off rates for an individual polymer A from the operator. The trend is the reverse of that from Figure 4-5a.

investigate a model in which the fluctuation in rate parameter occurs only when the dimer $A_2$ is unbound from the operator $O_b$, but after dimer $A_2$ binds to the operator it is fixed in one conformational change.

Throughout, the average value that an individual rate constant draws from is chosen to be 5. We find that, upon increasing the standard deviation in the distribution that a rate constant k chooses from over a range from less than one to ten, the mean first passage time to transition from a state of high A to low A (including those in homodimers and those bound to the operators) is significantly increased (Figure 4-5a).

The physical explanation behind the dramatic increase in mean first passage time is that, as we increase the standard deviation of the underlying lognormal distribution that an individual rate parameter draws from, we change the skew of the distribution. The consequence is that the most probable value (mode) of the probability distribution an off rate of $A_2$ is drawn from decreases to lower and lower values. This in turn means that a polymer $A_2$ will remain bound for a longer time on average, and thus does a better job at repressing the production protein $B$. Mathematically, if we
specify a mean $\mathbb{E}[k]$ and variance $\text{Var}(k)$ for an individual off rate $k$, we determine the parameters of the lognormal distribution by the inversion

$$\sigma = \sqrt{\ln \left( \frac{\text{Var}(k)}{\mathbb{E}[k]^2} + 1 \right)}, \quad \mu = \ln(\mathbb{E}[k]) - \frac{\sigma^2}{2}$$

(4.1)

The parameter $\sigma$ increases monotonically with $\text{Var}(k)$ and the skewness of the lognormal distribution (given by $(e^{\sigma^2} - 1)\sqrt{e^{\sigma^2} - 1}$) grows monotonically with $\sigma$. The parameter $\mu$ decreases monotonically with increasing $\sigma$, and the mode of the lognormal distribution, given by $e^{\mu - \sigma^2}$ decreases monotonically with $\text{Var}(k)$. Therefore, mathematically we shift the most probable values of the off rate to values that are much lower than the average $\mathbb{E}[k]$.

As expected, initializing the system in the low $A$ basin (using Monte Carlo sampling of the network to pick the initial point) and measuring the mean first passage time to transition to a state of high $A$ follows the opposite trend noted above. Namely, increasing the variance of the lognormal distribution serves to decrease the mean first passage time for this reverse transition (Figure 4-5b).

Indeed, studying the probability mass function (PMF) for the Toggle switch model with dynamic disorder shows that the basin of low $A$ and high $B$ is destabilized upon increasing the variance in the off rate of an individual polymer $A_2$. (Figure 4-6).

Of course, for a model in which the polymer does not switch when it is bound to the operator, we cannot see much interesting behavior with respect to the parameter $\lambda$. As long as the average time for an individual polymer to rebind is larger than $1/\lambda$, the polymer will on average have resampled its kinetic off rate before rebinding. Upon increasing $\lambda$ any further, the statistics of the network should be unchanged since it will not matter whether the polymer has resampled its kinetic off rate once or multiple times.

To examine a model that is more sensitive to $\lambda$, we simulated the same network but instead let the polymer conformation fluctuate when it is bound to the operator. What we see in this case is also fairly intuitive. When the switching rate is very low and $\sigma$ is large, a polymer that has just sampled a kinetic off rate that is far below the
average value will get frozen at that particular value for the duration of its time on
the operator. It will then, on average, remain bound for a long time and do a better
job at inhibiting the production of protein B. If we increase the switching rate \( \lambda \), we
expect that the system will begin to behave as if it was acting at its average value.
This can be shown in more mathematically precise language using renewal theory
[110]. Therefore, as we increase the switching rate \( \lambda \), the mean first passage time for
transition from high A to low A decreases. (Figure 4-7).

### 4.3.2 Continuous jumps

To demonstrate that the results are robust to the type of model we consider, we also
look at a continuous model in which the kinetic off rate evolves continuously following
a Langevin dynamics of the following form:

\[
k_t = \exp(\tilde{k}_t), \quad \mathrm{d}\tilde{k}_t = \lambda(\mu - \tilde{k}_t)\mathrm{d}t + \left(\sqrt{2\sigma^2\lambda}\right)\mathrm{d}Z_t
\]  

That is, the rate parameter is an exponential of an Ornstein-Uhlenbeck (OU) process.
We let \( \tilde{k}_t \) denote the value of the OU process at time \( t \), and \( k_t \) the value of the actual
Figure 4-7: Mean first passage times to transition from $A \geq 7, B = 0$ to $A = 0, B \geq 7$ are computed for various values of $\lambda$ and $\text{Var}(k) = 10, 25$. We do the calculation for both a discrete jump model and a continuous jump model, labeled DJCS,CJCS respectively. In these parameter regimes, the mean first passage times are comparable for both models. Importantly, increasing $\lambda$ allows faster transitions as the system behaves closer to the mean value. Increasing $\text{Var}(k)$ increases the skewness and therefore decreases the MFPT. For the $\lambda = 10^3$ value in the continuous jump model, a time discretization of $10^{-4}$ is used.
kinetic parameter. This process is chosen since the steady state distribution of the OU process is a normal distribution, and thus the stationary distribution of the $k_t$ is lognormal, which will mimic the stationary distribution sampled in the previous model. The value $\lambda$ plays functionally the same role as it did in the previous case. Though it does not appear in the overall stationary distribution of the OU process, it does modulate how rapidly the value $k_t$ changes in an instant of time $dt$.

Simulating the above process is much more computationally expensive. Now, we must discretize time by picking a $dt$ such that the sum of all rates for various reaction events in the continuous time Markov process simulated by the Gillespie process above is small relative to $dt$. Let the rate for reaction event $i$ be $\rho_i$. Then, in an instant of time $dt$, we first ask whether a reaction occurred or not, where the probability for the reaction to occur is the cumulative distribution function (CDF) of the exponential distribution with parameter $E > \rho_i$. If a reaction did occur, we choose its value from the multinomial defined by the $\rho_i$. Importantly, $dt$ must be sufficiently small such that the probability for more than one reaction event to occur is essentially zero.

We find qualitatively the same results as before. Increasing the value of $\text{Var}(k)$ at a fixed $\lambda$ will increase the mean first passage time from high $A$ to low $A$, while increasing $\lambda$ at a fixed value of $\text{Var}(k)$ will decrease the mean first passage time from high $A$ to low $A$ (Figure 4-7). Quantitatively, the mean first passage time for both the continuous and discrete model is very similar, with the continuous model appearing to have a slightly larger MFPT for low $\lambda$. We explore this in more detail in Appendix A.

4.4 Conclusion

In this chapter, we have studied how kinetic switching between stable states in biological networks is influenced by dynamic disorder or conformational fluctuations in the rate coefficients. We carried out simulation and analysis based on a general genetic toggle switch model. In the model, the unbinding rate of polymers from the operators fluctuates with the switching rate, $\lambda$. 

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First, we have studied a model where the rate parameter samples from a discrete probability distribution. The model is simple enough for us to study the optimal transition paths and the minimum actions by using large deviations theory and the geometric minimum action method (gMAM). We varied the switching rate over three orders of magnitude to reflect fluctuations of enzymatic rates of catalysis observed in single molecule experiments. Under different conditions with different switching rates, we obtained two stable states for each case by solving chemical rate equations. As the switching rate increases, the stable states shift in the direction of less $A$ and more $B$. We demonstrate that the optimal transition paths converge to the average path obtained by solving mass action ODEs. At a fixed switching rate, the smaller the concentration of $A$ is, the larger the deviation is. The reason for this is fewer $A$ makes the system more stochastic. Furthermore, according to the large deviation theory, the transition probability is a natural exponential function of the negative minimum action. We calculated minimum actions for different cases with different switching rates. The minimum action exponentially decays with respect to switching rate. The results indicate that dynamic disorder makes the system more stable.

We then considered the genetic toggle switch model in which the rate parameter draws from a continuous heavy tailed distribution. For this case, we have modeled the system first as a discrete Markov jump process and then under continuous Langevin dynamics. Both models produce qualitatively similar results. Under the condition of fixed switching rate $\lambda$, the mean first passage time from high $A$ to low $A$ increases with the variance $\text{Var}(k)$ in the distribution of off rates for an individual polymer $A_2$ from the operator. On the other hand, when we fix the variance $\text{Var}(k)$, the mean first passage time from high $A$ to low $A$ decreases as the switching rate $\lambda$ increases.

Overall, the regulation of the parameters modulating the dynamic disorder plays an important role in shaping the statistics of optimal transition paths, transition probabilities and the stationary probability distribution of the network.
4.5 Supplementary material

4.5.1 Time correlation functions for discrete and continuous models

As was shown in the main text, both the discrete and continuous models have quantitatively very similar mean first passage times. Here, we compare the two models further to understand why they behave so similarly. To compare the discrete and continuous models further, we will look here at the time correlation function $C(k_t, k_0)$ calculated as the correlation between the kinetic parameter $k_t$ and the time 0 value $k_0$. The continuous model follows an exponential of an OU process. We denote the OU process by $\tilde{k}_t$ such that $k_t = \exp(\tilde{k}_t)$. The transition density for the OU process is given by

$$f(\tilde{k}_t, \tilde{k}_0) = \sqrt{\frac{1}{2\pi \sigma^2(1 - e^{-2\lambda t})}} \exp \left\{ -\frac{1}{2\sigma^2} \left[ \frac{(x - \mu(1 - e^{-\lambda t}) - \tilde{k}_0 e^{-\lambda t})^2}{1 - e^{-2\lambda t}} \right] \right\}$$

$$= \mathcal{N} \left( \mu(1 - e^{-\lambda t}) + \tilde{k}_0 e^{-\lambda t}, \sigma^2(1 - e^{-2\lambda t}) \right)$$

(4.3)

(we will make use of the time dependent mean and variance of the $\tilde{k}_t$ later in calculating the correlation function). The stationary density of $\tilde{k}_0$ is of course $\mathcal{N}(\mu, \sigma^2)$. Using the mean and variance of a lognormal distribution allows us to calculate the time correlation function as

$$C(k_t, k_0) = \frac{\mathbb{E}[\exp(\tilde{k}_t) \exp(\tilde{k}_0)] - \mathbb{E}[\exp(\tilde{k}_0)]^2}{\text{Var}(\exp(\tilde{k}_0))}$$

$$= \frac{\mathbb{E}[\exp(\tilde{k}_t) \exp(\tilde{k}_0)] - e^{2\mu + \sigma^2}}{(e^{\sigma^2} - 1)e^{2\mu + \sigma^2}}$$

(4.4)

Note that we can actually calculate the integral $\mathbb{E}[e^{\tilde{k}_t} e^{\tilde{k}_0}]$ making use of a simple identity which can be proven easily by completing the square. For any random variable
$x \sim \mathcal{N}(\mu, \sigma^2)$ and any constant $\lambda$, one can show that

$$\mathbb{E}[e^{\lambda t}] = e^{\lambda \mu + \lambda^2 \sigma^2/2} \quad (4.5)$$

Breaking the integral up and carrying out the $\tilde{k}_i$ integrals first we get

$$\mathbb{E}[e^{\hat{k}t_i e^{\hat{k}_0}}] = \int \left( \int e^{\hat{k}_t \mathcal{N}(\tilde{k}_t; \mu(t), \sigma(t)^2)} d\tilde{k}_t \right) e^{\hat{k}_0 \mathcal{N}(\tilde{k}_0; \mu, \sigma^2)} d\tilde{k}_0$$

$$= \exp \left\{ \mu(1 - e^{-\lambda t}) + \frac{\sigma^2 (1 - e^{-2\lambda t})}{2} \right\} \int e^{\hat{k}_0 \mathcal{N}(\exp(-\lambda t)+1; \mu, \sigma^2)} d\tilde{k}_0$$

$$= \exp \left\{ \mu(1 - e^{-\lambda t}) + \frac{\sigma^2 (1 - e^{-2\lambda t})}{2} + \mu(e^{-\lambda t} + 1) + \frac{\sigma^2 (e^{-\lambda t} + 1)^2}{2} \right\}$$

$$= \exp \left\{ 2\mu + \sigma^2 + e^{-\lambda t} \sigma^2 \right\} \quad (4.6)$$

This leads to the simple expression for the time correlation function given by

$$C(k_t, k_0) = \frac{\exp \left\{ e^{-\lambda t} \sigma^2 \right\} - 1}{e^{\sigma^2} - 1} \quad (4.7)$$

which we will denote by $C_{OU}(t)$.

We now compare the above model with the discrete jump model in which each rate $k_t$ remains at its value for a random exponentially distributed interval and then redraws from a prescribed distribution. In this case, the time correlation function is very simple to compute and does not depend on the parameters of the distribution, which we call below $\mathbb{E}[k], \mathbb{E}[k^2]$:

$$\mathbb{E}[k_t k_0] = \mathbb{E}_{k_0} \left[ \mathbb{E}[k_t|k_0] k_0 \right]$$

$$= \mathbb{E}_{k_0} \left[ k_0 e^{-\lambda t} + \mathbb{E}[k] (1 - e^{-\lambda t}) \right] \quad (4.8)$$

$$= \mathbb{E}[k^2] e^{-\lambda t} + \mathbb{E}[k]^2 (1 - e^{-\lambda t})$$

This demonstrates that the time correlation function of this behavior is $C(k_t, k_0) =$
\( e^{-\lambda t} \). For small \( \sigma^2 \), the exponentiated OU correlation function approaches

\[
\frac{1 + e^{-\lambda t} \sigma^2 - 1}{1 + \sigma^2 - 1} = e^{-\lambda t}
\]

(4.9)

and the two correlation functions are approximately equal. We expect the most difference between the two processes for large \( \sigma^2 \), when the OU correlation function is

\[
\frac{\exp\{e^{-\lambda t} \sigma^2\}}{e^{\sigma^2}} = \exp\{\sigma^2(e^{-\lambda t} - 1)\}
\]

(4.10)

and the correlations die off with a large magnitude negative exponent for the exponentiated OU process. The results are intuitive. Because the correlation function for the continuous process is a double exponential in \( \lambda \), it drops off more rapidly. For small values of \( \lambda \), we know that the discrete model will get frozen at a single parameter value for the duration of its dwell time, while the continuous model will still explore some of the surrounding values of its probability distribution \( p(k) \). This explains some of the differences seen in the mean first passage times from the continuous process and the discrete process (Figure 4-7).

To explain why the stochastic processes in the kinetic off rate do not lead to significantly big differences between the MFPT behavior for both networks (even at large values of \( \sigma \) when the above analysis demonstrates the two models to be most different), we compute the average dwell time for a polymer following both above behaviors (by Monte Carlo sampling). For each sample, we initialize the system with a single polymer on the operator and draw the initial kinetic parameter from the lognormal with \( \mu, \sigma \). For the exponentiated OU, we discretize time and in each instant \( dt \) update the value of \( k_t \), and also ask if unbinding occurred in the last increment, which does so with probability \( 1 - e^{-k_t dt} \).

For the jump Markov process, we simply simulate the continuous time Markov process. At any instant in time \( t \) when the parameter is \( k_t \), there are two competing exponentials: unbinding which occurs with rate \( k_t \), and parameter switching which occurs with rate \( \lambda \). We use the Gillespie method, and redraw the value of \( k_t \) when the reaction event ‘rate switching’ occurs. We see that the expected dwell time is
essentially the same for both models in the parameter regime that is shown to give bistability (Figure 4-8).

Figure 4-8: We compute the expected dwell time of a polymer A on the operator for the continuous (labeled Exp. OU) and discrete model (labeled Standard) by Monte Carlo sampling. We find that the expected dwell times are comparable in both processes, which explains why the mean first passage times computed in the main text are similar for both models.
Chapter 5

Kinetic bottlenecks improve sensitivity and robustness of peptide-MHC discrimination

T cell antigen receptors (TCR) discriminate between self and non-self ligands with high specificity and sensitivity. After the formation of the pMHC-TCR complex, ZAP-70 is recruited to the complex and phosphorylated. Activated ZAP-70 is essential for downstream signaling. Before ZAP-70 is fully phosphorylated, there is a step of transformational change for ZAP-70, acting as a kinetic bottleneck. In this chapter, we investigate how the introduction of the kinetic bottleneck affects the stability of the network of peptide-MHC-TCR-mediated ZAP-70 activation. We build two coarse-grained models with the only difference of that if there exists a transformational change of ZAP-70 before it is fully activated. In biochemical systems, molecule concentrations and rate constants fluctuate, which may eventually drive the system out of a stable state. Based on the coarse-grained models we build, we first examine the probabilities of the fluctuation-driven transitions in terms of minimal actions by using the geometric minimal action method. Furthermore, we study robustness of the pMHC-TCR-ZAP-70 network to these perturbations of concentrations of species and rate constants of biochemical reactions, and identify which model is more stable.
5.1 Introduction

T lymphocytes (T cells) orchestrate adaptive immune responses to infections[50]. T cell antigen receptors (TCRs) recognize peptide-major histocompatibility complex (pMHC) expressed on the surface of antigen presenting cells (APCs). One of the most intriguing aspects is that TCRs discriminate between foreign peptides and self peptides with extremely high sensitivity and specificity. Numerous experimental studies have shown that T cells can be triggered by only a few or even one single exogenous peptide-MHC molecule in a sea of endogenous peptide-MHC molecules. [108, 48, 83, 47] To explain these characteristics of self/non-self ligand discrimination, McKeithan developed a model for TCR signal transduction[71], inspired by Hopfield's kinetic proofreading mechanism[45] which was first used to explain the high precision in the process of DNA replication and protein synthesis. In essence, the outstanding discriminatory ability requires a number of intermediate steps, i.e., sequential recruitment and transformation of proteins, that provides a temporal lag between ligand binding and major downstream signaling. Compared with endogenous ligands, the agonist ligands have a higher binding affinity for TCR so that they are more likely to remain bound until these intermediate steps are completed and eventually downstream signaling is triggered. Following the binding of TCRs to peptide-MHC, these intermediate steps include: CD4 or CD8 coreceptor-associated Lck, a Src family tyrosine kinase, is recruited and then it phosphorylates immune receptor tyrosine based activation motif (ITAM). Subsequently, 70-kDa zeta-chain-associated protein (ZAP-70) binds to doubly phosphorylated ITAM. The ITAM-bound ZAP-70 molecules can be phosphorylated by Lck. And activated ZAP-70 molecules are essential to initiate further downstream signaling.

In vivo studies have shown that most of ITAMs are phosphorylated and a relatively large amount of ZAP-70 are bound to them [119], but a small portion of bound ZAP-70 molecules are phosphorylated. A modified model [111] explaining this observation has been put forth by the Chakraborty and Weiss groups on the basis of recent experimental data showing that ZAP-70 needs to be converted from its
inactive to active conformation before it is fully phosphorylated. ZAP-70's conformational change is modeled as one composite step consisting of Lck-mediated Tyrosine 319 phosphorylation on ZAP-70 and subsequent binding to this site with its SH2 domain. This relieves ZAP-70 from its auto-inhibited state. It has been suggested that this composite step plays a key role as a kinetic bottleneck in the network of peptide-MHC-TCR-mediated ZAP-70 activation.

In the present work, we investigate the effects of this composite step on the sensitivity and the robustness of self/non-self peptide-MHC discrimination based on two coarse-grained models derived from this model[111]. These two coarse-grained models distinguish from each other by that if there exists a composite step as a kinetic bottleneck. In biochemical systems, fluctuations of molecule concentrations and rate constants are ubiquitous, which may eventually drive the system from stable cellular states to other states. Based on our coarse-grained models, we first examine the probabilities of the fluctuation-driven transitions in terms of minimal actions by using the efficient geometric minimal action method (gMAM) [44, 87]. Moreover, we study how fluctuation-driven transitions in the pMHC-TCR-ZAP-70 network are influenced by perturbations in concentrations of some species and rate constants of the biochemical reactions.

5.2 Coarse-grained models of ZAP-70 activation

To focus on investigating the effect of the kinetic bottleneck in the network of the peptide-MHC-TCR-mediated ZAP70 activation, we build two coarse-grained computational models with the only difference of the presence of the kinetic bottleneck. The kinetic bottleneck is represented as an "opening process" of ZAP-70 molecules. The rate of the "opening process" is denoted as $k_{open}$. The reaction scheme is depicted in Figure 5-1. Here, we ignore some details of the formation of peptide-MHC-TCR complex, namely, the binding/unbinding of co-receptors and recruitment of Lck, etc, which will not qualitatively affect our conclusions. After the formation of peptide-MHC-TCR complex, ZAP-70 can bind to ITAM or TCR. In Model I, once bound to
ITAM, ZAP-70 can be phosphorylated. To the contrary, in Model II, after binding, ZAP-70 needs to undergo an "opening process" first, otherwise it could not be fully phosphorylated. More details of the reaction scheme and corresponding parameters are described in supplementary material at the end of this chapter.

![Figure 5-1](image)

**Figure 5-1**: The coarse-grained models of pMHC-TCR-mediated ZAP70 activation. After the formation of peptide-MHC-TCR complex, ZAP-70 can bind to the complex. In Model I, once bound, ZAP-70 can be phosphorylated. By contrast, in Model II, after binding, ZAP-70 needs to undergo an "opening process" first, otherwise it could not be fully phosphorylated.

### 5.3 Few ZAP-70 molecules bound to ITAMs are phosphorylated

We first examine if our coarse-grained models correctly reproduce the key results of the in vivo experiments, specifically, that there are a certain amount of ZAP-70s bound to double phosphorylated ITAMs, but few of them are phosphorylated. Disregarding stochastic fluctuations, we obtain the theoretical steady states of the model systems by solving ordinary differential equations (ODEs. See the supplementary material). Then, we calculate the percentage of ZAP-70 molecules that bind to peptide-MHC-TCR complex, the percentage of bound ZAP-70 molecules that are phosphorylated,
and the percentage change of phosphorylated ZAP-70 upon the introduction of agonist peptides. We vary the value of off-rate (i.e. unbinding rate) $k_{off}$ of self peptide from $2s^{-1}$ to $24s^{-1}$ while keeping off-rate of foreign peptide fixed at $1s^{-1}$.

The results shown in Figure 5-2 indicate that there is nearly no difference between Model I and Model II for the percentages of ZAP-70 molecules that are bound to ITAM. But the mechanism of Model II inhibits the phosphorylation of ZAP-70 compared to that of Model I. Additionally, as seen in Figure 5-2c, after a certain amount (2%) of agonist peptide-MHC molecules are introduced into the system, in Model II, the change of the percentage of phosphorylated ZAP-70 is large, but not in Model I. If we keep increasing the amount of agonist peptide-MHC, the change of phosphorylated ZAP-70 becomes more dramatic (see Figure 5-2d). These results are consistent with the experimental observations and the computational results obtained from Thill's model [111]. And it shows Model II is better at discriminating foreign peptides against self peptides.

5.4 Transitions due to stochastic fluctuations

As seen in Figure 5-2c, the number of phosphorylated ZAP-70 increases due to the introduction of foreign ligands. Stochastic fluctuations in cellular systems, actually, can also drive self-ligand-only biochemical networks out of stable states, that results in spurious responses. In this section, we study the transition from the stable state to another state with more phosphorylated ZAP-70 resulting from stochastic fluctuations instead of the introduction of agonist ligands. We choose the steady state under the condition of no agonist ligands we obtained from ODEs as an initial state, represented by the concentration vector $\vec{c}$, and the steady state of the system with agonist ligands as an final state, $\vec{c}'$.

According to large deviation theory, the transition probability of a rare event is a negative exponential function of the minimal action, $K \propto A \exp(-VS^o)$, where $S^o$ is the minimal action and $V$ is the volume of the system [38, 113]. The larger a minimal action is, the harder it is to transit. The minimal action, therefore, can be evaluated
Suppose there are $N_R$ reactions in a biochemical system with $N_S$ species. The system transits from an initial stable state, $\vec{c}^i$, to another state, $\vec{c}^j$, following a particular
path \(\tilde{v}(t)\) in the course of time \(\tau\). The corresponding action can be calculated by

\[
S(\tilde{v}, \tau) = \sum_{i=1}^{N_R} \int_0^\tau dt (\tilde{v}_i \ln \frac{\tilde{v}_i}{v_i} - \tilde{v}_i + \nu_i) \tag{5.1}
\]

where, \(\nu\) is the deterministic propensities. Remarkably, this formula partitions the total action into contributions \((S_i^o)\) from \(N_R\) reactions, that will aid our calculations and analysis of sensitivities to each reaction in the following sections. To obtain the minimal action \(S^o\), we need to numerically determine the most probable transition path \(\tilde{v}^o\). It can be obtained by carrying out brute-force simulations which will be very time consuming [7, 20, 121]. Here we implement a very efficient method, called geometric minimal action method (gMAM)[44]. And then we substitute the optimal path into the formula of the action above.

The minimal actions at different off-rate of pMHC to TCR (from 12 \(s^{-1}\) to 24 \(s^{-1}\)) for both Model I and Model II are shown in Figure 5-3. As expected, the minimal action of Model II is larger than that of Model I, which means Model II is more stable than Model I. Also, it is true for both of models that increasing off-rate increases the minimal action. But it increases more dramatically in Model II. The results are intuitive because the kinetic bottleneck in Model II improves the stability of the network. And the larger off-rate of pMHC to TCR gives less waiting time for ZAP-70 to get phosphorylated, and it makes the system hard to be activated.

5.5 Robustness of the network

The rate of fluctuation-driven transition from a stable state to an activated state can be influenced by either changes of molecule concentrations or that of reaction rate parameters. Here, we apply a semianalytical framework developed in Chakraborty group[38] to obtain sensitivities to perturbations of molecule concentrations and rate constants.
Figure 5-3: Minimal actions for the transitions from the stable states to the other states with more phosphorylated ZAP-70 due to stochastic fluctuations. Results for Model I and Model II are indicated by black circles and blue crosses respectively.

The sensitivity to the perturbation of concentrations $c_i$ is

$$\frac{\partial \ln K}{\partial c_i} = V \sum_{j=1}^{N_R} \int_0^{\tau^o} E_{ij} \frac{\bar{\nu}_j^o - \nu_j^o}{c_i} dt$$

(5.2)

and the sensitivity to the perturbation of rate constants $k_i$ is

$$\frac{\partial \ln K}{\partial \ln k_i} = V \int_0^{\tau^o} (\bar{\nu}_i^o - \nu_i^o) dt$$

(5.3)

where $E_{ij}$ is the stoichiometric matrix and $V$ is the volume of the system. With the optimal path $\bar{\nu}^o$ obtained by gMAM, we can easily calculate the sensitivities.

5.5.1 Perturbation of concentrations

The fold changes of the transition rate due to adding one more ZAP-70 molecule and pMHC molecule are plotted in Figure 5-4. For the perturbation of ZAP-70, the fold changes of the transition rate in Model I are larger than that in Model II, which implies that Model II is more robust than Model I to the change of numbers of ZAP-70 molecules. However, the network of Model II is more sensitive than Model I to
the change of pMHC according to Figure 5-4b. To understand the result, we recall that Figure 5-2d shows activated ZAP-70 increases more dramatically in Model II than that in Model I due to the increasing agonist peptide-MHC. This deterministic result and stochastic perturbation analysis on the sensitivity to pMHC coincide with each other. It is of interest that Model II is sensitive to the external signals but not sensitive to the perturbations of the internal molecules. This kind of mechanism benefits discrimination between correct and spurious signals while avoiding stochastic noise-induced cellular activation.

![Graphs](image)

**Figure 5-4:** a).Sensitivities to the change of number of ZAP-70 molecules. b). Sensitivities to pMHC. Results for Model I and Model II are indicated by black circles and blue crosses respectively.

### 5.5.2 Perturbation of rate constants

For the perturbed rate constants, we increase the parameter by one percent and then calculate the corresponding percentage change of the transition rate. In Figure 5-5, we plot the sensitivity to the off-rate of ZAP-70 with pMHC-TCR complex as a function of the off-rate of pMHC to TCR. The results illustrate Model I is more sensitive than Model II to the off-rate of ZAP-70 with pMHC-TCR complex. Also, in both Model I and Model II, the sensitivity decreases as the off-rate of pMHC to TCR increases. To elucidate the results, we first need to identify the key factors affecting the sensitivities...
to rate constants. We derive the minimal action to its second-order approximation: 
\[ S_t^\infty = \int_0^\tau \frac{(\nu_t^\infty - \nu_i^\infty)^2}{2\nu_i^\infty} dt. \] 
After comparing it with the equation of the sensitivity to rate constants, \( \frac{\partial \ln K}{\partial n_k} = V \int_0^\tau (\nu_t^\infty - \nu_i^\infty) dt, \) we can conclude that a biochemical network has a larger sensitivity to the reaction with larger deterministic propensity \( \nu_i^\infty \) given the same action \( S_t^\infty \). Thus when the off-rate of pMHC with TCR increases, the concentration of pMHC-TCR-ZAP-70 complex declines, that results in a decreasing propensity of the unbinding reaction of ZAP-70 to TCR and eventually decreasing the sensitivity.

![Figure 5-5](image)

Figure 5-5: a). Sensitivity to the off-rate of ZAP-70 with pMHC-TCR complex for Model I. 
b). Sensitivity to the off-rate of ZAP-70 with pMHC-TCR complex for Model II.

### 5.6 Conclusion

Here, we have studied how the introduction of a kinetic bottleneck affects the stability of a biochemical network, in particular, the network of peptide-MHC-TCR-mediated ZAP-70 activation. We build two coarse-grained models of ZAP-70 activation, with the only difference being that there exists a kinetic bottleneck, represented by an "opening process" of ZAP-70 molecules, in one case and not the other.

We have investigated steady states by solving ODEs based on these two models. Comparing Model I and Model II, fewer ITAM-bound ZAP-70 are phosphorylated,
which is in agreement with experimental results. Model II is also better at discriminating self/non-self peptide-MHC after we introduce foreign ligands to the system.

In addition, we have applied the efficient geometric minimal action method to calculate the minimal action which is an indicator of the transition probability. According to large deviation theory, the larger the minimal action, the more rare stochastic transitions. The results show that Model II is more stable than Model I. Furthermore, we have implemented a semianalytical method developed in Chakraborty group to find the sensitivities to the perturbations of concentrations of proteins and rate constants in the peptide-MHC-TCR-ZAP-70 scheme models. The results demonstrate that Model II is less sensitive to change of expression of cellular molecules, but it is more sensitive to the change of external signal (peptide-MHC). Also, Model II is more robust to the perturbation of off-rate of ZAP-70 with pMHC-TCR complex.
5.7 Supplementary material

5.7.1 Computational models and steady states

The reaction scheme of Model I of pMHC-TCR-mediated ZAP-70 activation is listed in the following. The rate parameters with the 'bare' units of $s^{-1}$ are chosen according to the data in Thill's paper[111].

- **Binding of TCR and pMHC**
  \[
  \text{TCR} + \text{pMHC} \rightarrow \text{TCR} \cdot \text{pMHC} \\
  k_0 = 0.0052
  \]
  \[
  \text{ZAP} \cdot \text{TCR} + \text{pMHC} \rightarrow \text{ZAP} \cdot \text{TCR} \cdot \text{pMHC} \\
  k_0 = 0.0052
  \]
  \[
  \text{pZAP} \cdot \text{TCR} + \text{pMHC} \rightarrow \text{pZAP} \cdot \text{TCR} \cdot \text{pMHC} \\
  k_0 = 0.0052
  \]

- **Unbinding of TCR and pMHC**
  \[
  \text{TCR} \cdot \text{pMHC} \rightarrow \text{TCR} + \text{pMHC} \\
  k_1
  \]
  \[
  \text{ZAP} \cdot \text{TCR} \cdot \text{pMHC} \rightarrow \text{ZAP} \cdot \text{TCR} + \text{pMHC} \\
  k_1
  \]
  \[
  \text{pZAP} \cdot \text{TCR} \cdot \text{pMHC} \rightarrow \text{pZAP} \cdot \text{TCR} + \text{pMHC} \\
  k_1
  \]

- **Binding of ZAP-70/pZAP-70 and TCR**
  \[
  \text{ZAP} + \text{TCR} \cdot \text{pMHC} \rightarrow \text{ZAP} \cdot \text{TCR} \cdot \text{pMHC} \\
  k_2 = 0.0075
  \]
  \[
  \text{pZAP} + \text{TCR} \cdot \text{pMHC} \rightarrow \text{pZAP} \cdot \text{TCR} \cdot \text{pMHC} \\
  k_2 = 0.0075
  \]

- **Unbinding of ZAP-70/pZAP-70 and TCR**
  \[
  \text{ZAP} \cdot \text{TCR} \cdot \text{pMHC} \rightarrow \text{ZAP} + \text{TCR} \cdot \text{pMHC} \\
  k_3 = 0.5
  \]
  \[
  \text{ZAP} \cdot \text{TCR} \rightarrow \text{ZAP} + \text{TCR} \\
  k_3 = 0.5
  \]
  \[
  \text{pZAP} \cdot \text{TCR} \rightarrow \text{pZAP} + \text{TCR} \\
  k_3 = 0.5
  \]
  \[
  \text{pZAP} \cdot \text{TCR} \cdot \text{pMHC} \rightarrow \text{pZAP} + \text{TCR} \cdot \text{pMHC} \\
  k_3 = 0.5
  \]

- **Phosphorylation of ZAP-70**
  \[
  \text{ZAP} \cdot \text{TCR} \cdot \text{pMHC} \rightarrow \text{pZAP} \cdot \text{TCR} \cdot \text{pMHC} \\
  k_4 = 1
  \]

- **Dephosphorylation of pZAP-70**
  \[
  \text{pZAP} \cdot \text{TCR} \cdot \text{pMHC} \rightarrow \text{ZAP} \cdot \text{TCR} \cdot \text{pMHC} \\
  k_5 = 0.1
  \]
  \[
  \text{pZAP} \cdot \text{TCR} \rightarrow \text{ZAP} \cdot \text{TCR} \\
  k_5 = 0.1
  \]
  \[
  \text{pZAP} \rightarrow \text{ZAP} \\
  k_5 = 0.1
  \]
We fix the total number of peptide-MHC, TCR, and ZAP as $\alpha = 100$, $\beta = 700$, and $\gamma = 300$ respectively.

We denote

$x_0$ : number of phosphorylated ZAP-70
$x_1$ : number of ZAP-TCR complex
$x_2$ : number of pZAP-TCR
$x_3$ : number of TCR-pMHC
$x_4$ : number of ZAP-TCR-pMHC
$x_5$ : number of pZAP-TCR-pMHC

The deterministic evolutions of the biochemical species are governed by the ordinary differential equations:

\[
\begin{align*}
\dot{x}_0 &= -k_2 x_0 x_3 + k_3 x_2 + k_3 x_5 - k_5 x_0 \\
\dot{x}_1 &= -k_0 x_1(\alpha - x_3 - x_4 - x_5) + k_1 x_4 - k_3 x_1 + k_5 x_2 \\
\dot{x}_2 &= -k_0 x_2(\alpha - x_3 - x_4 - x_5) + k_1 x_5 - k_3 x_2 - k_5 x_2 \\
\dot{x}_3 &= k_0(\beta - x_1 - x_2 - x_3 - x_4 - x_5)(\alpha - x_3 - x_4 - x_5) - k_1 x_3 - k_2(\gamma - x_0 - x_1 - x_2 - x_4 - x_5)x_3 \\
&\quad - k_2 x_0 x_3 + k_3 x_4 + k_3 x_5 \\
\dot{x}_4 &= k_0 x_1(\alpha - x_3 - x_4 - x_5) - k_1 x_4 + k_2(\gamma - x_0 - x_1 - x_2 - x_4 - x_5)x_3 - k_3 x_4 - k_4 x_4 + k_5 x_5 \\
\dot{x}_5 &= k_0 x_2(\alpha - x_3 - x_4 - x_5) - k_1 x_5 + k_2 x_0 x_3 - k_3 x_5 + k_4 x_4 - k_5 x_5
\end{align*}
\]

The reaction schemes of Model II are listed here:

- **Binding of TCR and pMHC**
  
  \[
  \text{TCR} + \text{pMHC} \longrightarrow \text{TCR} \cdot \text{pMHC} \quad k_0 = 0.0052
  \]
  
  \[
  \text{ZAP}^c \cdot \text{TCR} + \text{pMHC} \longrightarrow \text{ZAP}^c \cdot \text{TCR} \cdot \text{pMHC} \quad k_0 = 0.0052
  \]
  
  \[
  \text{pZAP}^o \cdot \text{TCR} + \text{pMHC} \longrightarrow \text{pZAP}^o \cdot \text{TCR} \cdot \text{pMHC} \quad k_0 = 0.0052
  \]

- **Unbinding of TCR and pMHC**
  
  \[
  \text{TCR} \cdot \text{pMHC} \longrightarrow \text{TCR} + \text{pMHC} \quad k_1
  \]
\[
\begin{align*}
ZAP^c \cdot TCR \cdot pMHC & \rightarrow ZAP^c \cdot TCR + pMHC \\
ZAP^o \cdot TCR \cdot pMHC & \rightarrow ZAP^c \cdot TCR + pMHC \\
pZAP^o \cdot TCR \cdot pMHC & \rightarrow pZAP^o \cdot TCR + pMHC \\
\end{align*}
\]

- **Binding of ZAP-70/pZAP-70 and TCR**
  \[
  \begin{align*}
  ZAP^c + TCR \cdot pMHC & \rightarrow ZAP^c \cdot TCR \cdot pMHC & k_2 = 0.0075 \\
pZAP^o + TCR \cdot pMHC & \rightarrow pZAP^o \cdot TCR \cdot pMHC & k_2 = 0.0075 \\
\end{align*}
  \]

- **Unbinding of ZAP-70/pZAP-70 and TCR**
  \[
  \begin{align*}
  ZAP^c \cdot TCR \cdot pMHC & \rightarrow ZAP^c + TCR \cdot pMHC & k_3 = 0.5 \\
ZAP^c \cdot TCR & \rightarrow ZAP^c + TCR & k_3 = 0.5 \\
ZAP^o \cdot TCR \cdot pMHC & \rightarrow ZAP^c + TCR \cdot pMHC & k_3 = 0.5 \\
pZAP^o \cdot TCR & \rightarrow pZAP^o + TCR & k_3 = 0.5 \\
pZAP^o \cdot TCR \cdot pMHC & \rightarrow pZAP^o + TCR \cdot pMHC & k_3 = 0.5 \\
\end{align*}
  \]

- "**Opening**" of ZAP-70
  \[
  \begin{align*}
  ZAP^c \cdot TCR \cdot pMHC & \rightarrow ZAP^o \cdot TCR \cdot pMHC & k_4 = 2 \\
\end{align*}
  \]

- "**Closing**" of ZAP-70
  \[
  \begin{align*}
  ZAP^o \cdot TCR \cdot pMHC & \rightarrow ZAP^c \cdot TCR \cdot pMHC & k_5 = 2 \\
\end{align*}
  \]

- **Phosphorylation of ZAP-70**
  \[
  \begin{align*}
  ZAP^o \cdot TCR \cdot pMHC & \rightarrow pZAP^o \cdot TCR \cdot pMHC & k_6 = 1 \\
\end{align*}
  \]

- **Dephosphorylation of pZAP-70**
  \[
  \begin{align*}
  pZAP^o \cdot TCR \cdot pMHC & \rightarrow ZAP^o \cdot TCR \cdot pMHC & k_7 = 0.1 \\
pZAP^o \cdot TCR & \rightarrow ZAP^c \cdot TCR & k_7 = 0.1 \\
pZAP^o & \rightarrow ZAP^c & k_7 = 0.1 \\
\end{align*}
  \]

where \(ZAP^c\) and \(ZAP^o\) represent ZAP-70 at the state of close and open respectively.

We denote

- \(x_0\) : number of phosphorylated ZAP-70
- \(x_1\) : number of \(ZAP^c\)-TCR complex
\(x_2\) : number of pZAP\(^0\)-TCR  
\(x_3\) : number of TCR-pMHC  
\(x_4\) : number of ZAP\(^c\)-TCR-pMHC  
\(x_5\) : number of ZAP\(^0\)-TCR-pMHC  
\(x_6\) : number of pZAP\(^0\)-TCR-pMHC

The ordinary differential equations are

\[
\begin{align*}
\dot{x}_0 &= -k_2 x_0 x_3 + k_3 x_2 + k_3 x_6 - k_7 x_0 \\
\dot{x}_1 &= -k_0 x_1 (\alpha - x_3 - x_4 - x_5 - x_6) + k_1 x_4 + k_1 x_5 - k_3 x_1 + k_7 x_2 \\
\dot{x}_2 &= -k_0 x_2 (\alpha - x_3 - x_4 - x_5 - x_6) + k_1 x_6 - k_3 x_2 - k_7 x_2 \\
\dot{x}_3 &= k_0 (\beta - x_1 - x_2 - x_3 - x_4 - x_5 - x_6) (\alpha - x_3 - x_4 - x_5 - x_6) - k_1 x_3 \\
&\quad - k_2 (\gamma - x_0 - x_1 - x_2 - x_4 - x_5 - x_6) x_3 - k_2 x_0 x_3 + k_3 x_4 + k_3 x_5 + k_3 x_6 \\
\dot{x}_4 &= k_0 x_1 (\alpha - x_3 - x_4 - x_5 - x_6) - k_1 x_4 + k_2 (\gamma - x_0 - x_1 - x_2 - x_4 - x_5 - x_6) x_3 \\
&\quad - k_3 x_4 - k_4 x_4 + k_5 x_5 \\
\dot{x}_5 &= -k_1 x_5 - k_3 x_5 + k_4 x_4 - k_5 x_5 - k_6 x_5 + k_7 x_6 \\
\dot{x}_6 &= k_0 x_2 (\alpha - x_3 - x_4 - x_5 - x_6) - k_1 x_6 + k_2 x_0 x_3 - k_3 x_6 + k_6 x_5 - k_7 x_6
\end{align*}
\]

We vary off-rate of TCR to pMHC from 2 to 24 s\(^{-1}\) and calculate the steady states by solving ODEs above. The results are plotted in Figure 5-2.

### 5.7.2 Minimal actions and sensitivities

Suppose a biochemical system transits from a stable state to the other state with a path \(\bar{\nu}(t)\), which is a reaction propensity at time \(t\). And \(\nu(t)\) is the deterministic propensity. According to the semianalytical technique Chakraborty group developed, the action of the path \(\bar{\nu}\) over \([0, \tau]\) is

\[
S(\bar{\nu}, \tau) = \sum_{i}^{N_R} \int_{0}^{\tau} dt(\bar{\nu}_i \ln \frac{\bar{\nu}_i}{\nu_i} - \bar{\nu}_i + \nu_i)
\]  

(5.4)
By using the geometric minimal action method, we can obtain the optimal path \( \tilde{\nu}^o \) in a very efficient way and then we can calculate the minimal action by using Equation 5.4.

We define the transition rate of the system is \( K \), thus the sensitivity to the perturbation of concentrations \( c_i \) is

\[
\frac{\partial \ln K}{\partial c_i} = V \sum_j^n \int_0^T E_{ij} \frac{\tilde{\nu}_j^o - \nu_j^o}{c_i} dt
\]  

(5.5)

and the sensitivity to the perturbation of rate constants \( k_i \) is

\[
\frac{\partial \ln K}{\partial \ln k_i} = V \int_0^T (\tilde{\nu}_i^o - \nu_i^o) dt
\]  

(5.6)

where \( V \) is the volume of the system and \( E_{ij} \) is the stoichiometric matrix.

The percentage changes in transition rate \( K \) shown in Figure 5-4 and Figure 5-5 can be calculated by using Equations 5.5 and 5.6 above with the input of the optimal path \( \tilde{\nu}^o \).
Chapter 6

Conclusion

In this thesis, we have conducted studies on the stochastic behavior of biochemical systems, especially in lymphocytes.

With experimental collaborators in University of California, San Francisco, we have investigated the functions of SOS and RasGRP in the process of ERK and P38 MAK kinases activation. Based on experimental observations in cell lines and primary cells, we have carried out modeling of the antigen receptor signal transduction network and stochastic simulations by implementing the Gillespie algorithm. The analysis and results are presented in Chapter 2. We have found that sensitive and bimodal ERK activation critically relies on the allosteric activation of SOS1. Different from ERK activation, P38 activation does not depend on SOS' allosteric pocket, although it relies on SOS more than on RasGRP.

In Chapter 3, we have examined two distinct pathways of P38 activation, the LAT-MAPK cascade-mediated classical pathway and the ZAP70-mediated alternative pathway. Our collaborators have carried out experiments by utilizing single-cell phospho-flow cytometry. We have done complementary in silico modeling and mathematical analysis on the network. Different contributions to P38 activation by two pathways have been identified. The alternative pathway allows rapid P38 activation by lowering the activation threshold, while the classical pathway determines the magnitude of response.

Fluctuations are ubiquitous in biochemical systems. Such noise may drive the sys-

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tem from one state to another. In Chapter 4 and 5, we have investigated fluctuation-driven transitions in two biochemical systems. The first system is the well-known genetic toggle switch with bimodality. We introduced dynamical disorder to the system. We have investigated how the dynamical disorder affect kinetic switching between stable states. The optimal transition paths are examined by implementing the geometric minimum action method. The corresponding minimum actions have shown that the dynamical disorders make the system more stable. The dynamical disorder is also crucial in shaping the stationary probability distribution of the network.

The self/foreign peptide-MHC discrimination is studied in Chapter 5. Here we have built two coarse-grained models with the only difference of an "opening process" of ZAP-70 molecules as a kinetic bottleneck for signaling. For these two models, we first compared the minimal actions as indicators for the transition probabilities. The results have demonstrated that the bottleneck in Model II stabilizes the system. We then compared the robustnesses of these two systems by calculating the sensitivities to concentrations of various species and rate parameters.

In summary, the methodology of the combination of in silico simulation, theory and biological experiments can help unveil stochastic regulation of important biochemical networks.
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