Dynamic regulation and information transfer in intracellular-signaling pathways

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Abstract

MARCELO S. BEHAR: Dynamic regulation and information transfer in intracellular-signaling pathways
(Under the direction of Timothy Elston)

Cells contain myriads of specialized sensors that allow them to react to changes in the environment by activating pathways comprised by layers of signaling proteins. Cell survival requires these pathways to reliably propagate signals containing qualitative and quantitative information about their environment. They achieve this by modulating the enzymatic activity of signaling proteins. In this work we analyze some of these regulatory mechanisms, with emphasis on those that can be used to encode and decode quantitative information. In particular, we demonstrate that cells have the tools to encode quantitative information as signal-duration, and that by regulating pathways dynamics in a concerted way, they can ensure signal specificity in the presence of shared components without the need for cross-inhibition or scaffold proteins. A strategy termed **kinetic insulation** is introduced, which routes signals according to their temporal profile. We show that such a system can be built with regulatory motifs commonly observed in signaling pathways. We also present experimental evidence indicating that duration encoding is being used in the yeast pheromone response pathway. Computational modeling is used to analyze possible encoding mechanisms and key experiments are proposed that can distinguish among them. Finally, the importance of studying signals as dynamic events is discussed.
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Esta tesis esta dedicada a mis padres, Leonardo y Yoli Behar. Su cariño y esfuerzo han sido la fuerza que me ha impulsado durante este camino.

This thesis is dedicated to my parents, Leonardo and Yoli Behar. Their love and efforts have been the driving force along this path.
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<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration at which the effect is 50% of the maximum</td>
</tr>
<tr>
<td>FRE</td>
<td>Filamentous Response Elements</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>GPCR</td>
<td>G Protein Coupled Receptors</td>
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<tr>
<td>Kd</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>Mitogen Activated Protein Kinase Kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen Activated Protein Kinase Kinase Kinase</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical Density at 600nm</td>
</tr>
<tr>
<td>ODE</td>
<td>Ordinary Differential Equation</td>
</tr>
<tr>
<td>PDE</td>
<td>Partial Differential Equation</td>
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<tr>
<td>PRE</td>
<td>Pheromone Response Elements</td>
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<tr>
<td>RGS</td>
<td>Regulator of G Protein Signaling</td>
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Chapter 1

Basic concepts and review of the field

1.1 Overview

Cells are able to adapt and thrive in changing environments thanks to complex biochemical signaling networks. These systems take environmental information gathered by receptors at the membrane, and through the activation of multiple levels of signaling proteins, they transfer it to the cellular actors that will produce the adequate response. In many cases, the signaling proteins are unevenly distributed inside the cell and external challenges often result in complex, but well-organized spatiotemporal activity patterns. Malfunctions in a signaling pathway or one of its components often result in disease or death. Knowledge of the design principles underlying these systems and the regulatory mechanisms controlling the flow of information, would not only deepen our understanding of biological systems, but also provide valuable tools to those developing therapies to treat disease.

Understanding intra-cellular signaling pathways as whole entities constitutes a formidable biophysical problem and is the main subject of this work. This chapter introduces some notions from the fields of cellular and molecular biology as well as
biochemical and biophysical concepts necessary to understand the results presented later.
Readers from a biology background can skip this introduction. The second part of this
chapter provides a brief review of the field and some selected previous results are
presented. Chapters 2 to 4 contain the bulk the results. The broad significance of the
findings is considered in Chapter 5, in which we also speculate about their potential
generalization and suggest future directions for this research.

1.2 Biological background

This section is intended as brief introduction to the biological background relevant
to this work. For a more complete treatment of these subjects the reader is referred to the
books by Alberts et al. (1), or Goemperts et al. (2).

The ability to react to changes in the environment is a key aspect shared by the
majority of living cells. In early development, stem cells specialize in part because of
morphogenic cues present in the environment. Simple organisms are capable of
exquisitely sensing gradients and move towards regions with high levels of nutrients or
away from adverse environments. Cells in different parts of multicellular organisms
communicate with each other by releasing chemical messengers that can be sensed only
by specific kinds of target cells. Hormones, neurotransmitters, cytokines, and other
chemical messengers mediate the control of virtually every body function in mammals.
To stay alive, cells must detect these messages and process the information so the
adequate action is taken. Extensive networks of signaling pathways have evolved over
time just to do this. These pathways usually start with the activation of a sensor protein,
called receptor (in pathways devised to react to chemical cues), and involve the activation
of multiple signaling proteins organized to respond in a cascade-like fashion. Activation occurs when an upstream event modifies a target protein, endowing it with new enzymatic activity (or repressing a constitutive one), which in turns allows it to chemically modify a downstream substrate and so on. The activation of a signaling pathway can result in an increased or decreased expression (upregulation and downregulation) of groups of genes, a change in the permeability of the cell, the release of different effectors from internal storage, or the redistribution of proteins, lipids and other components of the cell, just to name a few possibilities. The sum of these processes ultimately drives the appropriate physiological responses such as morphological changes, cell division or differentiation, neurotransmitter release, etc.

To date, a large number of signaling pathways and components have been identified, and their study has revealed a number of common themes. Interestingly, similar designs have been observed in systems meant to operate in response to different cues or even in systems belonging to different species. This suggests that modularity may be a key feature of signaling pathways, and therefore the lessons learned from the study of a particular system can probably be extrapolated to other cases. For this reason and since it is impractical to describe the many known pathways, the rest of this section introduces only the elements that will be explicitly used later in this work with references to review articles when appropriate.

The term G Protein Couple Receptor (GPCR) is used to describe a large family of mostly membrane bound proteins (3, 4). Proteins in this group, also known has 7TM (7 Trans Membrane) receptors, are characterized by a common architecture consisting of seven trans-membrane helical segments. GPCR’s are widespread; more than 800 have
been identified in the human genome so far and more than 5% of the nematode *C. Elegans* genes encode them. These proteins usually act as sensors at the head of signaling cascades, becoming active when exposed to an *agonist* or *ligand*. Many receptors are capable of detecting chemical compounds with exquisite sensitivity and specificity. For example, the senses of smell and taste are initiated by odorants or flavonoids binding to GPCR’s. In multicellular organisms, cells depend on these receptors to *measure* blood concentration of hormones such as epinephrine (adrenaline) or other molecules such as glucose or nucleosides, and regulate organs function accordingly. Cells in the nervous system use GPCRs to detect some of the neurotransmitters (such as dopamine) that allow them to communicate with each other. Specialized GPCR’s are coupled to photosensitive compounds that undergo conformational changes when activated by light. These receptors are the basis of vision. In yeasts, GPCR’s are responsible for detecting the pheromone that induces these organisms to mate. The activation of most of the proteins that fall in this class is reversible, although there are groups of GPCR’s that are activated in an irreversible way when part of their extracellular domain is cleaved by an agonist. GPCR activity usually results from a conformational change induced (or stabilized) by the presence of the agonist. This change may expose intracellular catalytic or binding domains that are not accessible in the inactive state. Signaling by active GPCR’s can be terminated by the release of the agonist or the relaxation of the photosensitive species. GPCR activity can also be modulated by cytosolic species (residing in the cytosol) that bind to the receptor and prevent the activation of their intracellular targets (*arrestins*) or chemically modify it with the same result (*GRK* proteins). In some cases, receptor
activity is regulated by proteins that tag them for internalization (removal from the cell surface) often followed by degradation.

The primary targets of most G Protein Coupled Receptors are hetero-trimeric G Proteins (5, 6). These are usually the first layer of signaling proteins targeted by active receptors. Hetero-trimeric G proteins are complexes formed by the non-covalent association of three proteins, referred to as the α, β, and γ subunits. The α subunit has the ability to bind GDP and GTP (Guanidyl di/triphosphate). It also has hydrolytic activity, which allows it to remove a phosphate group from a GTP molecule and transform it into GDP. In the inactive state, the β and γ subunits are bound together to a GDP bound Ga. Active receptors induce Ga to exchange GDP for GTP. This change destabilizes the complex, prompting it to dissociate into a Gβγ complex and GTP-bound Ga. In most systems, the subsequent response is mediated by the action of the GTP-Ga moiety, which depending on the family, can activate or inhibit the action of other proteins such as adenyl cyclases, phospholipases, and ion channels. The Gβγ complex remains attached to the membrane, and it is usually thought to act as a regulator of Ga activity. As a matter of fact, constitutive pathway activity (that is, ligand-independent activity) is often observed in cells in which the stoichiometric balance between the subunits has been altered. In some cases, however, as in the yeast pheromone response pathway, it is the Gβγ complex that activates signaling. G protein signals are terminated as Ga converts GTP back into GDP thus allowing the hetero-trimeric complex to re-form. A family of GTP-ase proteins called Regulators of G Protein Signaling (RGS) can accelerate this process. In addition to hetero-trimeric G proteins, other GTP-ases exist and are commonly encountered in signaling networks, often playing mayor regulatory roles. Because these proteins do not
explicitly participate in the specific pathways considered in the next sections, they will not be discussed here. Interested readers should consult the references mentioned above.

Another group of important signaling molecules are the Mitogen Activated Protein Kinases (MAPKs) and their upstream effectors (MAPKK and MAPKKK) (7, 8). Upon phosphorylation, MAPKKK, MAPKK, and MAPK proteins gain kinase activity and become competent to phosphorylate their substrates. These proteins constitute the core of many signaling pathways, operating in cascades in which a MAPKKK phosphorylates and activates a downstream MAPKK, which in turns phosphorylates and activates a MAPK. MAPKKK and MAPKK proteins are usually very specific, with each being able to efficiently phosphorylate a very limited set of substrates, often only one. The source of this specificity are docking motifs that permit a MAPKKK to efficiently bind only to its target MAPKK. MAP kinases on the other hand can phosphorylate a variety of downstream targets, including transcription factors proteins that interact with the DNA processing machinery to induce or repress gene expression. The MAPK family is just one of the many protein groups with kinase activity. In fact, phosphorylation is one of the main mechanisms used by cells to regulate protein activity. There are many known MAPK proteins. The human genome encodes for at least 30 (including MAPKK and MAPKKK proteins). They are all part of networks that allow cells to relay information about a plethora of different stimuli. How specificity is maintained in these networks, that is, how different stimuli manage to elicit specific responses, is a matter of intense study and is the motivation for Chapter 3.

Phosphorylation is a reversible process, at least in principle. Phosphatases are a group of enzymes that can remove the phosphate groups from phosphorylated residues.
(9). Tyrosine phosphatases are specific for that kind of amino acid residue. Threonine-serine phosphatases can dephosphorylate threonine and serine residues. Dual specificity phosphatases dephosphorylate threonine, serine and tyrosine residues. Comparatively less is known about phosphatases and how their activity is regulated than about kinases. There are many examples of transient kinase phosphorylation in response to a signal, which indicates that at least some phosphatases must be constitutively active or quickly activated. Phosphatases often localize to specific parts of the cell. For example, phosphatases Ptp2 and Ptp3 are nuclear and cytosolic proteins respectively. Phosphatases can be promiscuous and operate on a number of substrates, or can be specific to a particular protein or protein family.

Signaling pathways target a number of systems inside cells, such as ion channels, ATP producing proteins, DNA binding proteins, etc. These systems then elicit the response necessary to cope with the conditions that triggered the signal. In many cases, the response includes the upregulation or downregulation of specific genes or group of genes. Signaling pathways affect gene expression by activating transcription factors, repressors, or other proteins that modify the interaction between the DNA encoding the gene and the machinery in charge of reading it. Pathways also can affect protein levels by modifying messenger RNA, either stabilizing it or making it more labile. Ultimately, it is the combination of these processes that causes the phenotypic response.

Many signaling pathways have been discovered during the past four decades, yet characterization of most of them is still in its infancy. Thanks to advances in light microscopy and genetic manipulation this is changing rapidly. There is currently great interest in the study of the organization and function of the signaling and information
processing systems operating inside the cell. The pharmacological potential of such understanding is huge. Mastering the design principles behind signaling networks associated with a given disease would have immediate repercussion on the way the disease is treated and provide a valuable guide for developing novel therapies. This is a complex task that requires the combination of a variety of technologies, skills, and perspectives in order to be successfully tackled. Biophysical modeling is one of these tools. Because it is extensively used in this work, the next section describes some of the approaches common in this field as well as some selected results.

1.3 Modeling intracellular processes

Computational modeling is an established component of the biophysical sciences. Early applications include the analysis of pattern formation driven by reaction-convection-diffusion systems and the use of the theory of dynamical systems to the study of problems from ecology (10). Modeling at the organism and cellular level is routinely used today in a number of applications (11, 12), ranging from neurophysiology (the study of neural activity and neurotransmitters), to pharmacokinetics (the study of drug distribution and elimination in the body). Many of these applications use a high level of abstraction to describe the system under consideration. For example, neuroscientists often model neurons as electrical circuits in which the cellular components play the role of capacitors, resistors, and batteries (12). The advent of modern molecular and cellular biology experimental techniques, such as the invention of monoclonal antibodies and fluorescent protein markers during the past decades, has provided the ability to observe intracellular processes in great detail. These new tools prompted renewed efforts to
model these processes at lower levels of abstraction. However, this wealth of new information brings new levels of complexity that can easily overwhelm anyone trying to understand the underlying mechanisms driving cellular behavior. Determining the key players and interactions in any given system has become a central problem for biologists and biophysicists. Computational modeling is slowly becoming the tool of choice to integrate, analyze, and ultimately make sense of the vast amount of new data coming daily from the labs.

Perhaps the most notable success story in the field of computational biophysics is the elucidation of the processes governing cell cycle regulation. The pieces of the puzzle, that is, the genes involved, were discovered in the 90’s, but it was not until the efforts by John Tyson and James Ferrell that the whole picture emerged. These researchers used a combination of computational and experimental work to show that the regulation of the cell cycle occurs as a result of a combination of bistable switches (13-18). These discoveries led to a number of studies about the properties of this regulatory network architecture (19, 20), and spurred a search for multi-stable systems underlying other biological processes. This case exemplifies how knowledge of the components and interactions cannot, on its own, produce understanding of how the system actually works. It took diligent modeling efforts to reveal inconsistencies in the experimental data, point to knowledge voids (hence focusing experiments), and to ultimately put the pieces together and provide a system level picture of cell cycle regulation.

The success behind the elucidation of the logic driving cell cycle regulation is mostly due to the module-based approach taken. The researchers built a complete model from smaller subunits that performed a specific function and could be characterized
almost independently. This approach not only made this large system manageable, but also allowed for the experimental validation of the individual modules. In most cases, however, it is not evident what the modules are or even if the system can be decomposed this way. A number of statistical-based methods, such as principal component analysis (PCA) have been used in these cases and their popularity is gaining momentum (21-23). The caveat is that these methods usually require a significant amount of quantitative experimental data of the type that is commonplace for genetic expression but rarely available for signaling pathways. These methods are very promising, and it is easy to predict that they will become widespread as high throughput techniques find their way into the signaling field.

Computational biophysics does more than just integrate data and provide a systems level picture of the system under consideration. Mathematical and computational approaches have been used to try to understand why cells are wired the way they are. The search for underlying principles guiding the design of genetic networks, that is the complex set of interactions between the genes in an organism, has been going on for some time. The availability of complete genomes, allowed researchers pursuing this area to look for the patterns and motifs that are commonly used by nature to regulate the expression of different genetic programs with amazing results. Some researchers are approaching this fascinating problem from a synthetic perspective, trying to build artificial genetic networks, hoping to gain insight by facing the same design challenges faced by nature (24). In contrast, the search for the design principles behind signaling networks has advanced more slowly. This is in part because of the lack of suitable experimental tools compared with the ones available to study genetics, but also because
much less is known about how signals are regulated and therefore there is little base to draw general conclusions.

Even with this limitation, a number of studies from have shed light on many of the questions posed by signaling systems. For example, it has been shown kinases tend to affect the downstream signal amplitude, whereas phosphatases affect both amplitude and signal duration (25) and that amplification often is at odds with propagation speed (26). Others have shown how signaling networks may use component sequestration or cross-inhibition to prevent signal leakage (cross-talk) to parallel pathways (27-29). Counterintuitive effects such as back-propagation in feedback loops (30), oscillations and bistability in simple biochemical systems has been studied (31-34). Considerable modeling efforts have been devoted to understand how signaling networks cope with noise and stochastic variation in their components (35-37). Many of these studies apply some variation of Metabolic Control Analysis, a branch of control theory developed specifically to study biochemical and metabolic networks (38). Its methods are more adequate to study steady state properties and perform local sensitivity analyses in the region of the parameter space around the steady state. However, some efforts have been made towards extending the formalism to time dependent problems (39, 40).

Despite experimental limitations several groups have produced detailed models of important signaling networks, some more successful than others. Some notable examples are the modeling of the networks targeted by Epidermal Growth Factors (EGF) (41-43). A beautiful combination of experimental and computational work on the dynamics of ERK signaling was published by Sasagawa et. al. (44). Other examples include studies on the dynamics of the G-protein regulation (45) as well as osmoadaptation (46-48) in yeast,
Her signaling regulation (49), and pathways involved in the inflammatory response in mammals (50, 51) just to name a few. The computational part of most of these examples usually involves simulating protein interactions and transport effects using ordinary differential equations or partial differential equations, depending on whether the system can be analyzed as a well stirred reactor or if the spatial distributions of the components plays an important role and thus must be taken into account. Stochastic simulations are also often used, especially for systems containing small number of molecules per cell.

Despite the insight these efforts provided about the particular system under study, it could be argued that little has come out in the form of principles that could be generalized to other systems. This is in part because in many cases, the experimental data available for any given pathway is insufficient to properly constrain the models, and therefore a number of architectures are compatible with the observations. In other cases, the modelers try to capture the whole complexity of the system all at once, and the results are as difficult to interpret and analyze as the real cell. The success stories (e.g. cell cycle regulation) demonstrate that the most promising path to understand a signaling network is by deconstructing it into –ideally- simple modules that can be analyzed independently and then assembled to produce the whole. This is the guiding philosophy of this work.

The following pages analyze a number of aspects important for the understanding of signaling networks. Key to what follows is the understanding that signaling is not about steady states but rather is a dynamic process that is better understood in terms of transient dynamics. The backbone of this work is based on the experimentally observed ability of cells to measure and reliably propagate information about the extra-cellular environment to the effectors that ultimately produce the adequate phenotypic response. It is often the
case that signaling pathways not only relay qualitative information (e.g. the presence or absence of a stimulus), but also quantitative information about the stimulus strength. In order to do so, this information has to be encoded and transferred across the network without interfering with other pathways. The fact that many signaling pathways share components makes this a delicate task and raises a number of questions regarding how cells manage to maintain signal specificity. In this work we demonstrate that dynamic modulation of pathway activity can play a fundamental role in ensuring that information is properly transferred down a pathway. Chapter 2 studies the different encoding strategies available for living cells to transfer quantitative information. The focus is not in a particular metric or measurement of pathway bandwidth (a very interesting problem on its own), but in the general properties of different encoding schemas as well as their relative advantages and drawbacks. A large portion of the chapter is devoted to adaptive systems, that is, systems that upon stimulation produce a signal and then return to pre-stimulation levels even in the presence of a sustained stimulus. These systems are pervasive in signaling networks and as we show, have the potential to endow pathways with very flexible signal modulation capabilities. A particular encoding strategy, duration encoding, is analyzed in more detail in the second part of the chapter. Evidence will be presented in Chapter 4, supporting the case for duration encoding as an information transfer vehicle used in a real yeast pathway. Chapter 3 demonstrates how the dynamic control of a signaling pathway activity can be used to route signals through branching points avoiding cross-talk between pathways sharing components. This novel strategy does not requires cross-inhibition or scaffold proteins to achieve specificity. Finally, in Chapter 4, we show how these concepts can be applied to gain a systems level
understanding of signaling in yeast. Given that many of the components of the signaling pathways described in that chapter are well preserved across species, it is plausible that the insight obtained from studying this system can be extrapolated to higher organisms, providing insight into the general design principles that underlie signaling networks.
Chapter 2

Information transfer in signaling pathways

2.1 Quantitative information transfer

2.1.1 Overview

Many substances such as hormones, cytokines, nucleosides, and a variety of drugs, affect cellular behavior by binding to membrane receptors and triggering a cascade of intracellular signals. These signals are processed by different parts of the cellular machinery, eventually leading to very specific responses. In many cases, the magnitude or nature of the response must be commensurate to the intensity of the external stimulus, forcing the cell to be aware of its environment in both qualitative and quantitative terms. This quantitative knowledge may come in the form of thresholds separating distinct response regimes, or involve a full graded scale with responses proportional to the stimulus strength. For example, the yeast *Saccharomyces cerevisiae* behaves differently when exposed to different concentrations of mating pheromone. At low concentrations, cells do not appear to respond and continue to grow and divide normally. At intermediate concentrations, cells undergo a transition to a filamentous-like growth pattern (52, 53).
Finally, at high concentrations, cells undergo growth arrest and the *bona fide* mating response with its characteristic morphological change is observed (Figure 2-1) (54). This behavior indicates that the signaling pathways involved are capable of “measuring” and relaying information about the presence of at least three concentration levels of mating pheromone. In fact, gene induction studies have demonstrated that this pathway is capable of relaying quantitative information in a fully graded fashion as shown in Figure 2-1. The curve shows a graded increase of the amount of reporter gene product expressed under the control of the mating specific gene *FUS1* promoter in response to different concentrations of pheromone as measured by the standard beta-galactosidase assay (55). Further examples of fully graded responses can be observed in the action of a variety of agonists on their specific Seven Trans-membrane Receptors (7TM) and other systems.

**Figure 2-1** Yeast response to pheromone. A) Cells exposed to a gradient of mating pheromone in a microfluidics chamber. Vegetative growth, pseudo-filamentous growth, and the mating response can be seen from left to right. (Picture by S. Nayak - UCSD) B) Receptor occupancy (blue, data from (56)) and *FUS1* gene induction (green) measured by the Lac Z assay (data from (45)).
There are two general strategies cells can use to generate stimulus intensity-dependent responses. One is for the cell to continuously monitor the effect of the response and use this information for regulatory purposes. For example, in some species, hyper-osmotic conditions cause mechanical stresses to accumulate in the cell wall, which if left unchecked can result in damage (57). These cells can alleviate the stress by producing compatible osmolytes to restore the osmotic balance. The amount of osmolyte necessary to restore balance depends on the magnitude of the osmotic stress. In principle, to cope with this situation, a cell only needs to monitor the presence of wall stress and keep producing osmolytes until it is eliminated. In this case a signaling pathway that indicates the presence or absence of stress in the cell wall in a binary manner suffices to produce a response that is stimulus strength-dependent. In control systems jargon, this is a biological example of a closed loop system. For this strategy to be viable, the effect of the response has to be dependent on the stimulus intensity. In this example, the effect of a given amount of osmolyte on the wall stress depends on the osmotic imbalance. However, this is not always the case and cells also use open loop mechanisms. In these cases, membrane receptors or other entities measure the magnitude of a stimulus, information that is subsequently encoded and relayed to the appropriate cellular machinery eliciting a pre-programmed response. Of course, nothing prevents a closed loop system to take advantage of quantitative stimulus intensity information as well, and in fact this dual approach is observed in one of the branches of the hyper-osmotic shock response in yeast (47). In general, quantitative information transfer does not necessarily have to be part of a homeostatic control system. Cells respond to many stimuli, most
notably hormones or other biochemical messengers, in preprogrammed ways that in many cases are dose dependent. The yeast pheromone response described above is a clear example. The gene expression data of Figure 2-1 indicates that the receptor and the signaling pathway behave in a graded fashion, and therefore are capable of producing an internal representation of the concentration of pheromone. This information is decoded and binned in three dose regimens that correspond with the three distinct physiological responses observed in the experiments.

The ability to encode and relay quantitative information about a stimulus is the topic of this chapter. More precisely, this chapter focuses on the strategies available to a cell for encoding, relaying, and decoding quantitative information about stimulus intensities, regardless of whether the information is used in a closed loop control system or to elicit a pre-programmed response, genetic or otherwise. This particular issue is part of the more general area dealing with the dynamic control of intracellular signaling, and therefore, considerable attention is devoted to the mechanisms of signal adaptation and modulation. In Chapter 3 we demonstrate that the dynamics in a given pathway can play a significant role helping cells to avoid crosstalk between pathways with shared components. Dynamic control and information encoding in some of the yeast signaling pathways is the subject of Chapter 4.

2.2 Encoding strategies

2.2.1 Linear pathways and amplitude encoding

The previous section introduced the concept of quantitative information transfer in general terms, but a more explicit definition is: A system is capable of performing
quantitative information transfer if, in response to stimuli of different intensity, it produces distinct internal representations that can, in principle, be interpreted by downstream effectors. In other words, a signaling pathway has to enable its downstream effectors to discriminate between stimuli of different strength. This property can apply to a pathway as a whole, or just to a section of it, although where a pathway begins and where it ends is often context-dependent. Accordingly, the concept of stimulus is used here in a broad way, meaning the input to a given pathway stage. Inputs include external cues, but could also refer to intracellular elements operating upstream of the section of interest.

The diagram in Figure 2-2 represents a simple linear signaling network with a membrane bound receptor at its head. Ligand binding confers the receptor with enzymatic activity and allows it to activate the enzymatic activity of a second downstream signaling protein. The active second signaling protein then activates the next component and so on. A prevalent example is a Mitogen Activated Protein Kinase cascade, in which each stage acts as a kinase able to phosphorylate the species immediately downstream of it. At the bottom of the pathway, the activation of a transcription factor triggers a genetic response, represented here by the enhanced expression of gene Z. The input for this pathway is the concentration or dose of a ligand, such as mating pheromone, which binds to the receptor. The output at each stage is the fraction of active signaling enzyme relative to its total concentration. The physiological, or cellular response in this example is the expression level of the hypothetical gene Z. The terms input, output, and physiological response will be used consistently throughout this text to refer to the concepts just described.
Figure 2-2 **A linear pathway.** Upon ligand binding, a receptor becomes active and catalyzes a chemical reaction that leads to the activation of a signaling component A. Active A in turns activates B, which in turns activate C. At the end of the pathway, the expression of gene Z is modified.

A stimulus with a level that remains constant for a time-scale that is long compared with the signaling time scales, is referred to as a *sustained stimulus*. When the stimulus appears quickly relative to the kinetic of the signaling events, a sustained stimulus can be accurately represented as a square step. When exposed to a square-step stimulus, a simple linear pathway such as the one depicted in Figure 2-2 can only relay quantitative information by encoding it in the enzymatic activity levels (from now on *activity level*) at each stage in the cascade. This is so, because under sustained stimulation and in the absence of other regulatory mechanisms, the activation reactions making up the pathway (phosphorylation, hydrolysis, etc.) will reach their steady state levels and operate at them for the remainder of the signaling event. Therefore, the stimulus dose
must be accurately represented by the steady state properties of the system, starting with the fraction of occupied receptors and followed by the enzymes activity levels as the signal propagates downstream. This mode of operation is termed amplitude encoding. This strategy is described in Figure 2-3.

Figure 2-3 Response curves in a linear pathway. The curves show the steady state activity levels normalized to their maximum realizable value. The horizontal axes correspond the range of activity levels achievable by the component immediately upstream. Curves for A and B show a graded response, whereas the curve for C illustrates a sharper, switch-like input-output relation.

The figure depicts the dose-response curves for the activation level at each stage versus the activity level of the species immediately upstream. For simplicity, activation level (e.g. percent phosphorylation) is equated here with the level of enzymatic activity (e.g. kinase activity). It must be stressed that this applies only to concentrations and not at the single molecule level, that is the higher the concentration of modified (e.g.
phosphorylated) enzymes, the higher its enzymatic activity in the solution, but each individual molecule is either active or inactive. The dose response curves of Figure 2-3 can be used to follow the resulting signal when the system is stimulated with a square-step profile of ligand. This system will also be used to introduce the mathematical framework used to describe some of the relevant biochemical processes that appear throughout this work. The first curve corresponds to the receptor occupancy and comes from considering the simple reaction system:

\[ R + L \xrightleftharpoons[k_2]{k_1} RL , \]  

(2.1)

where \( R \) and \( L \) represent the receptor and the ligand respectively. When there is no synthesis and degradation of receptor, the total amount of \( R + RL \) can be considered constant and the evolution of the RL complex can be described in terms of mass action kinetics by (2.2).

\[
\frac{d[R_L]}{dt} = k_1 \cdot [R] \cdot [L] - k_2 \cdot [RL] \quad (2.2)
\]

\[
\frac{[RL]_{ss}}{R_{total}} = \frac{[L]}{K_d + [L]} \quad (2.3)
\]

This equation is also valid in the common case when the production and degradation processes occur at a much slower time scale compared to the signaling time-scale. Under these conditions the number of receptors is conserved and therefore \([R]\) can be substituted in (2.2) by \( R_{TOTAL} - [RL] \), \( R_{TOTAL} \) being the total number of receptors on the cell membrane. The occupied receptor fraction at steady state (2.3) is obtained in a
straightforward manner by solving (2.2) for $[RL]/R_{TOTAL}$ with the time derivative (left hand side) set to zero. The ratio $k_2/k_1$ between the rate constants defines the equilibrium dissociation constant (Kd) for the specific ligand-receptor pair. This parameter will be used frequently in subsequent sections as a measure of the receptor affinity for the ligand.

The dose-response curves for the signaling components downstream of the receptor usually arise from the combined action of activating and deactivating enzymes. A typical system featuring an activation-deactivation cycle may look like the one depicted in Figure 2-3 for enzyme B, which can be described using Michaelis-Menten kinetics by the following equation (2.4).

$$\frac{d[B^*]}{dt} = \frac{k_1 \cdot [A^*] \cdot (B_{TOTAL} - [B^*])}{k_{1M} + (B_{TOTAL} - [B^*])} - \frac{k_2 \cdot [P] \cdot [B^*]}{k_{2M} + [B^*]}$$

(2.4)

The above equation assumes that B is conserved (that is no production or degradation occurs within the signaling time-scale). The constants $k_{1M}$ and $k_{2M}$ are the Michaelis constants of the enzymatic reaction, and their values determine the sigmoidal character of the steady-state dose-response curves. For small values of these constants, solving (2.4) for the active B fraction vs. the concentration of the upstream activator $[A^*]$ yields curves with sharp transitions like the ones depicted for $C^*$ in Figure 2-3. In contrast large values of Michaelis constants produce more graded responses like the ones depicted for species $A^*$ and $B^*$. Following pharmacological nomenclature, the point corresponding to 50% activation is referred to as the EC$_{50}$ value for the particular stage in the pathway.
The presence of reverse reactions is crucial for effective quantitative information transfer. Usually, enzymes present in the intra-cellular media can reverse the chemical modifications performed by the upstream species and lead to deactivation of the enzymatic activity. For example, phosphorylation caused by the action of an upstream kinase can be reversed by a phosphatase. The reverse reaction in (2.4), is catalyzed by a negative regulator P, which is constitutively present and active (i.e. independently of the presence of the stimulus). The equilibrium between a stimulus dependent forward reaction and a stimulus-independent backward reaction (or vice versa\(^1\)) is what allows the steady state level for the activated fraction \([B^*/B_{\text{TOTAL}}]\) in this architecture to be dose-dependent. An irreversible transformation leads to the saturation of the active species for any stimulus concentration, hence precluding any quantitative information transfer\(^2\).

A system of equations of the form given in (2.4) can be used to describe how information travels along a simple linear cascade. The signal propagates when the active form of the signaling enzyme immediately upstream (A*, for example) catalyzes the forward reaction converting its downstream substrate into its active form (B to B*). When the response curves of all the pathway stages are well matched, as for A and B in Figure 2-3, the result is amplitude encoding. Often times, however, some stages saturate before others and amplification may occur, usually accompanied by a reduction in the range of stimuli-intensity suitable for quantitative encoding.

\(^{1}\) If both, forward and backward reactions are promoted by the stimulus, the result is no longer a simple linear cascade but a feed-forward architecture. These systems are capable of producing complex dynamics and are discussed later in relation to adaptive systems.

\(^{2}\) This statement is strictly true for a linear pathway designed to respond to long-lived stimuli. In the presence of non-linear regulatory elements or for transient stimuli, irreversible modifications no longer hinder quantitative information transfer (see section 2.3).
2.2.2 Dynamic range and sensitivity

Three key properties that quantitatively characterize signaling pathways are: sensitivity, dynamic range, and robustness. A number of studies address sensitivity aspects (58-60) of signaling pathways, and some authors suggest that amplification of weak stimuli (increase of sensitivity) could be the main reason for the multi-tier architecture typically observed in these systems (61). Similarly, pathway robustness in terms of coping with parameter fluctuations and noise filtering has been widely analyzed (35, 37, 62). However, even though dynamic range is closely related to sensitivity, relatively little attention has been devoted to it (63).

The dynamic range for an intra-cellular signaling pathway can be defined as the range of stimulus intensities the pathway can detect, encode, and propagate in a form that is suitable for decoding by downstream components. While this definition is useful to characterize particular systems, it is less suited to study the more general case because it ties the intrinsic encoding capabilities of the pathway to the limitations of a particular set of downstream effectors. Therefore, for the purpose of this work, the dynamic range of a given pathway stage will be defined as the range of stimulus (or upstream activity) concentration that produces more than 1% and less than 99% of the maximum attainable activation at that stage. This operational definition is justified at the high end because it is unlikely that downstream effectors can distinguish between 99% and 99.9% activity of the upstream signaling enzyme. In some cases, high sensitivity of the downstream substrate to the presence of enzymatic activity, may justify the extension of the operational definition to lower input levels. However, the same sensitivity may cause the
downstream activity to saturate at low concentrations, causing the whole response curve to shift to the left and the dynamic range to remain constant.

In a simple linear pathway subject to a square-step stimulus, the dynamic range is initially determined by the properties of the receptor/sensor and its enzymatic activity. The range of ligand concentration a receptor can measure can be estimated from (2.3). A quick calculation reveals that a ligand concentration of 99 times Kd corresponds to 99% receptor occupancy, while a concentration of $10^{-2}$ Kd results in 1% occupancy. These are arbitrary limits, since in principle there is nothing preventing graded signaling to occur for receptor occupancy fractions below 1% or above the 99% occupancy level. Effectors immediately downstream of receptors such as G proteins, can be exquisitely sensitive to receptor activity and have the potential to stretch an initial four or five-decade dynamic range further to the left. On the other hand, due to the non-linearity of the receptor occupancy curves, systems are usually unable to discriminate receptor occupancy at high ligand concentrations, and in the absence of other regulatory mechanisms (discussed later) the receptor curve is an insurmountable barrier, preventing dose dependent signaling for ligand concentrations an order of magnitude above the Kd value.

The dynamic range of a pathway as a whole depends on the dynamic ranges of its component stages. To maximize the dynamic range in a linear pathway in the absence of feedback regulation, the response curves of the components must be properly matched. That is, the response curve of a given component must produce graded responses for the

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3 This scale is non-linear, but linearity is not a requirement for effective quantitative information transfer.
achievable output of the component immediately upstream, just like the example depicted in Figure 2-3.

![Diagram](image)

**Figure 2-4 Amplification.** As the steady state response curve at different levels shift to lower agonist concentrations, the signal is *amplified*. The curves are normalized to their maximum achievable value. Amplification does not compare the strength of the signals at different levels in the cascade, but the agonist concentrations sufficient to induce 50% of maximum activity.

When the response curves of the different tiers are not well matched, there are a number of scenarios that can cause the EC$_{50}$ concentration of the physiological response to shift to the left respect to the receptor Kd. Depending on the particulars, the dynamic range of the pathway may reflect that which is set by the receptor affinity, except for a displacement to lower ligand concentrations. This effect is termed *amplification* (Figure 2-4). It is important to stress that amplification in this context does not necessarily mean that successive stages generate more potent responses, but that the maximal observed response (or the EC$_{50}$) is reached at lower ligand concentrations as one moves down the pathway. A corollary of this observation is that amplification necessitates that the activity of at least one stage in the pathway, if not the physiological response itself, saturates before the receptor does. In other cases, mismatched curves can also result in a narrowed dynamic range.

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4 The absolute magnitude of each stage output is meaningful only in terms of the input-output properties of the stage immediately downstream. Hence the use of normalized response curves.
Figure 2-5 depicts three examples (columns) of response curves for each tier of the hypothetical pathway of Figure 2-3, illustrating different aspects of amplification. The first three rows in each column show the individual steady state response curves of the signaling species AP, BP, CP respectively (see Figure 2-3) as function of varying concentrations of their activators (occupied receptor, AP, BP, respectively). The first column shows a typical amplification case, in which each tier saturates at a low concentration of their respective upstream activators. For example, A reaches near maximal activity for a receptor occupancy fraction of 50%, active B saturates for an AP concentration of 0.5 (arbitrary units), and so on. The first two rows depict the activity of each tier as a function of the ligand concentration when the individual response curves are combined together with the receptor occupancy curve (black) to form a pathway. The fourth row indicates absolute values, whereas the fifth row shows the activity levels at the different tiers normalized to their respective observed maxima. The parameters used to generate the figure are listed in the Appendix. Amplification is clearly visible in the first column. Notice that the actual dynamic range remains unchanged, but it is just shifted to lower concentrations. The center column illustrates the opposite case, in which none of the individual response curves saturate for the achievable concentrations of the active upstream components, and therefore the dynamic range of the combined response is limited by the receptor. In this example, the response curves do not allow species A, B, or C to reach total activation because in each case there is not enough upstream activator, causing the different levels of activity observed in the fourth row. This information disappears when the curves are normalized respect to the maximum observed effect (fifth row). Interestingly, technical constraints usually prevent absolute concentration values to
be determined, and therefore experimental results often consist on only normalized combined curves. Finally, the third column depicts the hybrid case in which an intermediate component saturates at a low concentration of the upstream activator. Amplification in this case happens downstream of the saturated component (BP).

Figure 2-5 Three scenarios for a linear pathway. The top three curves are the individual steady-state responses for A (red), B (green) and C (blue) (Figure 2-3) as a function of their upstream activator levels. The combined responses as a function of ligand (L) concentration are depicted in the fourth and fifth (normalized) rows with the receptor occupancy curve in black. Right) Successive tiers saturating quickly lead to amplification, Center) Weak responses result in low activity levels for C. Because no component is fully activated, amplification is not observed. Left) The presence of one intermediate component with a narrow dynamic range results in amplification.
Figure 2-6 Effect of activation thresholds on a linear pathway. The rows are similar to the ones in Figure 2-5. Right) An intermediate component with an activation threshold and a narrow dynamic range limits the dynamic range of the whole pathway and changes the profile of the response curve. Center) A saturated upstream component produces amplification. Notice that C can be maximally activated. Left) The combination of switch-like responses produce systems with increasingly narrow dynamic range.

The cases illustrated in Figure 2-5 are all based on graded response curves (large Michaelis constants), and therefore the dynamic range of the overall pathway is
preserved, although shifted in some instances. Figure 2-6 illustrates the effect of components with sigmoidal response curves. The first column illustrates a dynamic range reduction due to an intermediate stage with a narrow dynamic range. The second column depicts a case in which an amplified signal stimulates a stage with a switch-like response curve, causing a reduction of the dynamic range and the displacement of the response to the left of the receptor Kd value. The third column illustrates a common phenomenon, in which the subsequent action of species with relatively graded response curves produce a pathway with a sharp switch-like response.

The examples above consider just a very small subset off all potential cases. It should be evident however, that maximization of the dynamic range a signaling pathway can respond to in a quantitative way sets potentially demanding requirements on the cell. However, it is important to keep in mind that maximizing the dynamic range does not need to be a design goal for every signaling pathway. In fact, the ubiquitous observation of amplification suggests that pathways often evolve to amplify low intensity signals rather than to allow quantitative responses to wide ranges of stimuli intensity. It is tempting to speculate that the need for amplification and a wide dynamic range may have driven the evolution of more complex signaling architectures. It should be also evident that the receptor occupancy curve sets an upper limit over which quantitative information transfer, and by extension, dose-dependent responses cannot occur in a simple linear pathway. However, this need not be the case in more complex networks containing non-linear regulatory elements. As demonstrated in the next section, the presence of these regulatory elements allows for alternative encodings that endow signaling pathways with
more flexible ways of transferring quantitative information without necessarily sacrificing dynamic range.

2.2.3 Alternative codes

The previous sections introduced the notion of *amplitude encoding*, which is the only viable route to transfer quantitative information in an ideal simple linear pathway. In reality, pathways are seldom linear and a plethora of regulated modules have been identified that involve feed-forward and feedback architectures. These non-linear regulatory elements are pervasive in real-life signaling networks. Even seemingly linear pathways are often made of regulated modules. Examples abound and include feedback or feed-forward activation/deactivation of the enzymatic activity of a signaling molecule (64-66), alterations in protein trafficking that may lead to degradation or sequestration (67-70), alteration of protein and messenger RNA stability (71), just to name a few. One thing all these mechanism have in common is that they can lead to rich dynamics, even in response to sustained stimulus. While a simple linear pathway can only use the steady state activity levels to represent stimuli intensity, these more complex networks can take advantage of the dynamics and use them as a vehicle for information transfer.

There are two main strategies for dynamics-based encoding cells can follow. The first is *frequency encoding*, in which the intensity of the stimulus regulates the frequency of oscillations in the activity of a signaling pathway. Biochemical oscillations have been documented for a number of systems including those behind circadian cycles (72), cell cycle regulation (20), hormone release (73), etc. We do not have to go further from our own hearts to appreciate the importance of cyclic behavior in biological systems.
However, the presence of oscillations in signaling pathways has been observed in just a few cases and the physiological relevance of these findings is still controversial (74). Perhaps the best-studied cases are calcium signaling (75, 76) and the NF-kB module mediating inflammatory responses in mammalian cells (51). It must be stressed that the study of signaling dynamics is still in its infancy. In particular, the investigation of oscillations in signaling pathways has been hampered by the fact that until very recently, most of the experimental work in this field was routinely performed integrating data from populations of cells, making oscillations at the individual level very hard to detect. Despite the experimental limitations, the possibility of oscillations and the information-transfer properties of frequency encoding have been studied from a theoretical standpoint and the reader is referred to (33, 34, 77) for a more extended review.

The second strategy for dynamics-based encoding is duration encoding, in which the intensity of the stimulus is codified in the duration of the signal rather than its amplitude. Duration encoding necessitates a downstream module with a decoder capable of interpreting the signal and producing the appropriate response based on its duration. Despite being conceptually simpler, duration encoding has not received the same attention as frequency encoding, and examples of this alternative have not been clearly documented until now. Experimental evidence for duration encoding in the yeast’s mating pathway is presented in Chapter 4. There is also evidence of duration encoding playing a role in the yeast’s osmotic response pathway as well (46). Signal duration, on the other hand has long been suggested to be an important parameter determining signal

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5 The oscillations the NF-kB module are damped oscillations occurring after the system has been stimulated with a square-step. The phenomenon is similar to the ringing observed in an under-damped oscillator. The physiological relevance of the oscillations in this system are the subject of considerable debate.
specificity. For example, in an often-cited review (78) the author shows that the duration of the active MAP kinase ERK activity underlies the cellular decision between differentiating or dividing when PC12 cells are exposed to Nerve Growth Factor (NGF) or Epidermal Growth Factor (EGF) respectively. In this case, the two stimuli (NGF and EGF) activate the same pathway, one in a transient manner whereas the other produces a more sustained response. The work of Marshall et. al. provides evidence supporting signal duration being a key determinant of the physiological response. This example does not show stimulus intensity being encoded as signal duration (although it is conceivable that both agonists activate upstream components with different efficiency), but explicitly demonstrates that signals of different duration can elicit specific genetic programs. A similar idea was introduced in order to explain signal specificity in yeast (79), however, the evidence supporting the case is incomplete.

The two strategies presented above share a number of qualities, in particular the need for regulatory mechanisms capable of modulating the propagating signals to encode and decode them. It turns out that the necessary steps can be achieved through simple architectures consisting of biochemical regulatory motifs commonly observed in living cells. In many instances, just the proper combination of reaction kinetics can produce significant changes, like for example converting a square-step stimulus into a slowly rising signal. These regulatory mechanisms are the subject of the next section, with particular emphasis on adaptive systems that produce a transient output in response to a sustained input. In section 2.4, the theoretical aspects of duration encoding and the mechanisms that underlie this strategy are further analyzed. Chapter 3 considers the challenges involved in maintaining signal specificity in signaling pathways that share
components and shows how dynamic regulation provides cells with flexible strategies for eliminating cross talk.

2.3 Adaptive systems

2.3.1 Adaptation in biological systems

Adaptation is a phenomenon commonly observed at all levels in biology, and signaling networks are no exception. At the molecular level, adaptation has been shown to play important roles in a number of systems. The best studied is perhaps the chemotropic response in the bacteria *E. coli* (80), in which a chemo-attractant receptor is dynamically modified by signaling components. The modifications (methylation) modulate the receptor activity, allowing the organism to adapt to the background level of chemo-attractant over a wide range of concentrations. A more familiar example comes from adaptation to a new smell after being exposed to it for a while. The sense of smell is mediated by membrane receptors and signaling pathways similar to those in yeast.

The defining property of an adaptive system is the ability of converting a sustained input into a transient output. A system is perfectly adapting if the output returns precisely to pre-stimulation levels. Adaptation is not always perfect and many systems show partial adaptation. Depending of the context, partial adaptation may be indistinguishable from perfect adaptation. Furthermore, some adapting architectures are well suited to produce oscillations. The ability to produce transient responses of variable duration or oscillations, make these systems prime candidates to function as encoders in signaling pathways. In this section we study the properties of these versatile mechanisms.
with special emphasis on those based on negative feedback. Their suitability for duration encoding is considered in the next section.

2.3.2 Three ways to adaptation

Broadly speaking, there are three approaches to adaptation: integral control, feedforward motifs, and negative feedback loops (Figure 2-7). Integral control uses the time integral of the difference between the response and its pre-stimulus level to regulate signaling, and it may rely on feed-forward or feedback regulation, although this is not strictly necessary. This control mechanism has been discussed extensively in the literature in the context of bacterial chemotaxis (81-84). Systems containing feed-forward motifs, in which two stimulus-dependent pathways converge on a common signaling component, also produce adaptation when the parallel pathways have opposing effects on the common component. This concerted action of a positive and a negative regulator is a required feature necessary for adaptation. Feed-forward motifs have received considerable attention in the literature and underlie the regulation of many genetic networks as well as signaling systems (44, 84-86). Negative feedback loops, the focus of this section, are pervasive in signaling systems, and many models of pathway regulation based on negative feedback have been proposed (64, 87-89). In these systems, adaptation is achieved when a signaling species initiates a feedback loop that negatively regulates its own activity either directly, or indirectly by targeting an upstream pathway component. Here, regulation occurs as a result of the propagated signal (output), whereas in feed-forward architectures, regulation is mediated by upstream components (input) independently of the pathway’s output. As a result, in feedback-based systems the
strength of upstream pathway inhibition is determined by the magnitude of the downstream response. For this reason, feedback is usually the method of choice for engineered systems in which control is exercised as a function of how the actual measured output compares to the desired target value. Unlike engineered systems, in which overshoots are often undesirable, intracellular adapting systems, especially those occurring in signaling pathways, critically depend on them. In order to propagate information, the activities of the signaling proteins have to increase and remain sufficiently high long enough to activate their downstream targets. For this to happen, usually the negative regulator has to operate at a slower time scale than the positive one.

Positive regulation can occur through chemical activation, translocation, or elimination of an inhibitor. Negative regulation in signaling cascades can assume many forms including deactivation (65), desensitization (47, 64), sequestration of an upstream species (70), spatial re-localization (90, 91), or stimulus-dependent degradation of a pathway component (92). Both integral control and feed-forward motifs are capable of strict perfect adaptation while negative feedback-based systems in general are not.

**Figure 2-7 Adapting systems.** A) This architecture can be modeled as an integral control system, B) Feedback based, and C) Feed-forward based adaptive architectures.
The regulatory processes mentioned above merit some clarification. In the context of this work, we define *deactivation* as a process by which a species is transformed (often aided by an enzyme) from its active to its original inactive form (e.g. a phosphorylated kinase being dephosphorylated by a phosphatase), such that it can be activated again if the stimulus is still present. Desensitization involves the conversion of a species (by formation or break down of a complex, (de)oligomerization, biochemical transformations, etc.) into a form incompetent for signal propagation. When the desensitized species can be transformed back to a competent state and this process is relatively fast, desensitization may in practice behave like a multi-step deactivation process. Sequesteration involves the removal of a species from the signaling pool by complex formation or spatial re-localization. For our purposes it can be considered a form of desensitization.

Adaptation in biological systems can occur over a wide range of temporal scales. In the cellular context, it can involve upregulation and downregulation of genes as well as chemical activation or deactivation of enzymes. Adaptation at the genetic level is usually a slow process because it requires the production of new proteins, a process that can take hours (albeit it is much faster in simple prokaryotes). Short-term adaptation in signaling pathways is more often mediated by the second kind of processes involving fast modulation of the signaling species activity through chemical modifications. Because of this reason, the analysis that follows is focused on short-term adaptation. This does not mean that changes in protein number are not considered. As a matter of fact, preferential degradation of the modified form of a signaling protein can occur quite fast and underlies adaptation in a number of systems (51). Changes in protein numbers can also occur from
regulation at the RNA level (71), which introduces the possibility of a signal-dependent enhanced protein production occurring on time-scales comparable to the ones typically associated to chemical modifications.

Adaptive systems can be characterized in terms of their ability to produce strong transient responses, their ability to adapt to sustained stimulus, their ability to remain responsive to changes in the environment after stimulation, and the recovery time needed before becoming competent to respond to new rounds of stimulation. To evaluate the potential of these systems to act as encoders or decoders of intracellular signals, it is of fundamental importance to understand the dose-response characteristics of both, their steady states and kinetic properties. These properties are analyzed in the following subsections for a variety of feedback and feed-forward adaptive modules, and general observations about adaptation are presented.

2.3.3 Negative-feedback and feed-forward based systems

2.3.3.1 Overview

Negative feedback and feed-forward adaptive systems share a number of properties, chief among them is the requirement for the coordinated action of positive and negative regulation in a stimulus-dependent fashion. The main difference is that while adaptation in feedback-based systems is driven by the output, feed-forward systems rely on a pre-programmed approach. In many cases however, this difference affects very little of the general properties of otherwise similar systems. Our observations indicate that the adaptation mechanism (deactivation, desensitization, degradation, etc.) and the stage in the pathway that is targeted place constraints on the system’s ability to signal and adapt
and are the main determinants of the system performance. To understand the advantages and disadvantages of these mechanisms, we analyze several simple negative feedback architectures; three based on feedback deactivation, Models I, II and III, and two that rely on desensitization or stimulus-dependent degradation, Models IVA-B. For the most part, the following results apply to feed-forward systems as well, although some differences occur and are pointed out in the text. The parameter values used to generate the figures in this and subsequent chapters are listed in the Appendix.

2.3.3.2 Feedback-mediated self-deactivation

In Model I the signaling component directly activates its own negative regulator (Figure 2-8). This mechanism is inspired by experimental evidence showing that the activity of some phosphatases can be increased upon phosphorylation by their substrate kinase (88). This feature suggests a scenario in which the stimulus leads to phosphorylation and activation of a kinase. In turn, the kinase regulates its activity level by phosphorylating and activating its own phosphatase. Note that rather than increasing the rate of deactivation of a pathway component, it is possible that the negative feedback decreases rate at which the component is activated. We investigated this scenario as well and found no significant differences between the two mechanisms. For the sake of clarity, in what follows the signaling species K will be referred to as the kinase and the negative regulator X will be referred to as a the phosphatase. However, the same mechanism can be generalized to other reversible modifications such as hydrolysis, methylation, etc. The signal in this model is carried by the activity of K*, which can activate some unspecified downstream substrate, and therefore the adaptive output considered is the concentration of K*. 
In its simplest form Model I, can be written in terms of two variables, \([K^*]\) and \([X^*]\), the concentrations of the phosphorylated forms of the kinase and phosphatase, respectively. The model is described by the equations (2.5) and (2.6).

\[
\frac{d[K^*]}{dt} = \frac{k_1 \cdot s \cdot (1 - [K^*])}{k_{1M} + (1 - [K^*])} - \frac{k_2 \cdot [P] \cdot [K^*]}{k_{2M} + [K^*]} - \frac{k_3 \cdot [X^*] \cdot [K^*]}{k_{3M} + [K^*]}
\] (2.5)

\[
\frac{d[X^*]}{dt} = \frac{k_4 \cdot [K^*] \cdot (1 - [X^*])}{k_{4M} + (1 - [X^*])} - \frac{k_5 \cdot [X^*]}{k_{5M} + [X^*]}
\] (2.6)

In the equations above, the total concentration of each species has been normalized to unity and is assumed constant. Under these conditions, this model consists of only two variables and its behavior can be understood by considering the phase plane with axes \([K^*]\) and \([X^*]\) (Figure 2-9). Information about the adaptation performance as
well as the dynamics of the system can be obtained from the nullclines, defined by the conditions $d[K^{*}]/dt = 0$ and $d[X^{*}]/dt = 0$.

![Figure 2-9 Phase space for a feedback deactivation-based system.](image)

Figure 2-9 Phase space for a feedback deactivation-based system. Point A corresponds to the initial state with a basal stimulation level. When the stimulus increases, the $K$ nullcline shifts to the right, and if the kinetics of $K$ activation are considerable faster than those of $X$, the system jumps to point B. Point C corresponds to the steady state after adaptation. (With permission from the Biophysical Journal)

Figure 2-9 depicts these curves for two stimulus levels. The $K^{*}$ nullcline can be interpreted as the signal-response curve for the activated kinase concentration for a given stimulus $s$, and as a function of the active phosphatase concentration. Similarly, the $X^{*}$ nullcline can be thought of as a dose-response curve for the active phosphatase concentration as a function of the active kinase concentration. Because the stimulus $s$ does not explicitly appear in (2.6), the $X^{*}$ nullcline is stimulus-independent. The
intersection of the nullclines represents the steady state of the system. In the absence of a stimulus (or under basal conditions), the steady state corresponds to point A in Figure 2-9. When the stimulus is present, the K* nullcline shifts to the right and the new steady state becomes point C. With the proper choice of parameters, the new steady state value of K* is very similar to its pre-stimulus level, and the system will be adaptive. This depends on the X* nullcline remaining close to the horizontal axis, which requires K* to be a strong activating agent for X (more on this later).

The conditions necessary for the system to produce strong transient responses can be also understood in terms of the structure of the nullclines and the trajectories available for the system to move from point A to C (the steady states before and after stimulus exposure). Upon exposure, the most immediate effect of the stimulus is to promote the activation of the kinase. Clearly if the phosphatase responds very rapidly to changes in the activity level of K, then the system evolves over the X nullcline until it reaches the new steady state. Because the X nullcline is monotonous, and good adaptation requires it to be relatively flat, the concentration of K* will not increase significantly above basal levels during the process. This is a desirable outcome for a homeostatic control system, but it is not suitable for signal propagation. In contrast, when the activation kinetics of X are slow compared to those of K, stimulation causes the concentration of K* to rapidly jump to its quasi-equilibrium value (point B). The increased kinase activity slowly activates X, and the system evolves towards point C closely following the post-stimulation K* nullcline. In this regime, as the system evolves, the concentration of K* remains in quasi-equilibrium respect the instant concentration of X*. In between these two limit cases (fast and slow X’s activation kinetics), the behavior of the system will
resemble one or the other depending on how far it operates from quasi-equilibrium. For the case in which the kinetics of the reactions are comparable, the resulting output may consist in damped oscillations around the final steady state.

The ability to generate strong transient outputs but still adapt close to pre-stimulation levels, makes the quasi-equilibrium regime with slow negative regulation the most effective one for signaling. The quasi-equilibrium condition has the added bonus of allowing the system to be analyzed in terms of dynamically regulated steady state response curves.

Figure 2-10 shows the steady-state response curve of K as a function of the upstream stimulus level in the absence and presence of the negative regulator X. The leftmost curve corresponds to the case in which X has been deleted. The curve to the right corresponds to the case in which X is maximally activated. It is evident from the figure that the effect of the negative regulator is to shift to the dose-response curve to higher active stimulus levels. The quasi-equilibrium condition allows us to think of the effect of stimulation as a gradual displacement of the response curve to the right.
Figure 2-10 Feedback deactivation response. A) Steady state response curve in the absence (right) and presence of maximally activated X (left). B) Time curve for K (black) and X (gray) in response to a square step stimulus. C) Trajectory in the phase space. D) The four operational regimes (see text). E) Typical responses on each regime. F) Peak (right) and adaptation amplitude (left) as a function of the stimulus strength. G) Signal duration (period between half maxima) as a function of stimulus strength.
In general, there is a repertoire of four operational stimulus dose regions available to this system. These are summarized in Figure 2-10D and E. Figure 2-10D again shows the steady state response curves in the absence (left) and presence (right) of the negative regulator. In this figure, the graph has been stretched for illustrative purposes. The four shaded regions shown on this graph correspond to the different operational dose regimes. The first regime corresponds to low stimulus concentrations. In this regime, stimuli of different strength result in transient peaks of increasing amplitude but roughly the same duration (Figure 2-10E, left panel). For each stimulus, the peak amplitude can be approximately determined by the dose-response curve in the absence of negative regulation. Increased upstream K activity increases the rate at which X is activated, and hence only a relatively weak dependence of the signal duration on the stimulus dose is observed. Regime II arises when the stimulus strength is sufficient to saturate K activity. In this regime, stimulus dose is transformed into output signal duration (Figure 2-10E, center panel). In Regime III, the stimulus level is high enough so that the negative regulator is no longer able to counteract the induced activation rate of K, even when X is maximally activated. In this regime the system begins to lose its ability to adapt (Figure 2-10E, right panel). If the stimulus level increases even further, the system operates in Regime IV and adaptation no longer occurs. In this regime, a sustained input produces a sustained output. Therefore, this pathway architecture is capable of acting as a switch; at low stimulus dose the response is transient, whereas at high levels the response becomes sustained. Physiological conditions and kinetic properties of signaling pathways may constrain some systems to operate in a subset of the theoretically possible regimes.
Figure 2-10F shows the measured peak activity (black) and adaptation level (gray) of $K^*$ for the parameters of the example, clearly illustrating the different regimes. Figure 2-10G shows the duration of the signal, defined as the distance between half maxima, as a function of the stimulus strength. The figure shows the typical response trends with signals duration becoming shorter at very low dose, then remaining relatively constant in region I. When adaptation becomes very poor (regions III and IV), the signal becomes sustained and signal duration as defined above is no longer meaningful.

The ability of the architecture to function on changing environments is strongly affected by the recovery time after the stimulus disappears and its signaling potential while already under stimulation. The recovery time for this model is intrinsically linked to the adaptation time. When the stimulus disappears, $K^*$ quickly return to basal levels but because of its slow kinetics, it takes a considerable time for $X^*$ activity to subside. In the best-case scenario, recovery can happen within the same time scale as adaptation. However, the constraints set by the requirements of good adaptation and strong signaling often necessitate the rate of $X^*$ deactivation to be significantly slower than that of its activation. This may result in long recovery times compared with the adaptation time-scale, potentially by orders of magnitude. Additionally, the potency of the negative feedback needed to produce adaptation means that signaling is strongly inhibited during most of the recovery phase while $X^*$ levels are still high, thereby generating a refractory period in which the system is not able to respond to a new challenge. When the system is exposed to a step-like stimulus, the response depends on the frequency of the concentration increases. If the period between stimulus increases is longer than or on the order of the adaptation time, the response consist on well defined signals of comparable
amplitude which is what we would expect based on the phase diagram (Figure 2-9). Increasing the dose generates adapting signals until the stimulus reaches the level that saturates the downstream negative regulator X. After that point, the signal fails to adapt and remains active until the stimulus disappears. The amplitude of the successive signals depends on the dose region (e.g. low dose regime with dose dependent amplitude or high dose regime with dose-independent amplitude), the size of the dose increases, as well as on the shape of the nullclines in Figure 2-9. As a result, the generated peak train can have increasing, constant, or decreasing amplitude, but in general strong signaling is possible after adaptation (Figure 2-11A). If the period between increases is short, the system starts to see the increases as a single stimulus and produces a single peak with an amplitude usually lower than the signal it would have generated had the stimulus been applied all at once (Figure 2-11B). This has important implications, because a bad choice of activation and adaptation time scales could render this mechanism blind to slow increasing stimulus (Figure 2-11C). This property will be exploited when considering the dynamic control of specificity in pathways with shared components.

![Figure 2-11 Response to multiple excitations. A) Model 2 response (clack) to multi-step stimulus (gray). B) Multi-step stimulus with time between steps on the adaptation time scale (gray). Signal that would result from a single square-step stimulus of the final amplitude (dashed). C) Accommodation. Stimulus grows in small increases (gray). Signal that would result from a single square-step stimulus of the final amplitude (dashed). (With permission from the Biophysical Journal)
The analysis above can be applied also to feed-forward based systems with two potentially significant caveats. Depending on the specific kinetic parameters, the post-adaptation steady state in feedback architectures contains a residual concentration of active K necessary to maintain the steady state of active X. As a result, feedback based systems usually settle in such a way that the steady state response curve after adaptation rests with its threshold just above the stimulus concentration (Figure 2-10) and therefore these systems do not show strict perfect adaptation. Furthermore, if the separation of time-scales between positive and negative regulation is weak, these systems will produce damped oscillations. Feed-forward based systems do not share this property. Since X is activated by the stimulus independently of the output, negative regulation can grow much larger than what is strictly needed for turning K activity off. Also, because the upstream species activity is sustained, there is no need to maintain residual K* activity, and strict perfect adaptation is possible.

2.3.3.3 Feedback-mediated deactivation of a positive regulator

A second strategy for producing adaptation through negative feedback is for the signaling component to deactivate an upstream element. The simplest architecture, albeit the least biologically realistic, is one in which the signaling molecule K is directly responsible for deactivating the pathway component KK located directly upstream (Figure 2-12).

As we show, this strategy usually results in poor adaptation. Its study however, highlights the specific benefits of more complex systems as well as the limitations of a feedback mechanism in which the same molecule that transmits the signal also directly inhibits pathway activity. A potentially more biological realistic scenario is one in which
the feedback effect of K is to decrease the rate at which KK is activated. Similar to Model I, this scenario does not produce qualitatively different results from the case of feedback deactivation.

Figure 2-12 Model II: Direct deactivation of an upstream component.

The simplest version of this model is very similar to the one studied in the previous section with the difference being that in this case the species K both propagates the signal and deactivates an upstream species KK* (the stimulus). Because of this reason, the analysis of this model will follow closely the one carried out in the previous section. Model II can be described in mathematical terms by the following equations:

\[
\frac{d[KK^*]}{dt} = \frac{k_1 \cdot s \cdot (1 - [KK^*])}{k_{1M} + (1 - [KK^*])} - \frac{k_2 \cdot [P] \cdot [KK^*]}{k_{2M} + [KK^*]} - \frac{k_3 \cdot [K^*] \cdot [KK^*]}{k_{3M} + [KK^*]} \tag{2.7}
\]

\[
\frac{d[K^*]}{dt} = \frac{k_4 \cdot [KK^*] \cdot (1 - [K^*])}{k_{4M} + (1 - [K^*])} - \frac{k_5 \cdot [K^*]}{k_{5M} + [K^*]} \tag{2.8}
\]
Figure 2-13 Phase space for direct deactivation of an upstream component. Point A corresponds to the initial state for basal stimulation. When the stimulus increases, KK nullcline moves up. If the kinetics of K activation are considerable faster than those of K, the system jumps to point B and then adapts to point C. (With permission from the Biophysical Journal)

Like in the previous cases, the concentrations of the species have been normalized to unity and are considered constant. The kinetic constants have been renormalized appropriately. Also, species KK, is deactivated by a constitutive regulator (P) in addition to the signal dependent effect of K*.

Figure 2-13 shows the phase plane for this model. The signaling species K* is on the vertical axis and the horizontal axis corresponds to the activating upstream kinase KK*. Analysis of the nullclines seems to indicate that adaptation could be possible if the K* nullcline (labeled as $d[K^*]/dt = 0$) is sufficiently switch-like as a function of [KK*].
However, further analysis shows that under these conditions, the system cannot fulfill the requirement of generating a significant response. To understand why, it is useful to consider the two limiting cases in which the kinetics of K are fast or slow compared to those of KK. When KK activation kinetics are fast, the stimulus causes KK* to rapidly rise from its basal level (A in Figure 2-13) to its quasi-steady state level (B) located on the post stimulus [KK*] nullcline. Next, K becomes activated causing the KK* level to decrease along the [KK*]-nullcline until it reaches the new steady state C. This scenario produces a monotonic increase in K* and no transient signaling occurs at this stage in the cascade. In a second limiting case in which the kinetics of K are fast compared to those of KK, the system evolves along the [K*] nullcline towards point C. Again, the increase in K* is monotonic in time. For the two limiting cases just described, the system is not capable of both adapting, and producing a significant response. In the intermediate regime, where the kinetics of K and KK are comparable the system is capable of showing some degree of transient signaling. With the right choice of parameters, the system can “overshoot” the equilibrium point C producing a transient increase in the [K*] level. However, this strategy has serious limitations. First, in the presence of the stimulus, the steady state K* concentration (C in Figure 2-13) has to be significantly higher than the basal level, thereby precluding significant adaptation. Second, this set up is very prone to oscillations. An analysis in terms of response curves is of little use in this case, in part because the quasi-equilibrium regime does not result in initial signaling and posterior adaptation. Figure 2-14A shows the peak response amplitude (Gray) and the steady-state level of K* after adaptation (black) as a function of the stimulus strength.
Figure 2-14 Dose response for Model II. A) Peak (gray) and adaptation (black) amplitudes as a function of the stimulus dose. B) Typical time courses for K* in response to different stimulus concentration. (With permission from the Biophysical Journal)

The time-dependent response for various doses is shown in Figure 2-14B. These figures clearly show how adaptation quickly disappears and the response turns into a small transient overshoot. In this model, response duration is not a relevant quantity because the response does not return to below its half maximum. The principal advantage of this system is its very fast recovery time after the stimulus has been removed. This is due to the fact that K* is responsible for both propagating the signal and deactivating K*K*, making activation and recovery times similar.

The difference between the behavior of Model I and Model II may appear puzzling, especially after realizing that both share essentially the same architecture. As a matter of fact, Model II is a “rotated version” of Model I, in which the negative regulator (X* in Model I, K* in Model II) is also the signal carrier. The key difference between the models is that in Model I, negative feedback occurs through an intermediate step, whereas in Model II the kinase deactivates its upstream activator directly. This difference is crucial because adaptation requires a sustained feedback that responds on a time-scale that is slower than that of activation. Model I can produce a transient response because
the activation of the signaling species K occurs faster than the negative feedback time-scale determined by the kinetics of the phosphatase. However, in Model II, activation of the signaling species K and the onset of the negative feedback are the same process and therefore occur on the same timescale. Based on these observations, we find that intermediate steps that separate the activation time-scale from that of feedback deactivation are a necessary feature for systems in which adaptation occurs as a result of deactivating a pathway component. Such intermediate steps allow for a strong negative feedback capable of returning the pathway output to near basal levels, while at the same time providing a time delay that enables a large transient response. A strong feedback is incompatible with fast kinetics because it prevents the development of the initial transient response. To further explore this observation, the next section considers more complex variants of Model II that include intermediate steps.

2.3.3.4 Effect of intermediate steps

To better understand the dynamics of feedback deactivation it is useful to consider two extensions of Model II. The first case (Model IIIA, Figure 2-15A) represents a scenario in which the signaling species K inhibits an upstream activator KK through activation of a negative regulator. This can be thought of as a terminal kinase K activating a phosphatase X, which in turns dephosphorylates the kinase KK directly upstream of K. In the second scenario (Model III B, Figure 2-15B), the signaling species K deactivates a pathway component two levels upstream. Even though the direct interaction assumed in this model is unlikely to appear in real signaling systems, the analysis of this case reveals the specific effects of the incorporation of multiple regulation
levels and targeting a component further upstream. Model IIIA and IIIB can be described by equations (2.9)-(2.11) and (2.12)-(2.14) respectively.

\[
\frac{d[KK^*]}{dt} = \frac{k_1 \cdot s \cdot (1-[KK^*])}{k_{1M} + (1-[KK^*])} - \frac{k_2 \cdot [KK^*]}{k_{2M} + [KK^*]} - \frac{k_5 \cdot [X^*] \cdot [KK^*]}{k_{5M} + [KK^*]} \tag{2.9}
\]

\[
\frac{d[K^*]}{dt} = \frac{k_6 \cdot [KK^*] \cdot (1-[K^*])}{k_{6M} + (1-[K^*])} - \frac{k_7 \cdot [K^*]}{k_{7M} + [K^*]} \tag{2.10}
\]

\[
\frac{d[X^*]}{dt} = \frac{k_3 \cdot [K^*] \cdot (1-[X^*])}{k_{3M} + (1-[X^*])} - \frac{k_5 \cdot [K^*]}{k_{4M} + [X^*]} \tag{2.11}
\]

\[
\frac{d[KKK^*]}{dt} = \frac{k_1 \cdot s \cdot (1-[KKK^*])}{k_{1M} + (1-[KKK^*])} - \frac{k_2 \cdot [KKK^*]}{k_{2M} + [KKK^*]} - \frac{k_5 \cdot [K^*] \cdot [KKK^*]}{k_{5M} + [KKK^*]} \tag{2.12}
\]

\[
\frac{d[KK^*]}{dt} = \frac{k_6 \cdot [KKK^*] \cdot (1-[KK^*])}{k_{6M} + (1-[KKK^*])} - \frac{k_7 \cdot [K^*]}{k_{7M} + [KK^*]} \tag{2.13}
\]

\[
\frac{d[K^*]}{dt} = \frac{k_3 \cdot [KK^*] \cdot (1-[K^*])}{k_{3M} + (1-[K^*])} - \frac{k_4 \cdot [K^*]}{k_{4M} + [K^*]} \tag{2.14}
\]

As in the previous cases, the total concentrations have been normalized to unity and are assumed constant.

**Figure 2-15 Intermediate steps.** A) Model IIA. Indirect deactivation of an upstream component. B) Model IIIB. Intermediate step between the source and target of the negative feedback.
Model IIIA is almost equivalent to Model I, except that the negative regulator X now acts on an upstream component rather than on the terminal signaling species K. Therefore, it is not surprising that the model is capable of near perfect adaptation and possesses dynamics and dose-response relationships very similar to Model I (not shown). In addition, the same analysis in terms of dynamically regulated response curves apply here. Figure 2-16 depicts the response produced by the system in the presence of various stimulus levels. As with Model I we can see that good adaptation is possible over a range of doses, but at some point the system loses the ability to adapt and a persistent response ensues. The square-pulse nature of the responses in this example is due the sharp response curve corresponding to the parameter values selected. This choice also highlights the decrease in response duration occurring at low stimulus levels. A key feature of this model is the relatively slow kinetics of the intermediate species X. This feature decouples the activation and feedback time scales allowing a sufficiently strong feedback for adaptation to a wide range of doses while at the same time producing a strong transient response. This model can also be seen as a variant of Model II in which a separate negative regulator mediates the deactivation of the upstream feedback target. The fact that this system adapts and signals well is testament to the importance of time-scale separation between the activating reaction and the action of the negative feedback.
Figure 2-16 Model IIIA. Typical responses for different stimulus concentrations. (With permission from the Biophysical Journal)

Figure 2-17 Model IIIB. A) Peak (gray) and adaptation (black) amplitudes as function of the stimulus dose. B) Time course for K* for different stimulus (square-step) doses. Insert: KKK* (black) and KK* (gray) dynamics for the same stimuli. (With permission from the Biophysical Journal)

Model IIIB (Figure 2-15B) resembles Model II in that the signaling species is directly responsible for feedback deactivation causing both timescales to be intrinsically linked. The key difference is that Model II now incorporates an additional step between the target of the negative feedback and the signaling species responsible for it. Because the time-scale of the negative regulator coincides with the activation of the messenger K, we could naively expect the system to show poor adaptation, much like Model II. While this is true, as the experiments represented in Figure 2-17 show, the inclusion of the intermediate step allows the systems to function much better because the feedback no
longer prevents the initial buildup of active K that constitutes the propagating signal. This is not an effect of the particular choice of parameters, but a more general property of these systems. Figure 2-17A shows the dose dependence of the response amplitude (Gray) and steady-state level (Black) when the intermediate species KK was adjusted to react slower than the upstream component KKK. Figure 2-17B shows responses produced at different doses as well as the [KK*] and [KKK*] responses (inset). The steady state level after adaptation is roughly similar to that of Model II. However, the presence of a slower intermediate step dramatically increases the amplitude of the transient response, especially at lower doses (compare with Figure 2-14). The slow deactivation kinetics of KK produces a delay between feedback-deactivation of KKK* and its downstream effect on K*. It is important to notice that the negative feedback starts acting as soon as K* levels rise and quickly deactivates the upstream element KKK, causing different components of the pathway to adapt on different times scales. This effect is a purely transient phenomenon, and as such cannot overcome the poor adaptation (a steady state property) observed with this model because the steady state level of K* has to be sufficient to deactivate KKK* in the face of the continuous presence of the stimulus.

It is interesting to contrast Models III A and B. In Model IIIA, the slow intermediate species delays the effects of the negative feedback while in Model IIIB what is delayed is the time at which the effect of the feedback reaches downstream components. As expected, adding additional levels to the pathway makes it easier to generate oscillatory responses that may be undesirable in actual signaling networks. As a matter of fact, the combination of negative feedback loops and delays is a classic recipe
for oscillations (31, 33). In all our examples, the strength or the slow time scale of the feedback mechanisms precluded any significant oscillations. However, low amplitude ringing (very over-damped oscillations) was observed in some cases.

As expected, Model IIIA’s response duration and recovery time are very similar to Model I. On the other hand, Model IIIB inherits Model II’s fast recovery time. The only slow component in this model is the intermediate element KK, which by the time adaptation is reached, has already come back to near basal levels. Therefore, this mechanism provides a very fast adapting system, albeit one that produces good signaling and adaptation only at low dose levels.

2.3.3.5 Degradation and desensitization-based mechanisms

The reason adaptation is lost at high dose levels in the case of feedback deactivation (Models I-III) is because the species acted upon by the feedback immediately becomes available for reactivation. At high stimulus levels, the negative feedback (which is limited by the output) may not be strong enough to counteract this effect. Additionally, the dual role played by species K, both as a signaling molecule and negative regulator, requires it to be a very effective deactivator to counteract a persistent stimulus, but not so strong that it prevents transient signaling altogether. Feed-forward based systems can partially cope with this problem because the negative regulation is detached from the output (adaptive) signal. However, these architectures still must juggle the positive and negative regulation timing and strengths carefully in order to produce potent transients and good adaptation.
An alternative approach to adaptation is to use desensitization or degradation rather than deactivation as the feedback mechanism. In this scenario, the desensitized (or degraded) component is removed (transiently or permanently) from the signaling pool, thereby relaxing the need for a strong sustained feedback. This mechanism plays a role in Raf-1 regulation (64) and the yeast pheromone response (92) among others. Desensitization and degradation based systems display behavior markedly different from feedback deactivation. To illustrate these differences, we focus on the two models depicted in Figure 2-18.

**Figure 2-18 Degradation and desenzitization-based adaptive systems.** A) Model IVA. Preferential degradation of the active form of species KK. B) Model IVB. Desensitization of species KK.

In Model IVA (Figure 2-18A) feedback regulation targets an upstream component of the pathway for degradation, thereby permanently removing it from the signaling pool. A possible scenario is one in which a kinase K feedback-phosphorylates the upstream kinase KK making it a target for ubiquitination and degradation. Because this mechanism relies on protein degradation, it is necessary to include protein synthesis in the model to maintain a finite concentration of KK. Model IVB (Figure 2-18B) involves a feedback mechanism in which the active form (K*) of the upstream signaling component is transformed to a desensitized form (K-) incompetent for signaling. This transformation is
reversible with the desensitized component eventually reentering the signaling pool. If
the rate at which desensitization is reversed becomes very small, we recover model
Model IVA. If on the other hand the recovery rate is fast compared with the signaling
time scale, model II is recovered. In theory there are two scenarios for how the
desensitized element is reintroduced into the signaling pool. In the first case, removing
desensitization causes the protein to reenter the active state. In the second scenario the
desensitized component must pass back through the inactive state before it can become
active again. We recently proposed this mechanism to describe a branch of the osmotic
response in the yeast (47). Under normal conditions, the Sho1 osmosensor exists as an
oligomer (KK) that, when exposed to osmotic stress, initiates a signaling cascade that
results in the phosphorylation of the kinase Hog1. Phospho-Hog1 then feedback-
phosphorylates Sho1 causing the oligomer to dissociate and signaling to stop. The Sho1
monomers (KK') must then be unphosphorylated before regenerating the signaling-
competent oligomers (KK). The models are described by equations (2.15)-(2.17) and
(2.17)-(2.20) (Model IVA and B respectively). In these equations, the concentration of
the messenger species K is normalized to unity and its concentration assumed constant.
The amount of upstream kinase KK is variable and depends on the regulation of its
degradation. Protein degradation involves enzymatic steps, and, therefore should also
follow some form of saturable kinetics. In this work, these steps are modeled using
Michaelis-Menten kinetics just to account for this fact.

\[
\frac{d[KK]}{dt} = k_0 - \frac{k_1 \cdot s \cdot [KK]}{k_{1M} + [KK]} + \frac{k_2 \cdot [KK^*]}{k_{2M} + [KK^*]} - k_s \cdot [K]
\]  (2.15)
\[
\frac{d[KK^*]}{dt} = \frac{k_1 \cdot s \cdot [KK]}{k_{1M} + [KK]} - \frac{k_2 \cdot [KK^*]}{k_{2M} + [KK^*]} - \frac{k_6 \cdot [KK^*]}{k_{6M} + [KK^*]} - k_1^* \cdot [K^*][KK^*]
\] (2.16)

\[
\frac{d[K^*]}{dt} = \frac{k_1 \cdot [KK^*] \cdot (1 - [K^*])}{k_{3M} + (1 - [K^*])} - \frac{k_4 \cdot [K^*]}{k_{4M} + [K^*]}
\] (2.17)

\[
\frac{d[KK]}{dt} = \frac{k_6 \cdot [KK^-]}{k_{1M} + [KK]} - \frac{k_1 \cdot s \cdot [KK]}{k_{1M} + [KK]} + \frac{k_2 \cdot [KK^*]}{k_{2M} + [KK^*]}
\] (2.18)

\[
\frac{d[KK^*]}{dt} = \frac{k_1 \cdot s \cdot [KK]}{k_{1M} + [KK]} - \frac{k_2 \cdot [KK^*]}{k_{2M} + [KK^*]} - \frac{k_5 \cdot [K^*][KK^*]}{k_{5M} + [KK^*]}
\] (2.19)

\[
\frac{d[KK^-]}{dt} = \frac{k_5 \cdot [K^*][KK^*]}{k_{5M} + [KK^*]} - \frac{k_6 \cdot [KK^-]}{k_{6M} + [KK^*]}
\] (2.20)

Models IVA and B work in a similar fashion producing good adaptation regardless of the stimulus strength. The mechanism of adaptation in these systems can be understood in terms of the dose-response curves of the components KK and K as shown in Figure 2-19A and B for Model IVA. In the figure, the activation curve of K* as a function of KK* concentration (Figure 2-19A) has a sharp threshold below which virtually no activation occurs. Figure 2-19B shows the dose-response curves for KK (dashed gray line) and KK* (solid black line) when feedback is present and for KK* (dashed black line) when the feedback loop is absent. When exposed to a sufficiently high stimulus level, the maximum amplitude of KK* (Figure 2-19B, solid gray line) transiently rises over the threshold value (dotted gray line, Figure 2-19A and B) needed for activating K and triggers feedback degradation. The systems adapt because the negative feedback is sufficient to maintain the steady-state level of KK* below the K* activation threshold. This architecture results not only in the adaptation of the signaling
species K*, but also of the active upstream kinase KK* (Figure 2-19E inset). An ultrasensitive K* response curve is not essential in order to achieve adaptation. However, for a system with a graded K* response curve to show good adaptation, the feedback must reduce the level of active KK* considerably, which in practice may mean degrading or desensitizing virtually all of KK.

Figure 2-19C shows the response amplitude (gray curve) and steady state level (black curve) for K* as a function of the stimulus strength. For comparison also shown in the figure is the maximum response of K* in the absence of feedback (dashed black curve). The response duration (black curve) and time to reach the maximum K* amplitude (gray curve) are depicted in Figure 2-19D. The figure clearly illustrates how the response’s duration becomes shorter as the dose increases, to eventually become dose-independent at high enough doses. Figure 2-20A and B show the respective curves for Model IVB. Figure 2-19E and Figure 2-20C show typical time series for Models IVA and B, respectively. In both cases adaptation is very good and unlike the case of feedback deactivation it is not necessarily lost as the stimulus level increases.
Figure 2-19 Model IVA. A) Steady state response curve for K*. Dotted line indicates the activation threshold. B) Response curves for KK* in the absence of feedback (black dashed), peak amplitude in the presence of feedback (gray), adapted amplitude (black). The dotted line corresponds to the activation threshold in A. C) Maximum peak amplitude in the absence (dashed) and in the presence of feedback (gray). Adaptation amplitude (black). D) Signal duration (time between half-maxima) (black), and time to peak (gray). E) Time courses for K and KK(inset) for square-step stimuli if different strength. (With permission from the Biophysical Journal)
The reason why the response duration initially becomes shorter with increasing stimulus to eventually become dose independent, is that since roughly the same amount of upstream activator has to be degraded or desensitized, a stronger signal results in a faster deactivation of the pathway. At high stimulus levels $K^*$ activity saturates and the rate of degradation or desensitization becomes signal independent. As a result, in the high dose regime, these models generate a strong transient response, with amplitude and duration independent of the stimulus strength. This behavior is in stark contrast with the one observed for feedback deactivation in which the response length is dose independent.
at low stimulus levels and increases as stimulus strength grows. The time series for Model IVA (Figure 2-19E) illustrate two interesting phenomena associated with the sharp \( K^* \) response curve. The complex decay observed at different doses is caused by the interplay between \( KK^* \) degradation and \( K^* \) deactivation kinetics. At high doses, elevated \( K^* \) levels cause the rapid degradation of \( KK^* \) (see inset in Figure 2-19E) without producing a significant drop in \( K^* \). As \( KK^* \) activation decays beneath the \( K^* \) activation threshold, \( K^* \) levels rapidly fall, causing \( KK^* \) degradation to slow down. At the final stage, \( K^* \) and \( KK^* \) activation levels slowly decline until reaching steady state. The initial and final phases are dominated by \( KK \) kinetics, whereas the intermediate stage is dominated by \( K^* \) deactivation kinetics. At lower doses, \( KK^* \) levels are not sufficient to fully activate \( K^* \), and the initial phase is missing. The second effect is a delay in the onset of signaling observed at low stimulus doses because of the time it takes \( KK \) to reach \( K^* \)’s activation threshold. This phenomenon is not exclusive to model Model IVA and often occurs when elements with ultrasensitive response curves are involved in signaling.

![Diagram](image)

\textit{Figure 2-21 Effect of intermediate steps on Model IVA and B. A) Intermediate stage between the source of and the feedback target. B) Mediated feedback.}
The addition of intermediate steps in Models IVA and B can be accomplished in two ways: 1) extra steps can be placed between the upstream activator and the signaling species K (Figure 2-21A), or 2) extra steps can be placed in the feedback loop (Figure 2-21B). Both architectures add new features to the models and increase the likelihood of generating oscillations. In the first case, the addition of an extra step again introduces a transient memory in the system that delays the downstream effect of the feedback desensitization (or degradation) of KK*. As clearly illustrated in Figure 2-22, this effect allows the system to achieve better sensitivity at low doses. This figure compares the maximum response for Model IVB and the model shown in Figure 2-21A. The figure was produced using similar parameter values for both models with the exception of the feedback strength (k5 in (2.20)), because the delay produced by the extra step allows a stronger feedback without compromising the ability to produce a strong response. Note that this increased sensitivity does not require the signal to be amplified by the pathway.

Like in the previous cases (Model III), the introduction of an intermediate step in the feedback loop (Figure 2-21B) allows a separation of time scales between signal initiation and attenuation. In general, the introduction of intermediate components endows these systems with more flexibility and permits the dynamics of species K to differ from that of KK, producing different response profiles which has potentially interesting biological implications (as discussed later in Chapter 3).
For both models recovery is slow, because good adaptation requires slow protein production (Model IVA) or slow recovery from the desensitized state (Model IVB). The recovery time can be improved in Model IVA by a proportional increase in both the production and degradation rates. However, the time scales for these processes are constrained in living cells. Model IVB recovery can be accelerated if the feedback desensitization not only acts on KK* but also on the inactive form KK. This additional depletion of KK permits a faster KK− to KK turnover allowing for quicker recovery. When re-stimulated following the removal of the signal, we observed that significant signaling was still possible even before recovery was complete. This effect is strongly dose dependent and depends on the amount of activator still available as well as the production and re-sensitization rates. However, when compared to the deactivation-based systems, the degradation or desensitization of a pathway component resulting from the initial challenge severely reduced the ability of systems based in these mechanisms to respond to subsequent increases in the stimulus level.
2.3.4 Adaptive systems: General remarks

Signaling systems that adapt to sustained stimuli must meet two requirements. The pathway must generate a response of sufficient strength and duration to elicit the correct response, while at the same time returning to basal levels upon continued exposure to the stimulus. We analyzed different strategies of adaptation that revealed several general principles. First, when the signaling molecule is also a direct negative regulator of an upstream pathway component, the time scales for signaling and feedback inhibition are linked limiting the dynamic properties of the pathway. This intrinsic connection is the reason Model II, which relies on feed back deactivation, cannot adapt or signal well. The near irreversible nature of feedback mechanisms based on desensitization and/or degradation (Models IV A and B) allows strong signaling and good adaptation without requiring a strong sustained negative feedback. The removal of the signaling species from the signaling pool means that a weak feedback is sufficient for adaptation. Even for these models, the addition of a feedback intermediary greatly adds flexibility by allowing different temporal dynamics at different levels in the signaling cascade. This could have interesting implications for the locations of branching points where signaling pathways feed into secondary pathways to elicit a complex cellular response. Different dynamics at different points along the pathway could allow for a variety of responses depending on where the secondary branches are connected.

It has been recognized that multiple level signaling cascades can amplify weak signals (25, 26, 30, 60), and it has been suggested such cascades provide a mechanism for increasing the rate of signal propagation (26, 93). Here we have shown a novel way in which intermediate steps in a signaling cascade can improve sensitivity to low stimulus
levels. In this mechanism, a long-lived intermediate step can store information about the activity level of an upstream component after feedback inhibition has terminated it. The result is prolonged and increased activity of any downstream components. An advantage of placing the delay downstream of the feedback target is that this architecture allows a rapid decrease in the activity of promiscuous upstream elements without a rapid attenuation of the response. In contrast, mechanisms that rely on slow intermediary steps in the feedback loop to decouple the signal and feedback timescales (e.g. Model I) must wait until the feedback acts, for the upstream target activation level to subside. This could have adverse effects potentially leading to cross talk if the upstream component is involved in multiple pathways.

Feedback deactivation has the ability to produce responses in which the response duration depends on the stimulus strength. In contrast, for sufficiently strong stimulus levels mechanisms that rely on desensitization or degradation, the response amplitude and duration become independent of dose. This effect is due to the finite amount and/or dose-independent production rate of the upstream activator that is the target of the feedback. Because of this feature, Models IVA and B are well suited for situations in which the stimulus strength is irrelevant for the response and/or an all-or-none response is desired. An interesting observation is that with the appropriate choice of parameters these models can be made into “signal repositories” with signal potency (area under the peak) regulated by the amount of activator “burnt” (degraded or desensitized) by the feedback in each event. No signal will result once the pool has been depleted, potentially avoiding multiple reactions to the same event.
Our study suggests that systems that adapt through feedback regulation are inherently slow to recover, often resulting in a refractory period much longer than the adaptation time. The notable exception is Model III B, which relies on an intermediate step delaying the effect of the feedback, to produce a transient response. This model is capable of fast recovery because the “slow” pathway component returns to its pre-stimulus level during the adaptation process. However, fast recovery comes at the price of a very limited range of stimulus strengths for which good adaptation is achievable. Obviously, slow recovery times may render these models unsuitable for pathways that must respond to time-dependent stimuli. Interestingly, the responses observed when the models are exposed to stepped increases in the stimulus level demonstrate that in general models based on feedback deactivation (Models I-III) do a better job responding to subsequent increases in the stimulus level than models based on degradation or desensitization (Models IVA and B). However, this effect is strongly dose dependent, and for a limited range of doses, any of the systems can generate a response to this type of stepped increase in the stimulus level. Interestingly, when the stimulation level increases in the adaptation timescale or faster, the results are often complex responses of lower amplitude than the response corresponding to the same final stimulus level applied all at once, potentially resulting in a sub-optimal cellular response. Furthermore, feedback-based adapting systems can produce strong responses only for stimuli that increase fast relative to the adaptation time scale, with slow rising stimuli becoming “invisible”. This phenomenon is observed in the response of neuronal tissue to neurotransmitters in the environment and has been known to neurophysiologists (12), who refer to it as *accommodation*. Taken together, these observations mean that adapting systems not only
must be tailored to elicit a response from their downstream targets, but also to receive particular temporal profiles from upstream activators. This limitation raises the interesting possibility that the redundancy present at the upper levels on some signaling networks (e.g. yeast’s osmotic stress response) (94) may have evolved to provide signaling capabilities at multiple time-scales. It also suggests that the parallel pathways found in many signaling systems are designed to deal with different temporal patterns of stimulation.

2.3.5 Adaptive systems and dynamic range

By regulating the activation or deactivation rates, the deactivation-based adaptive systems analyzed above are able to modify the EC$_{50}$ value of the species that is feedback-regulated. As a result, the effective dynamic range of that particular species can be stretched, potentially increasing the dynamic range of the whole pathway. In some cases, this can also have the seemingly puzzling effect of quantitative signaling occurring well beyond the point at which the steady-state response curve indicates the species activity saturates. Part of the answer to the puzzle is that the signaling event is just a transient and the response curve is constantly modulated during the event. A deeper understanding of this effect can be gained by looking at the way Model I above measures the stimulus. In fact, that system is a reaction rate-meter, because it determines how much stimulus is out there by measuring how fast the forward reaction (first term in (2.5)) can go. In other words, by gradually increasing the backward rate, the system is actually testing the activation rate of K not its amplitude of activation. More precisely, the system is measuring the maximum possible forward rate, which coincides with the initial rate (because the inactive K concentration is maximal just prior stimulation). When the
forward reaction cannot longer cope with the increasing backward rate, the system adapts and the time it took for this to happen constitutes the readout. In fact, upon stimulation, the forward rate quickly drops because most K has been consumed (activated). Then, as X becomes gradually activated, the increased K* turnover provides the forward reaction with inactive K at increasing rates thus allowing it to proceed faster, with rates that are ultimately limited by the concentration of the stimulus. Figure 2-23 shows a typical signaling event triggered by stimulation of Model I with a square step stimulus profile. Figure 2-23B shows how the absolute values of forward and backward rates. The quick decay of the forward rate is followed by a gradual recovery due to the increased availability of K brought about by the increasing backward rate. The backward rate in turns increases as more X is activated. At some point in time, the forward rate reaches its maximum (close to the initial rate), and cannot cope with the increasing backward rate causing the system to adapt.

Figure 2-23 Model I measures maximum rates. A) Species K (black) and X (gray) response to a square-step stimulus. B) Forward (gray) and backward (dash) reaction rates. Note how the system rapidly reaches its quasi-equilibrium state and the forward increases back to its initial level. At this point the forward rate cannot further increases and the system adapts.
2.4 Duration encoding and decoding

2.4.1 Overview

As discussed in section 2.2.3, duration encoding requires the propagated signal to act transiently. That is, at least one component of the pathway must return to its pre-stimulus level on a time-scale significantly shorter than that of the physiological response. This transient activity could result from the stimulus itself being transient or arise because the pathway contains regulatory elements that convert a sustained input into a transient output. The first case is commonly observed in inter-cellular signaling, where the duration of pathway activity often is regulated by the slow degradation of an agonist (95, 96). The guiding theme of the previous sections has been the second case, that is, intra-cellular adaptive systems with the ability to transduce information about the amplitude of the input signal into duration of the output signal even in the presence of a persistent stimulus. Figure 2-24 shows schematically how duration encoding works. In this example, a fixed agonist concentration quickly activates receptors in the plasma membrane. The steady-state level of active receptor (input) causes the activation of a signaling module (grey box) that generates a transient activation of the signaling protein A. In the ideal case, the output of the encoding module is a signal of constant amplitude but dose-dependent duration, such as the one corresponding to A* in Figure 2-24. At each stimulus dose, the amplitude of A* rapidly saturates, but information about the level of receptor occupancy is preserved in the duration of the signal. As already demonstrated, deactivation-based feedback mechanisms can produce such a transformation.
Figure 2-24 Duration encoding. Dose-to-duration encoding. The receptor occupancy level is proportional to the ligand concentration. An encoder transforms receptor occupancy into the duration of protein A activity. A* activates two downstream proteins, B and C. Because of its slow activation kinetics, B acts as an integrator transforming the duration of A activity into the amplitude of B activity. Protein C has fast kinetics and therefore its activity level mimics A* and information continues to be transmitted as signal duration.

Figure 2-24 shows two possible scenarios for how A* activates its downstream targets. In the first scenario, species B is slowly activated by A*. This causes the activity of B (B*) to increase over the entire period of A’s transient activation. If the kinetics for the deactivation of B also are slow, B activity remains elevated for a significant amount of time after A* has returned to its basal level. In this case B effectively works as a decoder, transforming the duration of A activity into the amplitude of B activity. In other words, slow kinetics makes B an integrator capable of measuring for how long the upstream signal has been on. In the second scenario depicted in Figure 2-24, species C has fast activation and deactivation kinetics. As a result, the C* concentration closely
mimics the behavior of A* reaching a quasi-equilibrium level soon after the signal is received and returning to pre-stimulation levels once the A activity ceases. In this case, quantitative information about the stimulus is preserved even when C* is saturated because it is encoded as signal duration. Duration encoding does not restrict the temporal nature of the cellular response. For example, positive feedback acting downstream of either components B or C can be used to convert transient pathway activation into a permanent developmental switch (97), or other downstream modules can convert transient signals into responses of various temporal profiles (section 3.2.2).

2.4.2 Encoders

As mentioned previously, the focus of this chapter is cases involving sustained inputs. Therefore, in order to produce duration-encoded signals it is necessary to consider systems capable of adaptation or desensitization such as the ones analyzed in the preceding sections. That is systems that respond to a stimulus by generating a transient increase in pathway activity to then return to basal levels even if the stimulus persists for a long time. Ideally, in order to work as a “dose-duration” transducer, the duration of the transient output has to increase with the concentration of the stimulus. This is not a general property of adaptive systems, but as demonstrated in a previous section, deactivation-based systems have this ability.
Figure 2-25 Dose-duration encoders. A) Feed-forward architectures. B) Feedback-based architectures.

Figure 2-25 shows a number of architectures capable of producing the desired transformation. The two pathway architectures depicted in Figure 2-25A consist of incoherent feed-forward loops (98) in which the upstream stimulus activates both a positive and negative regulator of the signaling protein K. For the system to show transient activity, negative regulation must occur on a slower time scale than activation of K. As shown in the figure, this can be achieved if the negative regulation is mediated by an intermediate species X. This species can operate either by inhibiting activation of K by KK or by promoting deactivation of K. Figure 2-25B shows two simple pathway architectures involving negative feedback loops that can exhibit adaptive behavior. In these examples, the signaling molecule activates its own negative regulator. In the first case, the negative regulator X increases the deactivation rate of K and in the second case X decreases K’s activation rate. Both strategies produce qualitatively similar behavior. Similar to the case of feed forward regulation, adaptive behavior in these systems requires the negative feedback to operate on a slower time scale relative to activation of K. The two keys for the dose-duration transformation are that the activation rate of K is proportional to the stimulus concentration (KK* concentration in the model under consideration) and that the activation of the negative regulator occurs with slow kinetics.

---

6 This model is the same as Model I discussed at length in section 2.3.3.2
Figure 2-26 Modes. A) The four operational regimes for Model I. B) Typical responses on each regime.

All these systems can potentially operate in the dose regions discussed in section 2.3.3.2 and summarized again in Figure 2-26. Regime II arises when the stimulus strength is sufficient to saturate K activity but the negative regulation is potent enough to eventually cause it to subside. This is the regime where the dose-duration transformation occurs (Figure 2-26E, center panel). A property of these systems, and in particular this regime, is that it produces clearly different outputs for a range of stimulus doses well beyond the original (that is without feedback) dynamic range. A downstream module capable of decoding these signals would endow the pathway with an overall increased dynamic range. In other words, by exploiting the dynamic properties of the system, rather than relying on steady-state characteristics, signaling pathways can increase their dynamic range.

When operating in Regime II, the temporal profile of K* resembles a square pulse (Figure 2-26C). This is because the dose-response characteristics of the system where
taken to be switch-like. It is important however, to study how the dose-duration transformation is affected when this assumption is relaxed. We start by observing that the steep dose-response curves result from the low values of the Michaelis constants used in the reaction rates \(k_{1M}, k_{2M}, \text{ and } k_{3M}\) in (2.5)), which means that the reaction rates saturate quickly. In particular, the small \(k_{1M}\) value causes the activation rate of \(K\) to be roughly independent of the \(K\) concentration except at very low levels. The opposite extreme occurs when the activation rate operates far from saturation (high \(k_{1M}\) limit). In this case the system can be described in terms of mass action kinetics. For the system to efficiently adapt, some degree of steepness in the response curve is still required. This can be achieved by manipulating the parameters involved in the negative regulation. For such cases, the system’s response to a sustained stimulus is no longer a square pulse, but shows a more gradual decay in time (Figure 2-27). However, the length of time required for the signal to decline below a given threshold still depends on the strength of the stimulus, and therefore the stimulus concentration can still be encoded as signal duration.

The scenario discussed above is of particular interest because it relates to a situation in which the negative feedback loop acts at the level of the receptor. Figure 2-27 shows a schematic diagram of a model in which the ligated receptor activates a negative pathway regulator \(X\). The protein \(X\) inhibits the pathway by modifying the ligated receptor (phosphorylating it in this example) and decreasing its affinity for the ligand (Figure 2-27A). Equations (2.21)-(2.23) provide a mathematical description of this model.
Figure 2-27 Dynamic regulation of a receptor affinity. A) An active receptor activates a species X, which promotes the ligand release reaction. B). Response curves in the absence (right) and presence of maximally activated X (left). C) Typical responses on each of the four operational regimes.

\[
\frac{d[R_L]}{dt} = k_1[L][R_{TOTAL} - [RL] - [RL^*]] - k_2[RL] - \frac{k_0[X^*][RL]}{k_{0M} + [RL]} \tag{2.21}
\]

\[
\frac{d[RL^*]}{dt} = \frac{k_0[X^*][RL]}{k_{0M} + [RL]} - k_3[RL^*] \tag{2.22}
\]

\[
\frac{d[X^*]}{dt} = \frac{k_4([RL] + [RL^*])(X_{TOTAL} - [X^*])}{k_{4M} + (X_{TOTAL} - [X^*])} - \frac{k_5[X^*]}{k_{5M} + [X^*]} \tag{2.23}
\]

The steady-state receptor occupation curves in the presence and absence of the negative regulator X and the temporal responses for the four regimes of operation for this model are presented in Figure 2-27B and C. Note that that the loss of the square pulse-like transient output does not prevent duration encoding, because higher ligand levels still cause the signal to persist for a longer time. Furthermore, a square-pulse activity profile is
easily generated if the pathway contains an intermediate species with sharp dose-response characteristics. As shown in Figure 2-28 and described by equation (2.24), species B measures for how long the receptor occupancy remains above the activation threshold, thereby rectifying and transforming the gradual decrease in the occupancy level into a square-pulse response.

\[
\frac{d[B^*]}{dt} = \frac{k_6 \cdot ([RL] + [RL^*]) \cdot (B_{TOTAL} - [B^*])}{k_6M + (B_{TOTAL} - [B^*])} - \frac{k_7 [B^*]}{k_7M + [B^*]}
\]  

Because in this example the negative feedback acts on the receptor, an important consequence of this pathway architecture is that it allows for “signaling beyond saturation”. That is, the system responds in dose-dependent manner to ligand concentrations higher than the level that would be required to virtually saturate the receptor in the absence of the feedback. In other words, the dissociation constant of the
receptor is context dependent and as such can be dynamically modulated and exploited to expand the dynamic range of the signaling pathway.

The mechanisms of duration encoding discussed so far involved activation and deactivation processes that are reversible. Feedback or feed-forward degradation and desensitization also produce adaptive systems. However, in their simplest incarnations, these mechanisms of adaptation tend to function as dose to duration transducer only over a narrow band of stimulus concentrations, if at all (see section 2.3.3.5). For this reason we do not further analyze these adaptive systems here, although we want to stress that at least in principle and under the right assumptions they work as duration encoders as well.

There is, however, a special case, in which adaptation by degradation or desensitization can produce the dose-duration transformation in a trivial way. This occurs when the system directly degrades the stimulus. Duration encoding ensues because higher agonist levels require longer periods of time to be degraded. For example, when a messenger peptide is released to the intracellular space in the presence of a protease, the more peptide that has been released the longer it takes for the protease to degrade it, and as a result, the longer the pathway is active. The same concept can be applied to intracellular calcium release or hormones in the bloodstream. Selective internalization of ligated-receptors can also transform dose information into signal duration (See Chapter 4). Because the signal strength in the media is determined by measuring the time it takes to eliminate the agonist, it is evident that this approach works best when dealing with events involving localized transient release of the signaling molecule. The mechanism of negative regulation (protease, internalization, etc.) can be constitutively present or
feedback regulated. As we show in Chapter 4, this mechanism appears to play a role regulating signaling through the pheromone response pathway in yeast.

2.4.3 General remarks

While the analysis above focused on minimal models for duration encoding, more complex architectures involving multiple levels of regulation would allow a tighter control of the input-output properties of the signaling system (99). It is important to emphasize that duration encoding, or any other encoding method for that matter, does not have to function throughout the whole network. It is likely that multiple information processing strategies coexist at different levels (or even under different conditions) in a single pathway. These observations raise the question of what advantages duration encoding provides over using the amplitude of pathway activity to transmit information. One important advantage of duration encoding is that it can potentially increase the dynamic range of the signaling pathway. That is, duration encoding increases the range of stimulus concentration for which the pathway can respond in a dose-dependent manner. Duration encoding may also provide a more robust transmission mechanism than amplitude encoding, especially in multilevel networks where accurate quantitative information transfer using amplitude encoding would require the response characteristics of the individual components to be carefully matched. Duration encoding may also play a role preventing spurious activation of pathways that share components. In the next chapter we propose a strategy termed “kinetic insulation” for achieving pathway specificity by dynamically regulating pathway activity. This mechanism relies solely on the temporal profiles of the propagated signals to insure signal fidelity and avoid cross talk. This investigation suggests that pathways with shared components are good
candidates for duration encoding. The MAPK cascade in the yeast pheromone response is an example of such a system because several of the signaling proteins in this system (e.g. Ste11 and Ste7) are known to participate in the hyper-osmotic shock (94) and filamentous growth (100, 101) pathways (Figure 3-1). As a matter of fact, in Chapter 4 we demonstrate that this organism uses duration encoding to relay information down the pheromone response pathway.

The duration-encoding mechanisms presented here not only allow the system to overcome pathway components with a narrow dynamic range (e.g. switch-like dose response characteristics), but take advantage of them. Operation in Regime II (Figure 2-26), where the dose to duration transformation works optimally, requires that the activity level of at least one signaling component saturates in response to the stimulus. As we demonstrated, in this regime the system continues to respond in a dose-dependent manner to stimulus levels above those required to saturate the pathway in the absence of feedback or feed forward regulation. The explanation for this apparent paradox lies in the dynamic nature of the response curve for the pathway. As the signaling event progresses, negative feedback regulation (or feed forward) shifts the EC\textsubscript{50} towards higher stimulus levels, increasing the dynamic range of a stage of the pathway “on the fly”. This is in contrast to the effect of amplification in which a leftward shift of the EC\textsubscript{50} (Figure 2-4) increases sensitivity at low stimulus levels, often sacrificing dynamic range as a result.

When the negative feedback operates by allosterically regulating the receptor affinity, the situation is even more interesting because in this case Kd is dynamically regulated (Figure 2-27). This has the potential effect of shifting the EC\textsubscript{50} of the cellular response significantly to the right of the receptor occupancy curve. Depending on the
response of the downstream components, this can in principle increase the dynamic range of the whole system (the system response curve is not only shifted but also stretched). Because of this dynamic property, receptor occupancy curves measured by ligand-binding assays are potentially time-dependent quantities and need to be interpreted with care. Interestingly, Kd values determined in vitro or in reconstituted systems usually differ from those obtained in-vivo, and this discrepancy is often attributed to an abnormal conformation of the receptor in the artificial environment. The analysis presented here suggests that even if the microenvironment of an in-vitro experiment matches cellular conditions, the results of ligand binding assays might differ from their counterpart in-vivo due to dynamic regulation of the receptor. This could happen, for example, if the receptor occupancy curve is determined in a system in which a downstream element of the signaling pathway has been disrupted, thereby breaking the negative feedback loop.

Duration encoding and dynamically encoded signals have been observed in a number of systems. For example the intensity of light (number of photons) impinging on photoreceptors in rod cells is encoded as the duration of the transducin (G protein) mediated activity of the pathway (102). It has been shown that the RGS protein RGS9 plays a key role in determining the duration of the signal (103). The recent discovery that different temporal profiles of IkB kinase (IKK) activity in the NF-kB signaling module selectively activate different groups of target genes, further supports the notion that dose-to-duration encoding can play a significant regulatory role determining cellular responses (50, 51, 104). In this case, stimulation of murine embryonic fibroblasts with tumor necrosis factor-α produces a short transient peak of IKK activity whereas stimulation with polysaccharides results in a slower and more sustained response. This example
further illustrates how the temporal dynamics of the activity in a signaling pathway is not only important for quantitative information transfer but also in managing specificity.

Finally we note that depending on the nature of the stimulus, signaling systems can alternate between the operating regimes depicted in Figure 2-26. It is remarkable that very simple architectures can generate such a variety of responses determined only by the strength of the stimulus. The systems described in this section not only function as amplitude and duration-encoders, but can also act as biochemical switches that transition from transient to sustained outputs depending on the intensity of the stimulus. Such switches have been proposed to underlie cell fate decision process in a number of systems (44, 78, 79, 101, 105). Given the simplicity and flexibility of the systems considered here, it is likely that dose-to-duration encoding plays an important role in signal transduction. We predict more instances of dose-to-duration encoding will be discovered as the temporal properties of more signaling systems continue to be characterized.
Chapter 3

Alternative encodings for specificity control

3.1 The cross-talk problem

Intracellular signaling pathways that share components are ubiquitous in nature. For example, in yeast the mating and starvation response pathways involve a common kinase (Ste7) (6, 106), yet respond to both environmental cues in very distinct and highly precise ways. In higher organisms, the situation is even more complex and undesired cross-talk underlies many pathological conditions (107-110). Therefore, understanding the mechanisms responsible for pathway specificity is a fundamental problem in signal transduction. Several solutions to this cross-talk problem have been proposed. Scaffold proteins are thought to limit cross-talk by sequestering and enabling activation of signaling molecules unique to a given response pathway (28, 111-113). A classic example is the yeast scaffold protein Ste5, which is required for mating but not the starvation response. Another mechanism to achieve specificity is cross-inhibition, in which a downstream component prevents signal propagation through the inactivation of an inappropriate pathway. Cross inhibition is also used in the yeast mating response. In this case, stimulation with pheromone produces an increased degradation of a
transcription factor (Tec1) involved in the starvation genetic program (114, 115). Finally, in higher eukaryotes spatial localization of signaling molecules plays a role in pathway specificity as observed in the widely studied response of ERK kinase to different growth factors (reviewed in (116, 117)) or in cAMP signaling in cardiac myocytes (118). These mechanisms play important roles in achieving pathway specificity and therefore have been subject to a number of theoretical studies in recent years (27, 106, 119-121).

Most of the approaches described above do not take advantage of the dynamic nature of intracellular signals and relay mostly in steady-state properties to ensure specificity. Interestingly, it has been observed that the component pathways of networks with shared elements have very different dynamics and often operate on distinct time-scales. For example, the MAPK Hog1 is activated very quickly and decays with a characteristic time of a few minutes during the yeast’s hyper-osmotic shock response (46, 47). On the other hand, signaling in the mating response operates in a considerably longer time scale: MAPK Fus3 is activated over the period of tens of minutes or more (see Chapter 4). Furthermore, MAP kinase activity during the filamentous growth response to certain starvation conditions is thought to operate on even longer time-scales (79). What is interesting in this case, is that the three pathways (HOSR, MR, FG) share components, in particular MAP-KKK Ste11 (Figure 3-1).

The distinct dynamics exhibited by signaling systems led us to ask: could these dynamics be used to minimize cross-talk and ensure specificity in the presence of shared components. In this section, we describe a mechanism termed “kinetic insulation” which takes advantage of the distinct chemical kinetics and network architectures commonly present in pathways with shared components to do exactly that. The analysis that follows.
reveals that temporal dynamics can be exploited by cellular systems to route information through a common component. We show that this approach is sufficient to maintain specificity and prevent cross talk in a variety of scenarios. In addition, we discuss different strategies for encoding information that allows properties of the input stimulus (strength, rate of change, and duration) to be transmitted by the pathway and subsequently decoded by the cell.

Figure 3-1 Yeast pathways. A number of pathways share the MAP-KKK Ste11. MAPK Kss1 is activated in response to pheromone and during nutrient deficit induced invasive/filamentous growth. More shared proteins (Ste12, Dig1/2) can be found downstream in the pathway (not shown).

3.2 Kinetic insulation

3.2.1 Discriminating transient and slowly varying signals.

Figure 3-2 shows a schematic diagram of a hypothetical signaling system. The system consists of two response pathways, A and B, that share the intermediate component C. Signaling through each pathway is initiated when the ligand, SA or SB, binds to the appropriate receptor, RA or RB, respectively. The correct response to either stimulus
requires activation of the appropriate terminal kinase, KA or KB, without significant activation of the other. We demonstrate that when the upstream components of pathway A cause C activity to increase slowly, and the upstream components of pathway B cause activity to increase transiently, then simple downstream pathway architectures can be constructed such that the kinases KA and KB discriminate between these two inputs (see Figure 3-2B). We then show how the appropriate input signal to C can occur regardless of the temporal profile of the external stimulus. In Figure 3-2B kinase KA responds to a slowly increasing signal while remaining in the inactive state if the signal is transient. The simplest way to achieve this behavior is if KA has slow activation kinetics relative to the timescale of the transient signal. Thus KA acts as a “low-pass” filter ignoring the fast transient signal from pathway B. To achieve pathway specificity, KB has to be significantly activated only when C receives a fast transient input signal, but remains inactive if the input signal varies slowly in time. This is achieved using an adaptive system. Adaptive systems generate a transient response, which eventually returns to pre-stimulus levels, even in the presence of a sustained input signal. This behavior can occur through the action of feed-forward or negative feedback loops as discussed in Chapter 2.

There we demonstrated that in order to generate a large amplitude signal, an adaptive system must be excited by an input that increases rapidly with respect to the adaptation time scale. Otherwise, as the input signal increases, the system continuously adapts and the activity of the terminal kinase remains near its basal level, phenomenon termed “accommodation”. If the downstream components of pathway B form an adaptive system, then the pathway functions as a “high-pass” filter. That is, pathway B is unable to respond to the slowly increasing input signals resulting from the activation of pathway A,
but produces a strong response to the fast transient signals from pathway B. Figure 1B shows a schematic diagram of a pathway architecture involving negative feedback that produces such a system. In this system the kinase KB phosphorylates and activates a phosphatase (P*) that in turn dephosphorylates and deactivates KB (Model I studied in Chapter 2). A consequence of this design is that KB activation is also transient. However, as we show in the next section, a transient signal can be transformed into a sustained signal in a straightforward manner.

In general, the common pathway component C can play an active role in modulating the temporal response of the two branches. However, for simplicity we assumed that C undergoes stimulus-dependent phosphorylation and constitutive dephosphorylation. The rates of these two processes must be sufficiently fast so that C is able to respond to the transient input signal from pathway B without significant distortion of its temporal profile.

![Diagram](image)

**Figure 3-2 Kinetic insulation.** A) Pathways A and B share component C. The terminal kinases KA and KB, respond to external cues received by receptors, RA and RB, respectively. B) Slow kinetics prevents KA from being activated by a short transient signal. The adaptive nature of KB prevents its activation by a slowly varying signal. C) Temporal activation profile of KA and KB when C is exposed to a slowly increasing stimulus (inset). D) Temporal activation profile of KA and KB when C is stimulated with a square pulse lasting 45 minutes (inset).
Equations (3.1)-(3.4) describe the system in mathematical terms. The normalization criteria are the same applied in previous models.

\[
\frac{d[KA^*]}{dt} = \frac{k_9 \cdot [C^*] \cdot (1 - [KA^*])}{k_{9M} + (1 - [KA^*])} - \frac{V_{10} [KA^*]}{k_{10M} + [KA^*]} \tag{3.1}
\]

\[
\frac{d[KB^*]}{dt} = \frac{k_6 \cdot [C^*] \cdot (1 - [KB^*])}{k_{6M} + (1 - [KB^*])} - \frac{V_7 [KB^*]}{k_{7M} + [KB^*]} - \frac{k_8 [P^*] \cdot [KB^*]}{k_{8M} + [KB^*]} \tag{3.2}
\]

\[
\frac{d[P^*]}{dt} = \frac{k_4 \cdot [KB^*] \cdot (1 - [P^*])}{k_{4M} + (1 - [P^*])} - \frac{V_5 [P^*]}{k_{5M} + [P^*]} \tag{3.3}
\]

\[
\frac{d[C^*]}{dt} = \frac{k_3 \cdot sa \cdot (1 - [C^*])}{k_{3M} + (1 - [C^*])} + \frac{k_1 \cdot sb \cdot (1 - [C^*])}{k_{1M} + (1 - [C^*])} - \frac{V_2 [C^*]}{k_{2M} + [C^*]} \tag{3.4}
\]

To test the ability of the system to discriminate between input signals with different temporal profiles, we performed a series of computational experiments in which C was activated using either a square pulse or slowly ramped input. The input signals and the temporal response of KA and KB are shown in Figure 3-2B and C. As can be seen, KA and KB only respond when C is activated with an input signal of the appropriate temporal profile. To test the range of input profiles the system can discriminate, we repeated the simulations varying the final amplitude and rise time for the ramped input (Figure 3-3A) and the signal duration and amplitude for square pulses (Figure 3-3B). The left panel in Figure 3-3A shows the activation level of KA reached after eight hours of stimulation whereas the right panel shows the maximum peak amplitude recorded for KB during that period. The figure clearly illustrates that, except for very low input amplitudes, KA is
strongly activated whereas KB is activated only in response to fast rising inputs (bottom right region of the right panel). Figure 3-3B shows the maximum activation level reached by KA (left panel) and KB (right panel) as a function of the amplitude (horizontal axis) and duration (vertical axis) of a square pulse input to the common component. As can be seen, KB is strongly activated over a wide range of amplitudes and durations, whereas KA shows modest activity for pulses of sufficient duration (~ 2 hrs).

The results presented above demonstrate that for pathways in which the signal must pass through a common element, simple downstream architectures can be used to selectively transmit a response based on the temporal profile of the input received by the shared component. We term this filtering mechanism “kinetic insulation”. We next considered what upstream architectures are required to convert receptor activity into appropriate input signals for the common pathway component. In the following section we describe mechanisms for encoding stimulus dose information into an appropriate temporal response.

![Figure 3-3 System response to various input profiles.](image)

(A) Species C is exposed to ramped inputs of various rise times and final doses. The gray scale indicates KA’s activity level after eight hours of stimulation (left) and maximum activation level of KB (right). (B) Species C is exposed to square pulses of different durations and doses. The gray scale represents the maximum activation level of kinase KA (left) and kinase KB (right). The gray scale is the same in the four panels with white corresponding to total activation and black to no activation.
3.2.2 Processing stimulus profiles into appropriate input signals.

Kinetic insulation requires that the common signaling component receive distinct temporal inputs from pathways A and B. However, depending on environmental conditions the cell can be presented with a variety of stimulus profiles. Therefore, a strategy must be in place to ensure that the appropriate signal is transmitted to the common component regardless of the temporal profile of the incoming stimulus. In our example, activation of pathway A requires that C receive a slowly increasing signal. However, the concentration of stimulus SA can vary slowly or rapidly in time. In the latter case pathway A must be able to convert the rapidly changing stimulus level into a transmitted signal that slowly increases in time. In the same way, pathway B must be capable of transmitting a transient input signal to C in response to arbitrary SB profiles. Therefore, below we consider strategies for generating transient and slowly increasing signals from three distinct stimulus concentration profiles: a) sustained (a fast rise followed by a sustained stimulus), b) square pulse, and c) ramped. Figure 3-4 depicts mechanisms that can be used to modulate pathway activity to generate the desired temporal profile for the six possible scenarios.

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

**Figure 3-4 Simple architectures designed to modulate the temporal profile of the input stimulus.** (A) Architectures that transform sustained, transient, and slow increasing inputs into a slowly increasing output signal. (B) Architectures that transform the same set of inputs into a transient signal.
Transforming a sustained stimulus into a ramped response is easily achieved by a signaling species with slow activation kinetics (Figure 3-4A, left column). The simplest way of producing a ramped response from a square pulse stimulus involves two steps, designated here as A and AA (Figure 3-4A, middle column). Initially, an upstream pathway component AA is rapidly activated by the transient stimulus to form the active and relatively stable AA*. The sustained level of AA* then slowly activates the species A generating the desired ramped response. The ramped stimulus can be transmitted directly as a ramped response. Or, if needed, the addition of an intermediate pathway component with slow activation kinetics can be used to further slow down the response rate (Figure 3-4A, right column).

A sustained stimulus is converted into a transient signal by using an adaptive system (Figure 3-4B, left column). The production of a transient response from a square pulse stimulus is straightforward and achieved by using a signaling species with fast activation/de-activation kinetics (Figure 3-4B middle column). Additionally an adaptive system can be used to guarantee a particular response duration, or make other output properties independent of the stimulus profile. Converting a ramped stimulus profile into a fast transient signal requires multiple steps because adaptive systems tend to be insensitive to slowly increasing stimulus levels. The simplest architecture able to perform this conversion contains a signaling species BB with fast activation and slow deactivation kinetics directly upstream of an adaptive system consisting of signaling molecule B (Figure 3-4B, right column). Species BB rapidly amplifies the slowly increasing stimulus level and passes a relatively sustained signal to B, which in turn is converted into a transient output by the adaptive system. The high sensitivity of the upstream component
BB makes this architecture very sensitive to noise. Using an ultra-sensitive switch (59) for BB helps avoid a spurious response because the system only responds once the stimulus level crosses a threshold value. This has the additional effect of introducing a delay between the time the stimulus level starts increasing and when the downstream transient signal is generated. This delay depends on the rate at which the stimulus is increasing and provides a mechanism for modulating the response depending on how fast the environmental conditions are changing. The above analysis is not meant to be comprehensive, but rather provides the basic building blocks necessary to kinetically insulate pathways with common components.

3.2.3 Preserving specificity through kinetic insulation.

As a proof of principle we combined the architectures shown in Figure 3-4 with the kinetic insulation mechanism described in Figure 3-2. The resulting model is shown in Figure 3-5. For illustrative purposes we designed the model in such a way that both branches produce the correct signal profile for each of three stimulus profiles discussed above: sustained, square pulse, and ramped. To make pathway A responsive to a transient stimulus in addition to sustained and ramped stimuli, it is built from an upstream component AA possessing fast activation and slow deactivation kinetics, followed by a slow reacting species A (Figure 3-4A center). Pathway B was designed with an upstream fast reacting ultra-sensitive species BB followed by an adapting species B (Figure 3-4B right). This endows pathway B with the ability to respond to ramped stimulus concentrations as well as to sustained and square pulse profiles. The model is described by equations (3.5)-(3.10) for the upstream section, and (3.1)-(3.3) for the terminal kinases. The equations for KA and KB remain unchanged from the previous model, but
the equation for the common species C now has been modified to account for the upstream branches.

![Equation Diagram](equation.png)

**Figure 3-5 A full model for kinetic insulation.** Upstream components of pathway A transform sustained, transient and slowly increasing SA levels into a slowly increasing output signal that is detectable by kinase KA, but unseen by KB. Upstream components of pathway B transform sustained, transient and slowly increasing SA levels into a transient output signal that is detectable by kinase KB, but unseen by KA.

\[
\frac{d[AA^*]}{dt} = \frac{k_{30} \cdot sa \cdot (1 - [AA^*])}{k_{30M} + (1 - [AA^*])} - \frac{V_{31}[AA^*]}{k_{31M} + [AA^*]} \\
\] (3.5)

\[
\frac{d[A^*]}{dt} = \frac{k_{32} \cdot [AA^*] \cdot (1 - [A^*])}{k_{32M} + (1 - [A^*])} - \frac{V_{33}[A^*]}{k_{33M} + [A^*]} \\
\] (3.6)

\[
\frac{d[BB^*]}{dt} = \frac{k_{20} \cdot sb \cdot (1 - [BB^*])}{k_{20M} + (1 - [BB^*])} - \frac{V_{21}[BB^*]}{k_{21M} + [BB^*]} \\
\] (3.7)

\[
\frac{d[B^*]}{dt} = \frac{k_{22} \cdot [BB^*] \cdot (1 - [B^*])}{k_{22M} + (1 - [B^*])} - \frac{V_{23}[B^*]}{k_{23M} + [B^*]} - \frac{k_{24}[M^*] \cdot [B^*]}{k_{24M} + [B^*]} \\
\] (3.8)
\[
\frac{d[M^*]}{dt} = \frac{k_{25} \cdot [B^*] \cdot (1 - [M^*])}{k_{25M} + (1 - [M^*])} - \frac{V_{26}[M^*]}{k_{26M} + [M^*]} \tag{3.9}
\]

\[
\frac{d[C^*]}{dt} = \frac{k_3 \cdot [A^*] \cdot (1 - [C^*])}{k_{3M} + (1 - [C^*])} + \frac{k_1 \cdot [B^*] \cdot (1 - [C^*])}{k_{1M} + (1 - [C^*])} - \frac{V_2[C^*]}{k_{2M} + [C^*]} \tag{3.10}
\]

The three types of stimulus profiles discussed above were used for the simulations shown in Figure 3-6. First these profiles were taken as the concentration of SA and used to activate the receptor RA (Figure 3-6A – C). The left panels show the responses of the intermediate species. As can be seen, all three stimulus profiles quickly activate the upstream species AA (AA*, dashed black line) generating a long-lived signal that in turn causes the slow activation of the second component A (A*, solid gray line). As a result C also is activated slowly (C*, dashed gray line) as required for effective kinetic insulation. The right panels show that in all three scenarios, only KA is significantly activated (gray lines), whereas KB is activated only very weakly (black line) even when the stimulus is very transient.

Figure 3-6D-F show the response when pathway B is stimulated with the same profiles used above. As expected, the fast kinetics of the intermediate species BB (left panel, dashed black line) and the adaptive nature of B (left panel, solid gray line) produce a transient input signal propagated downstream by C (left panel, dashed gray line). This causes significant activation of KB (right panel, black line) with just minimum activation of the slowly responding KA (right panel, gray line). The switch-like nature of the upstream component BB is evident in Figure 3-6F (left panel) showing the response to a slowly rising stimulus concentration. The right panel of Figure 3-6F clearly shows the resulting delay in the activation of KB.
Figure 3-6 System response to various stimulus. Input and response of pathway A (left two columns) and pathway B (right two columns). Panels A, B, and C illustrate the systems response to sustained, transient (45 minutes), and ramped stimuli, respectively, applied to pathway A. Temporal profiles for the stimulus SA and activity levels of AA, A, and C are shown in the left column. Temporal profiles for KA and KB activity are shown in the right column. Panels D, E, and F illustrate the systems response to the same sustained, transient, and ramped stimuli respectively, applied to pathway B. Times series for the stimulus SB and species BB, B, and C are shown in the left column. Time series for KA and KB activity are shown in the right column. Note the delay introduced by ultra-sensitive component BB in (F).

To test the model further we studied the response when both branches are stimulated concurrently. If both stimuli appear simultaneously, the result is a superposition of the individual responses with KB activation occurring transiently and KA activation levels rising slowly (Figure 3-7A). When the stimuli are not synchronized, the response depends on the order in which the branches are excited. When pathway B is stimulated first, pathway A remains competent for signaling and specificity is not affected. Alternatively, when pathway A is stimulated first, signaling through pathway B is possible only if C activation is not saturated. In this particular example, C saturation does not occur until a long time after pathway A was stimulated (Figure 3-7B-C). Interestingly, this limitation provides an effective cross-inhibition mechanism to prevent KB activation during the later stages of response A. However, it is possible to engineer
pathway A in such a way that it does not saturate C causing pathway B to remain competent for signaling.

![Figure 3-7 Specificity under simultaneous stimulation.](image)

- **Figure 3-7 Specificity under simultaneous stimulation.** (A) Application of a slowly increasing stimulus to pathway A and a square pulse lasting 45 minutes to pathway B. Temporal profiles of the stimuli and the shared component C (left panel) and the terminal kinases KA and KB response (right panel). (B) The same square pulse is applied to branch B after a four-hour delay. This demonstrates that stimulation of pathway B still elicits an appropriate response even when pathway A is active. (C) A similar square pulse is applied to branch B after ten hours. In this particular case, saturation of the common component C prevents signaling through pathway B.

### 3.3 General remarks

Different environmental stimuli are capable of producing characteristic cellular responses. However, most stimuli act through signaling components that are not unique to one signaling pathway. Component sharing is particularly prevalent among MAP kinase signaling cascades. For example the nutrient-response and pheromone-response pathways in yeast require the same MAP kinase kinase (Ste7) and the same MAP kinase kinase kinase (Ste11). Yet nutrient starvation and pheromone stimulation produce highly distinct and mutually exclusive cellular behaviors (106).
Component sharing has certain advantages for the cell, since it reduces the biological cost and complexity of assembling multiple signal transduction systems. However the prevalence of component sharing raises the fundamental question of how cellular pathways preserve signal specificity and avoid inappropriate cross activation. In the past signal specificity has been attributed to the scaffolded assembly of signaling proteins, as well as to cross-inhibition mechanisms that neutralize the activity of components in competing pathways (27, 111, 112, 115, 119, 122). Kinetic insulation is a novel mechanism for preserving pathway specificity in which signaling elements downstream of the common component are designed to respond only to specific temporal profiles of pathway activity. Our model is founded on the observation that signaling cascades exhibit distinct dynamical behaviors, including differences in the persistence of activation (transient versus sustained signaling) as well as variations in activation and deactivation timescales. Indeed it is well known that the dose and duration of an external stimulus can profoundly influence the magnitude or nature of the response. These observations led us to consider the possibility that cells use information encoded in the temporal profile of pathway activity to maintain signal identity. We reasoned that similar to modern communication devices that transmit multiple signals through a single channel, a cell might use biochemical networks to encode external cues into temporal patterns that can be received only by the intended target. Extending the analogy further, we designed simple architectures that can function as “filters” and combined them into a system capable of maintaining specificity under a wide range of conditions. The above computational experiments demonstrate how signal specificity can be achieved without
the need for scaffold proteins or cross-inhibition. Furthermore, we showed that kinetic insulation allows the activation of one pathway without neutralizing the other provided that the shared component is not saturated. This situation is easily achievable because the upstream portions of the pathways can be designed in such a way that saturation of the common component does not occur. Alternatively, saturation of the shared component by the slow pathway A provides an extra layer of control that can be used to prevent an undesired response to stimulus B once a response to stimulus A has been initiated.

MAP kinase signaling pathways are typically comprised of three or more protein kinases, acting in sequence. This level of organization has in the past been ascribed to a need for signal amplification. The kinetic insulation approach described here not only is congruent with the existence of multi-component pathways, but it necessitates such complexity. Our results indicate that this common network architecture may serve not only to amplify the signal, but also to modulate the temporal profile of pathway activity. For instance we show how the inclusion of additional upstream components (AA and BB in Figure 3-4) allows the system to respond appropriately to various stimuli profiles.

The observation above raises the possibility that cells can exploit the temporal modulation of a signal as it travels through the pathway to encode relevant information about the stimulus. This is especially important when the specific properties of the external cue, such as dose and duration, determine the magnitude or nature of the response. In these cases, this information must be encoded and preserved as the signal travels through the pathway. For example, yeast undergoes two developmental fates in response to pheromone. Low levels of pheromone lead to filamentous growth, whereas high concentrations produce a mating response (52). Interestingly, the different
modulation schemes studied above (Figure 3-4) also have important implications on how information about the stimulus profile is encoded. This suggests that information processing and signals specificity are two closely related issues. For example, information about a stimulus can be encoded as activation duration, activation level, or activation rate depending on the system’s kinetic requirements. In the case of pathway A, which requires slow increasing signals in order to produce a specific response, the activation rate is the only option. In pathway B, a transient signal is generated and therefore information about the stimulus is most efficiently encoded as duration, although amplitude encoding is also possible. The nature of the stimulus does not dictate the encoding strategy, as exemplified by the case of a sustained input generating a transient signal in pathway B.

In Chapter 2, we demonstrated that an adaptive system based on negative feedback could function as a signal transducer converting stimulus dose into response duration. We showed also that this mechanism allows information about the stimulus level to be transmitted even after a pathway component, such as the receptor, has been saturated. In this system, the ability to detect saturating stimulus concentrations results from the delay between receptor activation and the time needed for a negative feedback loop to become sufficiently activated to counteract the receptor activation rate. Another interesting case occurs when an ultrasensitive component responds in a switch-like manner to a slowly increasing stimulus thus creating a delay. This delay is determined by the rate of change of the stimulus. A pathway functioning in this way can be combined with another parallel pathway in a feed-forward fashion to produce signals of amplitude or duration proportional to the rate of increase of the stimulus concentration. In general
the relevant information can be transformed from one encoding scheme into another as
the signal is modulated by successive stages of the pathway. For example, in Figure 3-4A
(center) the duration of a transient stimulus is encoded in the activation level of AA,
which is then converted into activation rate of A.

As mentioned above, the addition of upstream components (AA and BB in Figure 3-4) allows the system to respond appropriately to various stimuli profiles. However, it
also tends to hinder the systems ability to encode information about the temporal
characteristics of the stimulus. This is obviously undesirable when the detailed nature of
the stimulus is important, and suggests that signaling pathways are likely tuned for a
specific subset of stimulation profiles. Therefore multi-branched signaling pathways
might result from the cells need to respond to different temporal profiles of the same
stimulus. For example, one environmental cue that can occur over a wide range of time
scales is osmotic stress. It is intriguing that the osmotic response of many simple
eukaryotes consists of multiple branched pathways (57, 123, 124). This pathway
architecture has been interpreted as providing either a backup system or a mechanism for
sensing a wide range of osmotic conditions. The recent discovery that the Sho1 branch of
the osmotic response of yeast is a rapidly adapting system (47) opens the possibility that
the other pathway branches are necessary to allow the cell to respond to slow changes in
osmolarity. Thus multi-component signaling architectures provide enormous flexibility in
transmitting detailed information about an incoming stimulus. In contrast, pathways
leading to all-or-none responses would need only to relay qualitative information about
the presence or absence of a stimulus, in which case a much simpler architecture would
suffice.
Finally, a kinetic approach to specificity has some interesting advantages. First, it is robust. Cross-talk is avoided not just by virtue of a specific interaction, but by the inherent kinetics of the signaling species involved combined with network level features of the upstream segments of the cascade. Additionally, kinetic insulation might reduce the biological cost and complexity of the system as compared to pathways that rely on protein scaffolds or cross-inhibition. Given the prevalence of multi-component signaling cascades and the use of shared signaling components, it seems reasonable to speculate that the kinetic insulation mechanism described here applies to a broad array on intracellular signaling systems.
Chapter 4

Dynamic regulation of signals in yeast

4.1 Overview of the mating process

The yeasts *Saccharomyces cerevisiae* (from now on yeast) is a unicellular eukaryote that can live stably either as a diploid or as a haploid. In the haploid state, yeast exist in two varieties usually referred to as $a$ and $\alpha$ cells. Under the right conditions, an $a$ and an $\alpha$ cell can mate to produce a diploid\(^7\). The mating response is triggered by the presence in the media of a pheromone of the opposite kind. These short peptides, denominated $a$-factor and $\alpha$ factor, bind to specific receptors on the membrane of $\alpha$ and $a$-cells respectively, initiating the cascade of signals that lead to mating. Yeast cells are not motile, therefore they can only mate with nearby neighbors. In order to do so, the cells must determine the location of the potential mating partner, and grow a projection (termed a *shmoo*) in the proper direction. For a successful mating to happen, the projections must reach the mating partner and the cells must fuse. The morphological change is just one aspect of a very complex response that includes growth arrest and

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\(^7\) When environmental resources are scarce, diploid cells form spores. When the conditions improve, these spores hatch and produce new generations of haploid cells.
changes in the expression of more than 400 genes (125). This response is induced by the activation of a number of messenger proteins and transcription factors usually grouped in what is called the yeast pheromone or mating pathway.

The mating pathway is activated when pheromone binds to a specific membrane receptor (Ste2 for α-factor and Ste3 for α-factor), thereby activating it. The active receptor promotes the GDP/GTP exchange in the α subunit of its cognate G-protein. This protein is actually a hetero-trimer, formed by an α (Gpa1), a β (Ste4), and a γ (Ste18) subunit. The GTP-bound form of the Gα cannot bind efficiently to the other subunits and, upon pathway activation the complex dissociates. The free Gβγ complex remains attached to the plasma membrane from where it recruits the scaffold protein Ste5, placing it in the proximity of the kinase Ste20. These proteins in turn recruit and activate a hierarchy of Mitogen Activated Protein Kinases (MAP kinases) formed by Ste11 (MAPKKK), Ste7 (MAPKK), Fus3 (MAPK) and Kss1 (MAPK). The MAP kinases Fus3 and Kss1 interact with two regulatory proteins (Dig1/2, also known as Rst1/2) in a process that results in the phosphorylation of the transcription factors Ste12 and Tec1. Tec1 does not play a role in the mating response and is quickly degraded8 following stimulation with pheromone. Homo-dimeric Ste12, is then able to bind to Pheromone Response Elements (PRE’s) and modify the expression of the early genes involved in the mating process.

Historically, the pheromone pathway denomination has been used to refer to events that are activated by the Gβγ complex. However, recent experiments have shown that the

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8 Tec1 degradation prevents activation of Filamentous Response Elements (FRE’s) and is thought to be an important specificity control feature.
α subunit may be playing a role activating the MAP kinases from the endosome membrane (126), and Intense efforts are underway to unveil the relative importance of this new branch. Because the relative contribution of this branch has not been characterized yet, we focus on the Gβγ branch.

![Figure 4-1 The pheromone pathway (see text).](image)

4.2 A MAPK code

As briefly mentioned in the Introduction, the developmental response initiated by yeast in response to pheromone depends critically on the concentration (52, 53) (Figure 2-1). In the presence of very low levels or no pheromone, cells continue to grow and divide normally. At intermediate levels, the cells become elongated and are capable of chemotrophic growth when presented with a pheromone gradient. High levels of pheromone produce a bona fide mating response, involving growth arrest and the emergence of the characteristic mating projections. It is clear that for this system to produce the proper response, quantitative information about the pheromone dose has to
be propagated down the mating pathway. Recent data generated in collaboration with Henrik Dohlman’s lab suggest that the mating response pathway is using duration encoding (see Chapter 2) to relay information about the concentration of pheromone in the medium and establish which developmental program is followed.

Figure 4-2 shows time course data for dual-phosphorylated (active) Fus3 and Kss1 in response to different doses of pheromone. The data were obtained from a population of a cells exposed to the indicated pheromone concentration. At different intervals, aliquots where taken from the culture, the cells were lysed, and the amount of phosphorylated Fus3 and Kss1 determined using an immuno-fluorescence assay with a specific antibody. The protocol is well established and the details are described in reference (79).

The transition from chemotropic growth to mating occurs between 3 and 10 µM, where there is a large increase in Fus3 activity. Note the qualitative similarity between the experimental results and the graphs in Figure 2-24 (compare pp-Fus3 to B* and pp-Kss1 to C*). The roughly dose-independent rate (slope) for Fus3 phosphorylation
suggests that its activation rate is saturated. This could happen if the level of upstream kinase activity is independent of the pheromone dose, whereas the duration of this activity is dose-dependent. Kss1 on the other hand, shows fast kinetics. Note that for high pheromone concentrations (10 µM), Kss1 seems to undergo two stages of phosphorylation with a second increase in phosphorylation starting around 30 minutes after the initial inoculation. If we ignore the second peak of Kss1 activity for the time being, then by virtue of its fast kinetics, Kss1 phosphorylation is providing a glimpse of the upstream signal dynamics. Furthermore, it is clear from the data that for the doses assayed, Kss1 operates in Regimes I and II (and perhaps III) depicted in Figure 2-26. These observations, combined with the very good correlation between the duration of the Kss1 and Fus3 signals (defined as the time at which phosphorylation begins to decrease), suggest that Fus3 and Kss1 phosphorylation are driven by a duration-encoded upstream signal (input).

To test this possibility we started by considering a simplified scenario in which Fus3 and Kss1 phosphorylation and dephosphorylation can be described in terms of Michaelis-Menten kinetics. Equations (4.1) and (4.2) define the evolution of the phosphorylated form of Fus and Kss1 respectively. In both cases, the dual phosphorylation is assumed to happen in a single step and the concentration of the species is held constant and normalized to 1.

\[
\frac{d[Fus^3*]}{dt} = \frac{k_1 \cdot s \cdot (1 - [Fus^3*])}{k_{1M} + (1 - [Fus^3*])} - \frac{k_2 \cdot [Fus^3*]}{k_{2M} + [Fus^3*]} \quad (4.1)
\]

\[
\frac{d[Kss1*]}{dt} = \frac{k_3 \cdot s \cdot (1 - [Kss1*])}{k_{3M} + (1 - [Kss1*])} - \frac{k_4 \cdot [Kss1*]}{k_{4M} + [Kss1*]} \quad (4.2)
\]
In this simplified scenario we sought to establish a single input profile capable of reproducing the experimental results for both Kss1 and Fus3. Specifically, we looked for a signal profile $s(t)$ that when used as input to (4.1) generates the Fus3 profile and when applied to (4.2) generates the Kss1 profile. The analysis produced the input signal and MAP kinases profiles shown in Figure 4-3A and B respectively.

The input signal was obtained by manually fitting the experimental data. The simplicity of the approach and the small number of parameters made more sophisticated optimization methods unnecessary. Because the experiment only provides the phosphorylation levels relative to each other, the ratio of phosphorylated to total protein is a free parameter. For the Kss1 case, we assumed that the concentration right after the first peak corresponds to saturation (all Kss1 present has been phosphorylated), with the assumption that the second peak arises from Kss1 being released from a second cellular pool. In the case of Fus3, the optimum correspondence was achieved when the maximum phosphorylated fraction (10uM and 1h) was half or less. Higher fractions significantly
affected the quasi-linear slope due to depletion of the unphosphorylated form. If the second peak of Kss1 phosphorylation is ignored, the coincidence between the simulated time-series and the actual experimental data is remarkable. This result suggests that the upstream signal driving Kss1 and Fus3 phosphorylation has a square pulse-like shape (Figure 4-3A), with higher concentrations being translated as longer duration. The model also suggests that at low concentrations, the input signal is operating in the amplitude-encoded region (region I, Figure 2-26) whereas for higher concentrations it moves to the duration-encoded region (region II).

The nature of the second Kss1 phosphorylation peak at high doses of pheromone can be attributed to several factors. A particularly attractive candidate is the pre-existence of more than one pool of Kss1 in the cells. As a matter of fact, Kss1 is known to form stable complexes with the transcription factor Ste12 and the regulatory proteins Dig1 and Dig2 (also known as Rst1 and Rst2). Furthermore, these complexes are regulated in response to pheromone and are thought to play an important role eliciting the signal (127-130). In particular, it has been postulated that MAP kinase dependent phosphorylation of Ste12, the Dig proteins or Kss1, causes the dissociation of the complex (127), presumably liberating Kss1 that could become a target for double-phosphorylation. An alternative source of fresh Kss1 can also be related to Kss1 moving from the nucleus to the cytoplasm as the signaling event progresses (131). To test the plausibility of these mechanisms as the source of the second peak, we replaced (4.2) with a simple model containing a complex between Kss1 and an unspecified protein D. Phosphorylation of Kss1 in the complex destabilizes it (131), causing it to break apart. When free, Kss1 can be phosphorylated directly by the upstream species represented by signal s. When in a
complex, phosphorylation of Kss1 is mediated by a species C, which is activated by the phosphorylated form of Kss1. In this model, Kss1 and C are part of a positive feedback loop: the signal s promotes free Kss1 phosphorylation, which in turns activate C, which phosphorylates the Kss1 in the complex (DKss1), destabilizing it and releasing more phosphorylated Kss1. The model is described by equations (4.3)-(4.7).

\[
\frac{d[Fus3^*]}{dt} = \frac{k_1 \cdot s \cdot (1 - [Fus3^*])}{k_{1M} + (1 - [Fus3^*])} - \frac{k_2 \cdot [Fus3^*]}{k_{2M} + [Fus3^*]} \tag{4.3}
\]

\[
\frac{d[Kss1^*]}{dt} = \frac{k_3 \cdot s \cdot (K_{TOT} - [Kss1^*] - [DKss1] - [DKss1^*])}{k_{3M} + (K_{TOT} - [Kss1^*] - [DKss1] - [DKss1^*])} - \frac{k_4 \cdot [Kss1^*]}{k_{4M} + [Kss1^*]} + \frac{k_5 \cdot [DKss1^*]}{k_{5M} + [DKss1^*]} \tag{4.4}
\]

\[
\frac{d[DKss1]}{dt} = k_6 \cdot (K_{TOT} - [Kss1^*] - [DKss1] - [DKss1^*]) \cdot (D_{TOT} - [DKss1] - [DKss1^*]) - k_7 \cdot [DKss1] - \frac{k_8 \cdot [C^*][DKss1]}{k_{8M} + [DKss1]} + \frac{k_9 \cdot [DKss1^*]}{k_{9M} + [DKss1^*]} \tag{4.5}
\]

\[
\frac{d[DKss1^*]}{dt} = \frac{k_8 \cdot [C^*][DKss1]}{k_{8M} + [DKss1]} - \frac{k_9 \cdot [DKss1^*]}{k_{9M} + [DKss1^*]} - \frac{k_5 \cdot [DKss1^*]}{k_{5M} + [DKss1^*]} \tag{4.6}
\]

\[
\frac{d[C^*]}{dt} = \frac{k_{10} \cdot [Kss1^*] \cdot (C_{TOT} - [C^*])}{k_{10M} + (C_{TOT} - [C^*])} - \frac{k_{11} \cdot [C^*]}{k_{11M} + [C^*]} \tag{4.7}
\]
Figure 4.4 Effect of a secondary Kss1 pool. A square-like input signal (A) applied to a model with a secondary pool of Kss1 reproduces Fus3 (B) and Kss1(C) phosphorylation data well. D) Dynamics of the pp-Kss1 (purple), Kss1 (blue), D-Kss1 (orange), and C* (gray) for each pheromone dose.

As the figure shows, there is good agreement between the model and the experimental observations. The model parameters were hand tuned. A simulated annealing optimization method was assayed but did not produce significantly better results. The total concentration of Kss1 was chosen to reflect the physiological levels (~5500 molecules/cell) as reported in (132), whereas the concentration of D (2000 molecules/cell) was chosen considering the physiological amounts of the possible inhibitory partners Dig1 (~1400 molecules/cell), Dig2 (1300 molecules/cell), Ste12 (1900 molecules/cell). The balance between the available amounts of D and Kss1 is important because it determines how much free Kss1 is available for phosphorylation before the complex is broken-down. Interestingly, the natural choice based on the concentrations of the Dig’s and Ste12, produces good agreement between the model and the experiment. This exercise demonstrates that the two-pool hypothesis can indeed explain the second
Kss1 phosphorylation wave. However, the model does not explicitly identify species D or C, and therefore the nature of the second pool and its regulatory mechanisms is still ambiguous and open to further investigation. In this respect, the analysis of the model can provide some guidance and hint at some properties the candidate mechanisms must have. Figure 4-4 shows the simulated time evolution of phospho-Kss1 (purple), free Kss1 (light blue), Kss1-D complex (orange), and active C (gray), in response to the same pheromone concentrations used in the experiments described before. The plots clearly show that the slow kinetics of species C activation introduce a delay that prevents the phosphorylation of Kss1 in the complex until after the upstream signal has been active for a while. Note that phosphorylation and release of Kss1 from the complex occurs only after a significant amount of C has been activated. In a way, species C resembles the activity profiles of Fus3 (compare with previous figure) and that suggest an alternative architecture in which Fus3 is responsible (most likely through an intermediary) for destabilizing the second pool of Kss1. In fact, Ste12 and Dig proteins have been shown to be substrates for Fus3 (130, 133-135). This model uses species C to create a delay and species D to sequester Kss1 in a second pool that is not immediately accessible for phosphorylation. It must be stressed that spatial sequestration of a fraction of Kss1 in the nucleus (131) could achieve similar results. In fact, since Ste12 and the Dig are nuclear proteins, and Fus3 is known to shuttle to the nucleus (136), it is likely that these two mechanisms both play a role generating the second Kss1 phosphorylation peak in response to high pheromone doses.
4.3 Encoding mechanisms

4.3.1 Overview

The analysis in the previous section provides evidence that yeast cells are using duration encoding, at least in the stage of the pathway upstream of Kss1 and Fus3. The first natural question is what is the mechanism responsible for encoding pheromone dose into signal duration. Unfortunately, the pheromone response pathway is more complex than the sketch in Figure 4-1 mainly due to a number of feedback reactions and the presence of the protease Bar1. However, there is a key fact that any mechanism has to account for, which greatly restricts potential mechanisms. This fact is that our experiments reveal a dose-dependent response to concentrations of pheromone up to 10uM, whereas the dissociation constant for the α-factor receptor has been determined to be anywhere between 4 and 15 nM (56, 137-140). This means that this pathway is responding in a dose dependent manner for concentrations that are virtually saturating the receptor. This should not come as a surprise, especially when the findings described in the previous sections are taken into account. Signaling beyond saturation is a hallmark of some of the dose-duration encoding strategies involving feedback regulation of the receptor affinity. Stimulus degradation, an alternative mechanism briefly discussed in section 2.4.2, can also explain this observation. In this case, dose is converted into signal duration by a system that measures how long it takes for the amount of agonist present in the media to be degraded. Regardless of the specific details, the observation of signaling beyond saturation indicates the mechanisms responsible for dose to duration transformation operates at the head of the pathway, and possibly on the ligand itself. The
following sections explore possible mechanisms for duration encoding using the know biochemistry of the pathway. The goal is to present a number of plausible scenarios and indicate the requirements for each of them to work as encoders. In most cases, the necessary assumptions constitute a basis for predictions that can be validated experimentally.

4.3.2 Protease Bar1

The EC$_{50}$ for the MAP kinases response is displaced considerably to the right of the receptor dissociation constant. This could indicate that the receptor affinity is being dynamically modulated or that the local concentration of pheromone seen by the cells is less than what was added. This last scenario could materialize if pheromone is quickly degraded after being added to the cell culture. This could be due to the presence of protease Bar1, which is secreted constitutively by yeast cells but also has been reported to be upregulated in the response to pheromone. The protease Bar1 has been shown to degrade pheromone but little is know about its potency. The observed dose-dependency however, would require different initial concentrations of pheromone to be quickly degraded to different final concentrations. Since there is no active pheromone addition after the initial inoculation, an equilibrium cannot be sustained making this possibility unlikely. A more plausible scenario is one in which pheromone is slowly degraded by Bar1. Because the pheromone amount in the growth media is fixed, the higher the initial concentration, the longer it would presumably take the protease to degrade it. Such a process could certainly account for the EC$_{50}$ shift as well as for the dose-duration transformation. Support for this mechanism comes from experiments looking at FUS1 induction. This gene is specific for the pheromone response and experiments using the
Lac-Z assay (55) in a BAR1 deletion background show dose response results consistent with a receptor Kd value of ~10nM (56).

The most efficient way to determine the effect of protease Bar1 is to repeat the dose-response experiments in cells on which the gene encoding this protein has been deleted. This mutant exists and has been extensively characterized. Figure 4-5B (dots) shows the temporal profiles for Fus3 and Kss1 double phosphorylation in response to different concentrations of mating pheromone.

![Figure 4-5 Effect of BAR1 gene deletion](image)

The experimental data shows that phosphorylation of these two species becomes saturated for pheromone concentrations over 10nM, much less that 30 uM observed in the
strain containing the *BAR1* gene. This is consistent with a receptor Kd of around 5nM. Interestingly, the phosphorylation profiles of Fus3 and Kss1 in a *BAR1Δ* strain still point to duration encoding taking place, so once again, we sought for a common signal that could reproduce these new data when feed to equations (4.1) and (4.2). The resulting input signal is shown in Figure 4-5A. The fitting was again performed manually. This time, the parameters for Kss1 and Fus3 were fixed to the same values determined in the previous experiment and only the input signal’s profile and the maximum Fus3 phosphorylated fraction were adjusted. The optimal value for this last parameter was very close to the one used to fit the wild-type data (~0.5). Considering these constrains, the quality of the fit is even more remarkable. The shape of the input signals resemble the profiles we would expect from the encoding mechanisms analyzed before for the case of a large $k_{1M}$ (e.g. with a relatively graded dose response curve, compare with Figure 2-27), although the resolution of the data makes the assignment of regions ambiguous. A difference observed in this case is that the signals do not seem to adapt all the way back to basal levels. A small residual activity is necessary to account for the non-zero phosphorylation of the MAP kinases at a later stage. It is important to notice that this could also be the case with the wild-type experiments, but they may not last long enough to see the effect clearly.

The above experiments show that Bar1 is a good encoding candidate for the dose-duration transformation observed in the wild type yeast strain. Slow pheromone degradation can produce a square like pulse downstream in the cascade in the presence of an intermediate stage with a sharp dose-response curve. Such a mechanism was discussed in section 2.4.2 in the framework of encoders based on receptor affinity regulation.
(Figure 2-28). The same approach can be used to rectify any slow decaying signal, regardless of the cause of decay. Even in the absence of this intermediate stage, it is possible for high concentrations of pheromone to saturate the protease capping the degradation rate and producing a signal resembling the predicted input in Figure 4-3. The results of a model making use of this mechanism are presented in section 4.3.5.

From the experiments we cannot tell whether the Bar1 effect is dependent on the upregulation of the \textit{BAR1} gene as part of the response. Protease Bar1 is released constitutively and it is quite possible that the amount of it already in the media before induction with pheromone is enough to cause the effect. This could be experimentally investigated by thoroughly washing and re-suspending the cells in fresh media before adding pheromone. Finally, this model makes a key prediction: the dose-duration encoding is dependent on the cell density, for more cells in the same volume would result in higher protease levels. This effect should be clearer when regulation occurs mainly because of the protease being constitutively released. Absence of a density-dependence may indicate that the concentration of extra-cellular Bar1 may be subject to a homeostatic control mechanism, that is, cells are able to sense and maintain a constant concentration of extracellular Bar1.

4.3.3 Modulation of the receptor affinity

Even though the case for Bar1-driven pheromone degradation is compelling, the possibility that the change in the Fus3 and Kss1 phosphorylation profiles is caused by an unexpected effect of the \textit{BAR1} gene deletion, rather than pheromone degradation, deserve to be considered. This is more so when we consider the physiological context in which mating takes place. In a natural setting, a pathway that is regulated by Bar1 would make
mating effectively a community decision because all the cells in the colony produce the protease. Furthermore, since pheromone is continuously re-supplied by cells of the opposite type, it is hard to foresee how the observed encoding could be achieved in a reliable way.

These considerations prompted us to consider an alternative mechanism based in the feedback regulation of the receptor affinity similar to the one discussed in section 2.4.2 (see Figure 2-27 and Figure 2-28). This mechanism can account for the displacement in the EC$_{50}$ to the micro-molar range and at the same time produce a duration encoded signal that can be rectified to generate the proposed input signal profile for Fus3 and Kss1. The proposed model assumes that the receptor is chemically modified in a signal-dependent fashion (e.g. phosphorylated) to a form with a lower affinity for pheromone. For simplicity we model the pheromone release and the reaction reversing the receptor modification as a single process, although it can be modeled in the necessary number of steps without affecting the results. The model is described by equations (4.8)-(4.13).

\[
\frac{d[RL]}{dt} = k_1[L](R_{TOTAL} - [RL] - [RL^*]) - k_2[RL] - \frac{k_0[X^*][RL]}{k_0 + [RL]} \tag{4.8}
\]

\[
\frac{d[RL^*]}{dt} = \frac{k_0[X^*][RL]}{k_0 + [RL]} - k_3[RL^*] \tag{4.9}
\]

\[
\frac{d[X^*]}{dt} = k_4([RL] + [RL^*])(X_{TOTAL} - [X^*]) - \frac{k_4[X^*]}{k_4 + [X^*]} \tag{4.10}
\]

\[
\frac{d[MK^*]}{dt} = k_5([RL] + [RL^*])(MK_{TOTAL} - [MK^*]) - \frac{k_5[MK^*]}{k_5 + [MK^*]} \tag{4.11}
\]
\[
\frac{d[ppFus3]}{dt} = \frac{k_{10} \cdot [MK^*] \cdot (Fus3_{TOTAL} - [ppFus3])}{k_{10}M + (Fus3_{TOTAL} - [ppFus3])] - \frac{k_{20}[ppFus3]}{k_{20}M + [ppFus3]}
\]

\[
\frac{d[ppKss1]}{dt} = \frac{k_{30} \cdot [MK^*] \cdot (Kss1_{TOTAL} - [ppKss1])}{k_{30}M + (Kss1_{TOTAL} - [ppKss1])] - \frac{k_{40}[ppKss1]}{k_{40}M + [ppKss1]}
\]

(4.12)

(4.13)

, where \( R_{TOTAL} = [R] + [RL] + [RL^*] \), \( Fus3_{TOTAL} = [ppFus3] + [Fus3] \), \( Kss1_{TOTAL} = [ppKss1] + [Kss1] \), and \( MK_{TOTAL} = [MK^*] + [MK] \).

Figure 4-6 shows a diagram of the model and the dynamics expected for each component. In order to focus on the encoding mechanism, the model does not include the second Kss1 pool responsible for the second wave of phosphorylation. The good agreement between the model and the experimental observation demonstrates that this mechanism can produce the dose-duration transformation and explain the Fus3 and Kss1 phosphorylation dynamics.

**Figure 4-6 Receptor affinity modulation.** A) Upon ligand binding, the pheromone receptor activates species X, which phosphorylates the receptor causing a conformational change that accelerates pheromone release. A cascade consisting on species MK, Fus3 and Kss1 is activated. B) Dynamics corresponding to the occupied receptor [RL] (top-right), active MK (top-left), Fus3 (bottom-right), and Kss1 (bottom-left). Curves are normalized respect to each species maximum concentration value, except for Kss1 in which the secondary peak has been ignored. Experimental data is shown for comparison.
A key question is what is the identity of species X? That is, what interaction is capable of modulating the receptor affinity for pheromone? The top candidate is the $\alpha$ subunit of the G protein (Gpa1). It has been shown in vitro that the dissociation rate of pheromone from the receptor changes in a GTP dependent manner (140). This effect has also been observed in mammalian receptors (141). This allows us to speculate that dissociation of the G-protein from the receptor may lead to a conformational change weakening the bonds with the pheromone molecule. The caveat is that this effect has been observed at high PH values (PH=8) and therefore the physiological relevance of this finding is still unclear. The existence of organelles with differing PH levels inside the cells raises the interesting possibility that regulation is achieved through interplay of receptor trafficking and affinity modulation. If this were the main encoding mechanism, then the experiments with the $BAR1$ deletion strain would indicate that Bar1 might be playing more than a simple proteolytic role. In a pure affinity based scenario we expect signal duration to be independent of cell density. However, Bar1’s effect on pheromone is clearly established and therefore we estimate that a combination of the two proposed mechanism is the most likely scenario. In this case, it is reasonable to expect cell density or pre-washing to have some effect, especially at low pheromone dose. In any case, experiments at different does and with fresh media will provide valuables clues regarding the relative importance of pheromone degradation and affinity modulation in the encoding process.
4.3.4 Intrinsic dose-duration encoding

4.3.4.1 A Bar1 independent encoder

Close examination of the MAP kinase phosphorylation profiles and the shape of the input signals in Figure 4-5 reveals that higher pheromone doses produce longer lasting signals, and therefore, quantitative information is still duration-encoded in the absence of Bar1. This means that there must be also a second, Bar1-independent mechanism generating a dose-duration transformation in the protease-deleted strain. At the moment of this writing, there is no evidence for the presence of another proteases capable of degrading pheromone. Furthermore, Jenness and Spatrick report no loss of pheromone activity under conditions similar to the ones used for our experiments (≈10^7 cells/ml) during an incubation period of 3 hours (142). These authors report a slow decay (t_{1/2}~80 minutes) at ten times the cell density used in our experiments and they attribute this effect to internalization of pheromone bound to the receptor (discussed in the next section). This information is important because it allows us to rule out the effect of a less potent protease that could potentially account for the Bar1-independent encoding observed in the deletion strand. Therefore, it is important to analyze what intrinsic mechanisms, that is, not dependent on pheromone degradation, could mediate this additional encoding.

4.3.4.2 Receptor internalization

Both, $\alpha$ and $\alpha$ receptors are constitutively internalized. During vegetative growth, constant production balances internalization and a constant number of approximately
10,000 receptors is maintained on the cellular surface (56, 137). The dynamics of the internalization process has been only partially characterized mainly because of the efforts of Duane Jenness, using radiolabeled pheromone in the 80’s. Thanks to these experiments we know that upon pheromone exposure, receptors disappear from the membrane on time scales of tens of minutes. This effect has been attributed to increased internalization of both, occupied and unoccupied receptors (142). After a period of time, receptors reappear on the cell membrane due to upregulation of the Ste2 (or Ste3) gene as part of the mating response. This typically occurs between 90 and 120 minutes after induction (142, 143). Receptor internalization can modulate the pheromone response pathway activity in two ways. The first one is through the elimination of pheromone from the media as the receptors drag it with them during internalization, in a variation of the protease-driven degradation theme observed in the wild type strain. The other one is through the reduction in the absolute number of active receptors as the signaling event progresses.

The first mechanism is easier to analyze in terms of existing data. Jenness and Spatrick used radio-labeled pheromone to determine the number of receptors on the cell membrane at different times after exposure to pheromone (142). In the presence of Cycloheximide (a chemical that rapidly blocks the synthesis of proteins), their data allows us to estimate a constitutive internalization rate of approximately 30 receptors/min\(^9\) (the uncertainty in this value cannot be determined from the data as published). This rate remained constant for 100 minutes. This value is consistent with the half-life value of 232 minutes obtained in Henrik Dohlman’s lab. In the presence of pheromone, the internalization rate can be estimated by fitting Jenness and Spatrick data

\(^9\) An initial load of 10,000 receptors was assumed to calibrate the cpm (counts per minute) data provided by the authors.
with an exponential decay. The half-life value obtained this way is 16 minutes. These observations allow us to write the following very simplistic model for receptor internalization:

\[
\frac{dR}{dt} = k_1 - k_2 \cdot R
\]  

(4.14)

The half-life of the receptor in the absence of pheromone (232 minutes) can be combined with the number of receptors at steady state \(R_{SS} = k_1/k_2\) to obtain values for \(k_1\) (\(k_1 = 43\) rec/min) and \(k_2\) (\(k_{2n} = 4.3 \times 10^{-3}\) 1/min). In the presence of pheromone, the half-life drops and \(k_2\) increases \((k_{2p} = 6.25 \times 10^{-2}\) 1/min). Assuming \(k_1\) does not change during the initial phase of the signaling event \((t < 1h)\), this equation can be solved to obtain the number of receptors on the cell surface at time \(t\):

\[
R(t) = \frac{k_1}{k_{2p}} \left[ \left( \frac{k_{2p}}{k_{2n}} - 1 \right) e^{-k_{2p} \cdot t} + 1 \right]
\]  

(4.15)

This equation allows us to calculate the number of receptors that are internalized as a function of time by integrating the second term in (4.14) \(k_{2p} \times R(t)\):

\[
R(t)_{\text{INTER}} = k_1 \cdot \left[ t + \left( \frac{k_{2p} - k_{2n}}{k_{2p} \cdot k_{2n}} \right) \cdot (1 - e^{-k_{2p} \cdot t}) \right]
\]  

(4.16)

Knowing the number of cells in the culture, and assuming 10,000 receptors per cell, this equation can be used to estimate how fast pheromone is internalized (and presumably destroyed) in our experiments. These experiments were performed at an optical density (600 nm) of 1.0, which it is estimated to represent a concentration of
2x10^7 cells/ml (144). Figure 4-7A shows the dynamics of receptor internalization obtained from (4.15), and the number of internalized receptors from equation (4.16). Figure 4-7B shows the estimated effect of the internalization in the concentrations of pheromone used in the BAR1 deletion experiments. These curves were calculated by assuming that each internalized receptor takes a pheromone molecule with it. However, at these concentrations the receptors are partially occupied, and when this is taken into consideration (assuming a conservative dissociation constant of 4nM) we obtain the curves depicted in Figure 4-7C.

**Figure 4-7 Effect of receptor internalization.** A) Number of receptors on the cell surface (black) and number of receptors internalized in response to pheromone (gray). B) Effect on pheromone concentration if each internalized receptor takes a molecule of pheromone. C) Effect on the pheromone concentration if only the fraction of occupied receptors at each concentration carries a pheromone molecule. Cell density is 2x10^7 cells/ml.

As the figures clearly demonstrate, receptor internalization only affects the lowest concentrations of pheromone. This effect becomes negligible when the occupancy fraction is taken into account. The reason why this approach fails to produce a significant decay in the pheromone levels is because the initial number of receptors is too low compared with the number of pheromone molecules. Further internalization is limited by the rate at which receptor is replenished and because of this, increasing the rate of pheromone-induced internalization has little effect. However, as mentioned before, the rate of receptor production increases during the response so it is important to consider a
more realistic model in which $k_1$ changes with time. To analyze the impact of an increasing production rate we numerically solved equation (4.14) for the arbitrary $k_1$ profile shown in Figure 4-8A. In the absence of detailed experimental information, this profile corresponds to a best-case scenario in which the rate of receptor production increases more than 10-fold during a period starting 20 minutes after pheromone exposure (reasonable time for gene upregulation to occur) and ending an hour into the response. Figure 4-8B shows the number of receptors on the cell surface recovering after an hour of exposure, consistent with experimental observations. Figure 4-8C and D illustrate the effect on the concentration of pheromone when the occupancy fraction is ignored or taken into account respectively. From the figure, we can see that if we assume each internalized receptor takes a pheromone molecule with it, then there is a significant increase in the rate at which pheromone is internalized when compared with the case with fixed production rate. However, when the occupancy fraction at the different concentrations is taken into account, the effect again becomes negligible in the time-scale relevant for signaling.

When the occupancy fraction is taken into account, changes in pheromone concentration are too small to account for the observed MAP kinase phosphorylation response. The internalization and production rates must be significantly higher than the experimentally derived ones to achieve a sizable effect. An interesting observation is that the number of cells in the culture has a significant effect on the pheromone internalization rate. The effect is illustrated in Figure 4-9 in which a cell density of $5 \times 10^7$ (OD$_{600}=1.57$) was used. This high cellular density is well outside the normal inter-experiment...
variability, however, this example shows that variations in cell density have measurable experimental effects that could be used to test the models.

Figure 4-8 Effect of receptor upregulation. A) Temporal profile of the kinetic constant describing receptor production (see text). B) Number of receptors on the surface (black) and number of receptors internalized (gray) after pheromone induction. C) Effect on pheromone concentration if each internalized receptor takes a molecule of pheromone. D) Effect on the pheromone concentration if only the fraction of occupied receptors at each concentration carries a pheromone molecule. Cell density is 2x10^7 cells/ml.

Figure 4-9 Effect of cell density. Effect of receptor internalization on pheromone concentration for the cases of Figure 4-8 when the cell density is 5x10^7 cells/ml (OD600~1.57).
Taken together, the results discussed above make pheromone internalization an unlikely candidate for the dose-duration encoding mechanism in the absence of protease Bar1. This conclusion depends heavily on the currently available parameter estimates. We should point out however, that a change in one order of magnitude in the parameters could render this into a viable scenario. Also, receptors are not internalized as single units, but aggregate near actin patches and internalized in endocytic vesicles (145), which could potentially result in increased pheromone uptake. Because of these caveats, at this point we cannot definitively rule out this mechanism until the experimental data on the cell-density dependence becomes available.

Receptor depletion from the plasma membrane can also regulate signaling because of the reduction in the absolute number of active receptors. In other words, when exposed to the same pheromone concentration, pathway activity will be more intense when there are more receptors to activate it. In this scenario, signaling activity rapidly increases in response to pheromone driven by the occupied receptors. As the receptors get internalized, this driving force slows down and pathway activity decays. For this effect to account for the Bar1-independent dose-duration encoding, the internalization of the receptors has to take longer for higher pheromone doses and occur rapidly when the dose is low. This requirement seems inconsistent with the role of pheromone as a promoter for internalization. However, there are a number of possibilities that could lead to slower internalization at high doses that are worth considering. For example, preferential internalization of unoccupied receptors, signal-mediated negative regulation of the internalization process, signal-driven receptor recycling, all can delay internalization in a dose dependent manner. At the writing of this thesis there is no evidence of preferential
internalization of either the occupied or unoccupied receptor. Experimental data show that both species are internalized with similar rates (142). It is worth stressing however, that there has not been independent verification of these data to our knowledge. Equally, there is no evidence of signaling slowing down the internalization process. In fact, Jenness and Spatrick show data supporting that the initial internalization rate increases with pheromone concentration. Furthermore, there is evidence that pheromone induced internalization of the α factor receptor does not require signaling through the Gβγ branch of the pathway (146). This situation contrasts with the case for the a factor receptor Ste3 (67). Also, even though signal-dependent recycling has been demonstrated for the a factor receptor (147) no evidence supporting recycling has been published for the α factor receptor Ste2. Again, it is important to stress that the only experiment measuring the dose dependence of the internalization process was the one using radiolabeled pheromone published by Jenness and Spatrick. To the best of our knowledge, no independent confirmation has been published. In summary, the current data does not support any of these mechanisms. However, given the lack of independent validation, we cannot rule out the possibility that new, more precise observations may change this conclusion.

Finally, receptor endocytosis poses a number of interesting questions. Chief among them is how can internalization be independent of the presence of a bound ligand and at the same time be independent of signaling through the G-protein branch? In other words, what prompts the unoccupied receptor internalization? One answer can be that receptors are clumped together and unoccupied receptors are internalized in a collateral manner. An alternative explanation is that there is a so far unrecognized alternative
signaling pathway activated during the pheromone response. Interestingly, the increased receptor internalization observed in response to pheromone is phosphorylation-dependent (146). The standard interpretation is that either the detachment of the active G-protein or a structural change in the occupied receptor allows hyper-phosphorylation to happen. Unfortunately, no experimental data supporting these explanations have been published and therefore the nature of the biochemical processes driving internalization remains unclear. More advanced techniques are available nowadays and an experimental revision of the internalization dynamics as a function of pheromone concentration would provide more conclusive evidence to rule out (or rule in) receptor internalization as the Bar1-independent encoding mechanism.

4.3.4.3 Regulated receptor affinity

The proposed input curves responsible for generating the MAPK activity profile (Figure 4-5) resemble the dynamics produced by an encoder based on the regulation of receptor affinity via a negative feedback such the one considered in section 2.4.2 (Figure 2-27). To test how well such a mechanism can reproduce the data, we built a simple model based on an hypothetical protein X, that when activated attaches to the receptor and reduces its affinity for pheromone. This model is similar the one considered in section 4.3.3, except that in the present case there is no need for the intermediate species MK to rectify the input profile and therefore, for simplicity, this component has been eliminated. Figure 4-10C shows a fit of the model described by equations (4.17)-(4.21) to the BAR1 deletion strain data.
\[
\frac{d[RL]}{dt} = k_1 \cdot (R_{TOT} - [RL] - [RL^*]) \cdot [L] - k_2 \cdot [RL] - \frac{k_0 \cdot [X^*] \cdot [RL]}{k_{0,M} + [RL]} \quad (4.17)
\]

\[
\frac{d[RL^*]}{dt} = -k_3 \cdot [RL^*] + \frac{k_0 \cdot [X^*] \cdot [RL]}{k_{0,M} + [RL]} \quad (4.18)
\]

Figure 4-10 Receptor affinity modulation in a BAR1 deleted strain. A) Upon ligand binding, the pheromone receptor activates species X, which phosphorylates the receptor causing a conformational change that accelerates pheromone release. For simplicity, the active receptor is used to activate Fus3 and Kss1. B) Response curves for the receptor without (right), and with full X activation (left). C) Dynamics corresponding to the occupied receptor [RL] (right), Fus3 (center), and Kss1 (left). Curves are normalized respect to each species maximum concentration level. The error bars reflect the variability observed in three experiments.

\[
\frac{d[X^*]}{dt} = \frac{k_4 \cdot ([RL] + [RL^*]) \cdot (X_{TOTAL} - [X^*])}{k_{4,M} + (X_{TOTAL} - [X^*])} - \frac{k_5 [X^*]}{k_{5,M} + [X^*]} \quad (4.19)
\]

\[
\frac{d[Fus3^*]}{dt} = \frac{k_6 \cdot ([RL] + [RL^*]) \cdot (1 - [Fus3^*])}{k_{6,M} + (1 - [Fus3^*])} - \frac{k_7 \cdot [Fus3^*]}{k_{7,M} + [Fus3^*]} \quad (4.20)
\]

\[
\frac{d[Kss1^*]}{dt} = \frac{k_8 \cdot ([RL] + [RL^*]) \cdot (1 - [Kss1^*])}{k_{8,M} + (1 - [Kss1^*])} - \frac{k_9 \cdot [Kss1^*]}{k_{9,M} + [Kss1^*]} \quad (4.21)
\]
Figure 4-10B depicts the effect of the negative regulator X on the receptor affinity. The curve on the left is the occupancy curve in the absence of X activity, whereas the curve to the right corresponds to $[X^*]=1$. From the figures, it is evident that the model captures the qualitative behavior of the system but fails to reproduce the data adequately. The cause of the poor correspondence is that, as written, the model allows minimal flexibility to set the slope for the receptor response curve. The shape of this curve plays an important role determining the relative amplitude of the responses to different pheromone doses and the lack of flexibility significantly hurts the fit. Notice that in order to reproduce the experimental data, the initial receptor dissociation constant has been set to $\sim$1.5 nM, which is low compared to the accepted experimental values (4-15 nM). However, the fact that the model captures the qualitative behavior (longer signals at higher doses) is not a small feat and the reason of the suboptimal fit could be related to its simplicity. In fact, the low Kd value points to the need for a downstream stage capable of amplifying the signal. Such a stage would add flexibility and allow the pathway to further modulate the signal before reaching the kinases Fus3 and Kss1. Many candidates can do this, including the G-protein subunits, the kinases Ste11 or Ste7, and the action of the protein Ste5. Any or all of these components can provide extra layers of modulation that could lead to the precise Fus3 and Kss1 dynamics observed in the experiments.

As mentioned in section 4.3.3, there are several candidates for species X. The most attractive is the G protein because existing data demonstrate that its state affects the receptor affinity in-vitro (140). As discussed before, receptor phosphorylation or interaction with other species (most notably Sst2) could also play a role. Until more is
know about the kinetics of these species, little else can be said about the likelihood of this encoding mechanism other that it has the potential, at least in qualitative terms, to account for dose-duration encoding.

4.3.4.4 Internal encoding

The mechanisms discussed above work at the head of the pathway. This was necessary to explain the wild-type response because of the observed dextral shift in the EC$_{50}$. The $BAR1$ deleted strain does not display this behavior. On the contrary, the dose-dependent response is limited to within the dynamic range of the receptor. Experiments determining FUS1 gene induction in the absence of Bar1, show a dose-response that in most cases\(^\text{10}\) overlaps with the receptor occupancy curve (56). There is no need in this case to restrict the encoding mechanism to the cell surface, and therefore a few other possibilities must be explored. To do this, we built a model postulating a hypothetical adaptive system similar to Model I (2.3.3.2) operating upstream of Fus3 and Kss1. Figure 4-11 shows a diagram of the model and the fit to the $BAR1$ deletion strain MAP kinase phosphorylation data. The model is described by equations (4.22)-(4.26).

\[
\frac{d[R_L]}{dt} = k_1 (R_{TOT} - [RL]) \cdot [L] - k_2 \cdot [RL] \\
\frac{d[M^*]}{dt} = \frac{k_{10} \cdot [RL] \cdot (M_{TOTAL} - [M^*])}{k_{10M} + (M_{TOTAL} - [M^*])} - \frac{k_{11} \cdot [M^*]}{k_{11M} + [M^*]} - \frac{k_{12} \cdot [P^*] \cdot [M^*]}{k_{12M} + [M^*]} \\
\frac{d[P^*]}{dt} = \frac{k_4 \cdot [M^*] \cdot (P_{TOTAL} - [P^*])}{k_{4M} + (P_{TOTAL} - [P^*])} - \frac{k_5 \cdot [P^*]}{k_{5M} + [P^*]} \tag{4.24}
\]

\(^{10}\) Some authors report a dextral shift in the EC$_{50}$ even in the $BAR1$ deleted strain (151)
\[
\frac{d[Fus3^*]}{dt} = \frac{k_6 \cdot [M^*] \cdot (1 - [Fus3^*])}{k_{6M} + (1 - [Fus3^*])} - \frac{k_7 \cdot [Fus3^*]}{k_{7M} + [Fus3^*]} \\
\frac{d[Kss1^*]}{dt} = \frac{k_8 \cdot [M^*] \cdot (1 - [Kss1^*])}{k_{8M} + (1 - [Kss1^*])} - \frac{k_9 \cdot [Kss1^*]}{k_{9M} + [Kss1^*]}
\]

(4.25) (4.26)

**Figure 4-11 Internal feedback.** A) Active receptors activate species M, which together with P is part of a negative feedback based adaptive system. M Activates kinases Fus3 and Kss1. B) Temporal profiles of active M, Fus3, and Kss1. Data obtained from a *BAR1* deleted strain is shown for comparison.

As the figure clearly demonstrates, a deactivation-based feedback mechanism can reproduce the experimental observations remarkably well. Based on the findings of Chapter 2, we expect a feed-forward deactivation-based mechanism to work as well. Again, the fraction of Fus3 and Kss1 that is being phosphorylated relative to the total amount in the cell is unknown. For this fit, we found that a maximum phosphorylated fraction of 0.5 or less for Fus3 produced good results. For Kss1 we assumed that all the
available protein is phosphorylated at 10nM. The natural question now is what is the precise nature of the adaptive system. More specifically, what is the identity of species M and P.

There are several negative regulatory mechanisms described for the yeast pheromone response pathway. Perhaps, the best characterized one is the regulation of the G-protein activity by the RGS (Regulator of G Protein Signaling) protein Sst2. This protein catalyzes the hydrolysis of GTP bound to Gpa1 (the G-alpha subunit), accelerating its conversion to GDP at least a hundred fold (148, 149). The GDP-bound form of Gpa1 is then free to bind to the other subunits and presumably terminate pathway activity. Sst2 is constitutively present but also the SST2 gene is upregulated as part of the response (45, 150).

In order for Sst2 to act as the negative regulator P, its activity has to be regulated in a signal-dependent way. Gene upregulation is a slow process and it cannot explain the quick signal decay at low pheromone doses. Although Sst2 can be phosphorylated, the enzymatic activity is not affected by this modification (151). There is some evidence however, that signal-dependent phosphorylation may stabilize the protein against degradation (45). This effect could provide a suitable regulated mechanism thanks to which Sst2 concentration increases rapidly, not because of stimulated production but because of reduced turnover. A different possibility is that Sst2 activity is regulated by the spatial relocation known to occur upon pheromone induction. Recent experiments demonstrated that Sst2 binds to the C terminal of Ste2 (the pheromone receptor) through a DEP domain (152). These experiments show that Sst2 activity is severely impaired in mutants that cannot bind the receptor and that phosphorylation of the receptor C-terminal
portion prevents binding. Taken together, these observations provide an elegant mechanism of Sst2 activity regulation: Sst2 is constitutively present, but it does not become active until pheromone binds to the receptor. To prevent constitutive activity we need to assume that either binding occurs only to occupied receptors, or that in the absence of pheromone, binding is prevented by the presence of the G-protein bound to the receptor (153). In this scenario, the regulatory role of receptor phosphorylation as well as its internalization remains unclear and must be addressed experimentally.

Deletion of Sst2 causes otherwise wild type cells to become hypersensitive to pheromone and brings the EC$_{50}$ in line with the receptor occupancy curve. This indicates that there must be significant amplification going on downstream of the receptor, since lower pheromone doses are able to elicit a full response. Also, the pheromone response pathway is constitutively active in cells carrying this mutation. However, these cells are large and resemble the characteristic mating phenotype. Because of this, some researchers propose that the observed basal activity is due to pheromone released by the small number of cells that changed mating type (142) rather than faulty regulation due to the lack of Sst2. In any case, Sst2 is playing an important negative role, as evidenced by the increased sensitivity of the deletion strains. Interaction with the receptor and phosphorylation provide two mechanisms capable of regulating Sst2 activity in a signal-dependent manner. Based on these observations, a scenario in which the G-protein plays the role of M and Sst2 plays P is indeed plausible and can be tested by observing the G protein association and dissociation dynamics.

There are at least six other negative regulatory mechanisms known to operate in this pathway: i) induced degradation of Ste7 (MAPKK) (154, 155), ii) degradation of
Ste11 (MAPKKK) (92), iii) feedback hyper-phosphorylation of Ste5 (156), iv) hyper-phosphorylation of Ste7\textsuperscript{11}, v) induction of phosphatase Msg5 (157-159), and vi) spatial reorganization of the MAP kinases. Very little is known about the dynamics and strength of any of these processes and therefore little can be said about the plausibility of any of them playing a role in the dose-duration transformation occurring in the absence of Bar1. However, some predictions can be made regarding what is needed to happen for these processes to produce the observed encoding.

The discussion in section 2.3.3.5 suggests that if induced protein degradation (e.g. Ste11 or Ste7) were to produce a duration encoded signal, then the process has to proceed in a heterologous manner. That is, the degradation machinery has to preferentially target the inactive protein because otherwise, degradation would halt the system faster as the pheromone dose increases. Successful duration encoding necessitates a degradation process that happens faster at lower doses but slows down at higher doses. Therefore, if experiments demonstrate that degradation of Ste11 or Ste7 is responsible for the encoding, we can predict that either degradation is heterologous or that there has to be an associated process slowing down degradation at high pheromone doses. This could happen for example, through RNA stabilization or gene induction.

The protein Ste5 is a complicated entity because it has a number of functions, not all of them understood. On the one hand, after being recruited to the plasma membrane, presumably by free G\(\beta\gamma\) subunit, it functions as a scaffold bringing the components of the MAP kinase cascade together (160-162). This re-localization to the membrane is part of a more complex behavior that includes shuttling in and out the nucleus, although the reason

\textsuperscript{11} Yildirim et al. Submitted for publication.
for this shuttling is still unclear (163). On the other hand, Ste5 is subject to regulation by phosphorylation (156, 162) by kinase Fus3. It has been recently shown that hyper-phosphorylation of Ste5 has a negative effect on Fus3 phosphorylation, thereby providing a negative feedback loop that could be used as part of a duration encoder. Kinase Kss1 phosphorylation, on the other hand, does not require Ste5. Another recent investigation shows that a mutation that inhibits Fus3 docking to Ste5 (Ste5\textsuperscript{ND}) has a significant effect on Fus3 dynamics\textsuperscript{12}, dramatically accelerating its phosphorylation in response to pheromone stimulation. Taken together, these experiments show that Ste5 functions both as a positive (Fus3 phosphorylation does not occur in its absence) and a negative regulator (Fus3 phosphorylation is slowed down). These observations suggest that Ste5 could be playing the role of species M with Fus3 acting as negative regulator as well as messenger molecule. This resembles Model II discussed in detail in section 2.3.3.3. Our findings demonstrated that this kind of architecture produces poor adaptation unless an intermediary compound separates the positive and negative regulation time-scales or delay the effect of the feedback. Therefore, if feedback regulation of Ste5 by Fus3 is responsible for the encoding we expect the existence of an intermediary.

The case in which regulation occurs through hyper-phosphorylation of Ste7 is closely related to the case of regulation by feedback phosphorylation of Ste5. It has very recently been shown that Fus3 hyper-phosphorylates Ste7, rendering it incompetent to phosphorylate Kss1\textsuperscript{13}. No effect has been reported on Fus3, so this mechanism for Kss1 regulation would require an additional module to control Fus3. Because our findings suggest that both Fus3 and Kss1 respond to a common upstream signal, this scenario is

\textsuperscript{12} Nan Hao, personal communication.
\textsuperscript{13} Hao, Yildirim, Dohlman, Elston, submitted for publication.
unlikely. It is possible for regulation of Ste7 by Fus3 to partially affect the dynamics of Kss1, but it is not likely to be the main driver.

The gene encoding phosphatase Msg5 is induced in response to pheromone. This phosphatase is thought to be a mayor regulator of MAP kinase activity (31, 157-159). Msg5 operates in conjunction with phosphatases Ptp2 and Ptp3. Production of a phosphatase can produce a model such as the one described above, with Msg5 playing the role of P. The caveat with this scenario is that protein production takes too long (typically on the order of an hour) to explain the quick decay observed at low doses. If upregulation of the MSG5 gene were to cause the encoding, then it must happen on a faster time scale. It is worth mentioning that Msg5 (and the other proteases) do regulate Fus3 and Kss1 phosphorylation in the sense that it is their enzymatic activity what de-phosphorylates the kinases after the upstream activity subsides.

Finally, there is a last scenario that is worth mentioning. After pheromone induction, a complex ballet takes place and all the proteins discussed above are redistributed between the cell membrane, the cytoplasm, and the nucleus. Each different environment has its own biochemistry (for example, the phosphatase Ptp2 resides almost exclusively in the nucleus) and it is possible for the encoding to occur when a signaling species moves to a different location and get exposed to a local negative regulator as a result. This process would not require the activity of the negative regulator itself being regulated. The effects of the cell spatial extension on signal regulation is something that has only very recently begun to be appreciated and more research is needed before a meaningful prediction of its effect on quantitative signal encoding can be made.
This section discussed some mechanisms that could account for the dose-duration transformation observed in cells lacking the protease Bar1. In particular, all the mechanisms analyzed are endogenous and do not rely on the degradation or internalization of pheromone. A key experiment that can distinguish whether encoding in the absence of Bar1 is of endogenous origin is the measurement of the cell-density effect. If the pheromone internalization or degradation is the main encoding mechanism, variation in the cell density should produce measurable differences in the MAP kinases phosphorylation profiles. The negative result, that is if the cell density has no effect, would provide strong support for an endogenous encoder.

4.3.5 Putting it all together

In the previous section we discussed several possible scenarios that can explain the dose-duration encoding observed both in wild type and BAR1 deletion strains. The question now is can these mechanisms work together and explain both sets of experimental data simultaneously? The answer is in fact positive and as a proof of concept here we present a combined model that can account for the two sets of experiments. In this model (Figure 4-12), the encoding observed in the wild-type strain is caused by the slow degradation of pheromone discussed in section 4.3.2 whereas an adaptive endogenous encoder (Section 4.3.4.4) was used to reproduce the effect observed in the BAR1 deleted strain. The model is described by equations (4.27)-(4.35).
\[
\begin{align*}
\frac{d[L]}{dt} &= -\frac{k_{60} \cdot [Bar1] \cdot [L]}{k_{60,M} + [L]} \\
\frac{d[M^*]}{dt} &= \frac{k_{10} \cdot [RL] \cdot (M_{TOTAL} - [M^*])}{k_{10,M} + (M_{TOTAL} - [M^*])} - \frac{k_{11}[M^*]}{k_{11,M} + [M^*]} - \frac{k_{12} \cdot [P^*] \cdot [M^*]}{k_{12,M} + [M^*]} \\
\frac{d[P^*]}{dt} &= \frac{k_{70} \cdot [M^*] \cdot (P_{TOTAL} - [P^*])}{k_{70,M} + (P_{TOTAL} - [P^*])} - \frac{k_{71}[P^*]}{k_{71,M} + [P^*]} \\
\frac{d[Kss1^*]}{dt} &= \frac{k_{3} \cdot [RL] \cdot (K_{TOT} - [Kss1^*] - [DKss1] - [DKss1^*])}{k_{3,M} + (K_{TOT} - [Kss1^*] - [DKss1] - [DKss1^*])} \\
&- \frac{k_{4} \cdot [Kss1^*]}{k_{4,M} + [Kss1^*]} + k_{5} \cdot [DKss1^*] \\
\frac{d[DKss1]}{dt} &= k_{6} \cdot (K_{TOT} - [Kss1^*] - [DKss1] - [DKss1^*]) \cdot (D_{TOT} - [DKss1] - [DKss1^*]) \\
&- k_{7} \cdot [DKss1] - \frac{k_{8}[C^*][DKss1]}{k_{8,M} + [DKss1]} + \frac{k_{9} \cdot [DKss1^*]}{k_{9,M} + [DKss1^*]} \\
\frac{d[DKss1^*]}{dt} &= \frac{k_{k} \cdot [C^*][DKss1]}{k_{8,M} + [DKss1]} - \frac{k_{9} \cdot [DKss1^*]}{k_{9,M} + [DKss1^*]} - k_{13} \cdot [DKss1^*] \\
\frac{d[Fus3^*]}{dt} &= \frac{k_{20} \cdot [M^*] \cdot (1 - [Fus3^*])}{k_{20,M} + (1 - [Fus3^*])} - \frac{k_{30} \cdot [Fus3^*]}{k_{30,M} + [Fus3^*]} \\
\frac{d[C^*]}{dt} &= \frac{k_{40} \cdot [M^*] \cdot (C_{TOT} - [C^*])}{k_{40,M} + (C_{TOT} - [C^*])} - \frac{k_{50} \cdot [C^*]}{k_{50,M} + [C^*]} \\
\frac{d[RL]}{dt} &= k_{1} \cdot (R_{TOTAL} - [RL]) - k_{2} \cdot [RL]
\end{align*}
\]
The model includes the mechanisms already discussed with the only difference that intermediary species C, responsible for phosphorylating the Kss1-D complex is now activated by active M. The same equations and parameters were used to model the wild type cells and the \textit{BAR1} mutant, except that k60 (pheromone degradation) was set to zero in the last case. The model does not include upregulation of the \textit{BAR1} gene and therefore a fixed amount is assumed present at the beginning of the experiment\textsuperscript{14}.

The parameters for the data fitting were estimated using a Monte Carlo algorithm. As Figure 4-12 B and C show, the composite model reproduces the wild type and \textit{BAR1} deletion data well, although some deviations occur, especially at low doses. This is not surprising given that the model does not contain any amplification step or other regulatory elements that may serve to fine-tune the response. As with previous models, the fraction of phosphorylated vs. unphosphorylated kinase cannot be determined from the experiment and therefore it was adjusted in order to get a good fit. This model does not consider the effects of receptor internalization due the current lack of knowledge about the process. This omission can also be in part responsible for the deviations. It is important to stress that this is not the only possible model consistent with the experimental data. The experimental signatures discussed before can be used to validate the model, especially the effect of the cell density on the signals.

\textsuperscript{14} The model is run to steady state in the absence of pheromone and then a fixed amount is added.
Figure 4-12 A complete model for MAPK activity. A) Active receptors activate species M, which is part of an adaptive system together with P. M activates Fus3, Kss1, and a putative species C. Kss1 exists partially as a complex with species D. This complex is phosphorylated by active C and separates as a result, causing the second phosphorylation peak observed for Kss1. Pheromone is degraded by Bar1 constitutively present in the media. B) Simulated (solid lines) and observed (dots) dynamics of active M, Fus3, and Kss1 in a wild type strain. C) Simulated (solid lines) and observed (dots) dynamics of active M, Fus3, and Kss1 in a strain lacking the BAR1 gene. Concentrations in the BAR1 deletion strain are relative to the WT ones. Data provided by Dr. Nan Hao.
4.4 General remarks

4.4.1 Information encoding in the pheromone response pathway

In the pages above, evidence was presented supporting the use of duration encoding in the yeast pheromone response pathway. The models discussed were mostly based on known, albeit partially characterized, biochemical processes. The effect of removing the *BAR1* gene on the encoded signal suggests this protease plays a prominent role. As we demonstrated, the most obvious mechanism by which this can happen is the Bar1 mediated degradation of pheromone. However, a deletion mutant cannot by itself rule out the possibility that Bar1 is affecting signaling in some unexpected way and that the encoding is actually happening at the receptor level. This last scenario is compelling because it would mean that regulation occurs at the individual cell level. A Bar1-driven encoding, on the other hand, would mean that the pathway activity is regulated by the community. There is no reason however, why both mechanisms cannot coexist. For example we can envision a scenario in which signaling at low dose is regulated by pheromone degradation (at 0.1 and 0.3 nM curves in Figure 4-3) and a slower feedback drives adaptation at higher pheromone levels for which degradation would take too long. This hybrid scenario is attractive because it could be expanded to explain the dose-duration encoding observed in the *BAR1* deletion strain.

If the protease Bar1 turns out to be the main encoding mechanism, a hypothesis that can be tested by analyzing the effect of cell-density on the signals, then this would require some reconsideration of how the mating process occurs in natural settings. One possibility is that the release of Bar1 is regulated in order to maintain a low background
pheromone concentration. The pathway then would get activated only in response to an episodic release of pheromone by a close-by partner. Such a strategy would prevent cells from responding to pheromone cues originating from cells that are too far away for mating.

An open question is what is the role of receptor internalization? This process is still not well understood. Current literature indicates that the receptor internalization is a homologous process (both occupied and unoccupied receptors are equally internalized) (142) and in the case of the α-factor receptor Ste2, the process is independent of signaling through the G-protein (146). This contrasts with the case for the a-factor receptor (Ste3), which undergoes signal dependent internalization (67). Another puzzling difference is that internalized Ste3 is partially recycled (147), process that has not been observed for the α-factor receptor. Elucidating the dynamics of this process is of crucial importance to understand signaling in this pathway. Signal-dependent recycling could result in the time for receptors elimination being proportional to the pheromone dose (dose-dependent recycling is equivalent to a dose-dependent increase in production), producing yet another encoding mechanism candidate. The nature of this process has important implications for the signals generated in response to low doses of pheromone. As the simple calculations above show, pheromone internalized together with receptors could produce results akin to pheromone degradation by protease Bar1. With the current estimates for the parameter values, this is unlikely. However, an increase in the rate of receptor production and degradation of an order of magnitude would result in this process having a sizable effect, especially at low doses. It is interesting to point out that receptors are internalized in vesicles that form in the plasma membrane, and therefore it is possible
for the ratio of internalized pheromone to internalized receptors to be larger than unity. In this scenario, pheromone internalization could play a role in the duration encoding observed in the absence of protease Bar1. An interesting idea is that once internalized, the receptors keep signaling for a while, and as more receptors are produced and subsequently internalized the pool of active receptors grows larger than the initial count. This could potentially provide an alternative explanation for the second peak of Kss1 phosphorylation observed in the experiments\textsuperscript{15}

However, the experimental data currently available points to the existence of an endogenous encoder, for which there are several candidates. The G-protein cycle is the best characterized process among the ones discussed in the previous section and it has all the elements necessary to produce duration encoding. Among the other mechanisms, feedback regulation of Ste5 or other pathway components by Fus3 or Kss1 is the most compelling because there is experimental evidence demonstrating increased Kss1 signals in cells lacking Fus3 (79, 164). A possibility that cannot be ruled out is that Fus3 acts indirectly as the negative regulator of Kss1 on top of one of the processes described above. Clearly, more detailed experiments in strains containing mutant versions of the FUS3 gene are necessary to investigate this possibility.

One interesting observation is that because of its slow kinetics and almost linear rate of phosphorylation, Fus3 seems to be acting as an integrator; timing for how long the upstream signal has been on. This information is being decoded back to Fus3 phosphorylation level and hence its activity. Furthermore, the combination of fast and slow kinetics exhibited by the two MAP kinases has the potential to form a feed-forward

\textsuperscript{15} This idea was proposed by Meng Jin in the Elston Lab.
adaptive system. In fact, it has been demonstrated that pheromone induced degradation of the transcriptional activator Ste12 requires Fus3, but not Kss1 (165). This system is similar to the one depicted in Figure 2-25A and may play a critical role in ensuring proper timing of the transcriptional programs required for chemotropic growth and mating.

Even though the models presented here do not identify the specific actors playing the key regulatory roles in this pathway, they considerably narrow down the possibilities. By providing a rational explanation of how the pathway processes information, our analysis has shown the features that should be present in the regulatory schema. This information provides a useful framework for the design of the future experiments that will lead to the elucidation of the design principles behind the pheromone response pathway.

4.4.2 Dynamic control of specificity in yeast?

The yeast pheromone response pathway does not exist in isolation. As described previously, this pathway is part of a complex network than includes the hyper-osmotic shock response pathway and also the pathway responsible for the filamentous growth (also referred to as invasive growth) observed under certain starvation conditions (164). All three pathways share component Ste11, and the MAP kinase Kss1 in the case of the mating and filamentous growth pathway (Figure 3-1). It is intriguing that the terminal kinases in the three pathways have very different dynamics. More specifically, Hog1, the kinase activated by the osmotic shock pathway is quickly activated and reaches its peak activity level within minutes to then quickly decay to pre-stimulation levels (46-48). Kinases Kss1 and Fus3 activities, on the other hand, are also transient but as shown
above, operate on slower time scales, especially Fus3. Finally, sustained Kss1 activity seems to be necessary for invasive growth (79). These observations suggest that, as already proposed by Sabbagh et al. for the mating and filamentous growth responses, specificity in this network is maintained through the careful regulation of each pathway dynamics. We can speculate that the quick shut down of Hog1 activity in response to hyper-osmotic conditions may be in place to prevent the activation of the other kinases. Supports this the substantial cross talk observed in cells lacking Hog1 (166), which is consistent with the role played by Hog1 in the quick feedback deactivation of the pathway (47). The protein Ste50, a partner of Ste11, has also been shown to play an important role preventing cross-talk among these pathways (167, 168) and recent evidence indicates that this protein may be subject to regulation by Hog116. Additionally, FUS3 mutants have been identified that are impervious to Hog1-mediated repression and cause growth arrest (a component of the mating response) when exposed to osmotic stress (169). Interestingly, this network appear to contain a branch with an adaptive system (Hog1) that relays information in the form of short pulses, a branch with slow activation kinetics (Fus3) leading to mating, and a branch that depend on sustained signaling (Kss1) to produce filamentous growth. Such a network fits neatly the kinetic insulation paradigm discussed in Chapter 3 with the addition of a transient-to-sustained switch that segregates the mating and the filamentous growth responses.

16 Nan Hao. Personal communication.
Chapter 5

Significance and perspectives

5.1 Dynamics of intracellular signals

The preceding chapters explored important aspects of signaling networks, with dynamic regulation and its effects as the driving theme. Signals are not simple events that prompt multistable systems to change from one steady state to another. As the yeast pathways demonstrate, signals can be complex transient events that must balance dynamic range, reliability, and speed. The key finding is that a host of new effects, some very counterintuitive, become possible when signals are studied as the dynamic events they are. These dynamics provide cells with enormous flexibility to overcome limitations imposed by components with narrow dynamic range or maintain specificity even in the face of shared pathway components. Therefore, in order to understand intracellular signaling networks and signals we must look beyond the steady states and tackle the much more difficult problem of understanding the transient responses as well.

In Chapter 2 we studied the multiple aspects that make adaptive systems suitable for signaling. Unlike their counterparts engineered for control, a significant overshot is a necessary ingredient for these biological signal modulators. There are a number of
reasons why a cell would want to convert a sustained input into a transient signal. On the one hand, the sustained activation of a pathway consumes more energy than a transient activation. Also, sustained activity may result in the undesirable activation of other pathways, especially in the face of shared components, which by definition have to be promiscuous. As we shown in Chapter 3, by modulating the pathway activity, cells can control information flow in a very precise fashion. That chapter shows that pathway specificity may not come from built-in specificity in the interactions between the pathway proteins, but may come from their activation patterns instead. Therefore, when looking for the sources of specificity in given pathway, the search has to include the dynamics of the components.

We show that different adapting network architectures have different properties, advantages, and drawbacks. Some adapt better than others. Some recover faster than others. Some are capable of reacting to multiple challenges in a short period of time while others a less suited for this kind of application. These observations suggest new ways of experimentally investigate pathway architectures by characterizing their dynamics. They also could provide answers to questions such as why evolution settled on different network architectures for different systems. In fact, the enormous advantage associated with the use of dynamic signals suggests that the need to control and modulate these dynamics is probably a mayor evolutionary drive. These observations could also shed light on why pathways branch where they do. Consider the case of the model used to illustrate the kinetic insulation mechanism (Figure 3-5). In that example it makes sense for the pathway to branch at the level of kinase C only if the goal is to select which branch to activate based in profile of the upstream signal. Now, if for example, the
response to signal B requires the activation of some cellular system that necessitates a long, persistent signal, then it would make sense to introduce a branch at the level of protein BB. On the other hand, if this secondary system needs to be activated transiently, then branching at the level of protein B would be more adequate. The corollary is that much can be learned about how a network works by studying the location and dynamics of the branching points. Conversely, this could also explain the evolution of these interconnected signaling pathways. Systems that need to be activated transiently probably are connected at levels where the pathway activity is transient and systems that require a longer excitation period are probably connected to branches that show sustained activity. Based on the results discussed above, it is safe to speculate that for signaling pathways, function, architecture, and dynamics are all interconnected and it is unlikely that they can be understood in isolation.

From the discussion above is should be clear that signaling pathways not only transfer but also process information. Understanding a pathway is synonymous to understanding the path information follows from the receptors to the cellular targets responsible for eliciting the different biochemical processes that constitute the physiological response. This information is often quantitative and in Chapter 2, different information encoding strategies were analyzed. Special emphasis was put on duration encoding because of the evidence presented in Chapter 4 supporting this as the strategy of choice in the yeast pheromone response pathway. This pathway has been studied for more than 30 years and is considered by many as the prototypical MAPK cascade. Yet until now, it was not known how information about the pheromone level was being encoded. This work answers that question and provides useful guidance for researchers
working to identify the main regulatory mechanisms in the pathway. In Chapter 4 we show that pheromone concentration is encoded as duration, at least upstream of the MAP kinases Fus3 and Kss1. Protein Fus3 then acts an integrator, decoding this temporal information into concentration. This time, concentration of phosphorylated Fus3. We also showed a number of possible mechanisms that can perform the encoding, and our experimental results indicate that even thought protease-driven pheromone degradation plays an important role, there must be another, Bar1-independent, encoding mechanism at play. More importantly, we suggested a number of key experiments that narrow down the field of possibilities. Confirmation of the degradation of pheromone by the protease Bar1 as the main encoding mechanism would represent an important advance. Such an outcome would imply that the decision between mating and chemotropic growth is based on the state of the colony, effectively making the pheromone response pathway into a more complex mating-quorum sensing mechanism. Important questions remain to be answered in this system. Chief among them is the role of receptor internalization. This process cannot be ruled out as the encoding mechanism, and as discussed before, this would set some requirements that could be tested experimentally. In a pathway that relays on external components outside of its control (Bar1) to turn off the signal, receptor internalization could provide reassurance that the pathway will in fact be turned off. Internalization may set the higher limit for signal duration.

The pheromone pathway is a prototype for many signaling networks across species. It is interesting to speculate about the possibility that the encoding observed here could be a more general feature shared by other systems. The plausibility of this will depend on the specifics of the encoding mechanisms, but the possibility of duration-encoding being
a general modus operandi for some family of GPCR/G proteins is intriguing. Such a discovery would be a significant contribution towards the understanding of the basic design principles behind signaling pathways. A thorough understanding of this phenomenon could also immediately translate into new therapeutic approaches. Most drugs in the market today approach disease from a steady-state perspective, for example, by completely inhibiting a kinase or blocking a receptor. Undesirable side effects are a common consequence of this approach. The findings in this work suggest that when the dynamics of a pathway are important, it should be possible to restore it to proper function modulating the kinetics of the relevant reactions. Knowing the logic behind the pathway operation in quantitative terms could provide guidance for the design of therapies with minimal side effects.

Our findings also raise interesting issues about the properties of the components downstream of a duration-encoded pathway, in particular, how is the encoded information used. Pheromone concentration is encoded as duration somewhere upstream of Fus3 and Kss1. Kinase Kss1 preserves this encoding while kinase Fus3 decodes it back to activity level. It was suggested that this combination is ideally suited to produce an adaptive system downstream of these kinases. However, the question remains of how is this code is interpreted and acted upon by the pathway targets. One clue may come from the discoveries made in the cell cycle control system. The progression through the stages of the cell cycle is driven by the slow production of a regulatory protein (14), which as it crosses a threshold level, causes a bi-stable system to switch to a new steady state. In a way, Fus3 recapitulates the cell cycle-control system. The pathway is taking a stimulus that was added all at once (a square step), and producing a slow raising species
(phospo-Fus3). Evidence of bistability in the pheromone response has been recently presented (53), providing further support for this intriguing possibility.

In this work we unveiled the semantics of the MAPK stage of the pheromone pathway, a MAPK code. We analyzed this code from a theoretical standpoint and illustrated how cells could use it advantageously to transfer quantitative information. A thorough analysis of a variety of mechanisms provided us with a general picture of how this system may work and what networks architectures we should expect to find. As a result of this analysis we generated models and made testable predictions. Even though the precise nature of the encoder has not been determined, these predictions will serve as a guide for further research in the field. Knowledge of the code allows us to ask meaningful questions, and more importantly understand the answers.

5.2 Future directions

The findings of this work illustrate that studying pathway dynamics provides great insight into the organization and design rationale of intracellular signaling networks. This work answered a number of questions but also raised new ones, some very specific and others of a more general nature. The most obvious examples of the first kind are related to the yeast pheromone pathway. The evidence presented for *duration encoding* as a MAPK code provides a new framework for the study of this system in terms of which new questions can be asked and answers interpreted. The following are some important questions and how we think they could be addressed:

- *What is the precise nature of the encoding mechanism?*

  Experimental observations suggest that protease Bar1 may be the key
player, but other possibilities cannot be ruled out. As discussed in Chapter 4, there are a number of key experiments that may strengthen the case for Bar1 or rule it out. Of particular importance is the determination of MAPK phosphorylation dynamics at different cell densities and in fresh media. A result showing signals being independent of the cell density would support a scenario in which encoding is due to a mechanism other than Bar1-mediated pheromone degradation. Such a result would also indicate that Bar1 is affecting the pathway in a novel way, and would justify a search for potential interaction partners. Additionally, much could be learned from a careful quantitative characterization of Bar1 potency. Interestingly, this issue has been investigated only in relative terms, which makes it difficult to gauge Bar1 effect on the concentration of pheromone. We can expect that a more definitive answer to the question of what is generating the encoding will arise as biosensors or specific antibodies become available for elements upstream in the pathway. A promising approach is the use of fluorescence resonance energy transfer (FRET) between the subunits of the G protein to monitor the dynamics at the head of the pathway. This technique was already applied (56) to this system but not enough observations were made. Our findings suggest that refocusing these experiments to determine the dynamics of G protein activity in response to different doses of mating pheromone could provide important information about the role of the G protein cycle.

- **How is the MAPK code decoded by the pathway downstream effectors?**

  We showed that Fus3 acts as an integrator, converting signal duration into phosphorylation levels. On the other hand, Kss1 phosphorylation remains
duration-encoded. Investigating the dynamics of the targets downstream of the MAPK (Ste12, Tec1, Far1) should provide some initial clues. Some data on Ste12 is already available (165) and shows a transient response. Considering that slow Fus3 and fast Kss1 kinetics are ideally suited to form a feed-forward based adaptive system, it would be instructive to test how the dynamics is affected in mutants lacking one or the other MAPK. Ultimately, the question that needs to be answered is how gene expression is regulated by the pathway, and what is the role being played by duration encoding.

• **What role does receptor internalization play?**

  Receptors are internalized on a time-scale similar to the one for signaling. Therefore, it is important to understand what regulatory role (if any) this process plays. It has been suggested previously in the text that internalization may serve as a way to limit the duration of a signal under environmental conditions with a continuous supply of pheromone. This raises the question of how duration encoding operates in such a scenario. A number of mutant strains defective in internalization are already available, and investigating signal dynamics on them can provide the answers. However, these mutants must be characterized, especially respect of the effects of the mutations on the receptor/Sst2 interaction (152), before interpreting the observations. Related to this question are the puzzling differences between Ste2 (α-factor receptor) and Ste3 (a-factor receptor) in terms of internalization. Most of the experiments from where this knowledge comes from have not been independently reproduced or validated. It would be valuable to apply modern optical technology to characterize the internalization
and recycling of both proteins. In particular, we should seek to establish beyond doubt whether internalization is homologous and if its dynamics are dose-dependent. Another related question is what prompts unbound Ste2 internalization. If this process is signal-independent, as it is though to be, how do the unoccupied receptors know that something is going on?

- **What is the physiological relevance of the MAPK code?**

  When in their natural environment, cells are exposed to variable concentrations of pheromone, which they do not control. The concentration of pheromone is affected by environmental conditions as well as by the protease secreted by other cells in the colony. This raises the question of how is this code used under such conditions. This can be investigated by measuring MAPK or other pathway proteins activity in microfluidic chambers. However, for optimal results, this would require the use of biosensors not currently available. Gene expression reporters, on the other hand, are easier to build and many are already available. Understanding the process by which the genetic machinery reads the duration code could permit to infer signal activity based on gene expression reporters. This in turns would allow investigating the pathway in a more natural setting.

  These are just a few of the questions inspired by this work. An extensive list can be made with further questions such as: Why does the cell use duration encoding? Is yeast using Kinetic insulation? Is the combination of Bar1 and pheromone being used as a population control mechanism?

  The existence of the MAPK code described in Chapter 4 also raises a number of more
general questions. The possibility of dose-duration encoding being a widespread strategy can be investigated by characterizing the dynamics of other signaling pathways, especially those mediated by GPCR’s and MAPK cascades. It is interesting to speculate that, just like as similar signaling modules are found in a variety of systems, perhaps their dynamics are carried over between systems and are modular as well. Comparing evolutionary related pathways in terms of signaling dynamics and location of branching points would reveal how new pathways were built into existing networks and provide important clues about how signal specificity is maintained. Examples of preserved interactions abound, so it is possible that the signals dynamics and the associated encoding strategies might be preserved as well. Looking at changes in the dynamics as species diverge can provide significant insight about how a pathway operates, and allow us to peek into the design principles behind signaling networks in general.

The ultimate function of a signaling network is to reliably process and propagate information. To understand a pathway we must understand these processes, and as this work shows, in order to understand these processes we must understand the underlying dynamics. By revealing the existence of a MAPK code, we have provided a new framework for studying signaling in yeast pheromone response pathway, a prototypical organism. Kinetic insulation may further guide this research by placing this pathway in the context of a more ample signaling network. Understanding the mechanisms that control information processing and propagation in this organism could be a first step towards the application of a dynamics-based approach to the study of signaling pathways.
Appendix:

This appendix contains the parameters used to generate the figures presented in this work.

Figure 2-5: Equation (2.3) was used for the receptor and equation (2.4) has been used for A, B, and C. In all cases $[P]=1$.  
1st column: (R) $kd=1$ (A) $k_1=50$, $k_{1M}=5$, $k_2=2$, $k_{3M}=5$ (B) $k_1=50$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$ (C) $k_1=50$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$.
2nd column: (R) $kd=1$ (A) $k_1=2$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$ (B) $k_1=2$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$ (C) $k_1=2$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$.
3rd column: (R) $kd=1$ (A) $k_1=2$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$ (B) $k_1=20$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$ (C) $k_1=2$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$.

Figure 2-6: Equation (2.3) was used for the receptor and equation (2.4) has been used for A, B, and C. In all cases $[P]=1$.  
1st column: (R) $kd=1$ (A) $k_1=2$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$ (B) $k_1=6$, $k_{1M}=0.05$, $k_2=2$, $k_{2M}=0.05$ (C) $k_1=2$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$.
2nd column: (R) $kd=1$ (A) $k_1=2$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$ (B) $k_1=40$, $k_{1M}=5$, $k_2=2$, $k_{2M}=5$ (C) $k_1=6$, $k_{1M}=0.05$, $k_2=2$, $k_{2M}=0.05$.
3rd column: (R) $kd=1$ (A) $k_1=4$, $k_{1M}=0.1$, $k_2=2$, $k_{2M}=0.1$ (B) $k_1=4$, $k_{1M}=0.1$, $k_2=2$, $k_{2M}=0.1$ (C) $k_1=4$, $k_{1M}=0.1$, $k_2=2$, $k_{2M}=0.1$.

Figure 2-10: Equations (2.5) and (2.6) with $[P]=1.$  
$k_1=3$, $k_{1M}=0.05$, $k_2=0.005$, $k_{2M}=0.01$, $k_3=0.25$, $k_{3M}=0.01$, $k_4=0.0001$, $k_{4M}=1$, $k_5=2 \times 10^{-6}$, $k_{5M}=1$.

Figure 2-11: Equations (2.5) and (2.6) with $[P]=1.$  
k_1=1$, $k_{1M}=5 \times 10^3$, $k_2=3 \times 10^3$, $k_{2M}=10^3$, $k_3=2.5 \times 10^3$, $k_{3M}=2 \times 10^3$, $k_4=1 \times 10^3$, $k_{4M}=5 \times 10^3$, $k_5=10^3$, $k_{5M}=1$.

Figure 2-14: Equations (2.7) and (2.8) with $[P]=1.$  
k_1=2 \times 10^3$, $k_{1M}=1$, $k_2=10^3$, $k_{2M}=5 \times 10^3$, $k_3=1.5 \times 10^3$, $k_{3M}=10^3$, $k_4=6 \times 10^3$, $k_{4M}=10^3$, $k_5=3.2 \times 10^2$, $k_{5M}=10^3$.

Figure 2-16: Equations (2.9)-(2.11).  
k_1=10^3$, $k_{1M}=1.5 \times 10^3$, $k_3=3 \times 10^3$, $k_{2M}=1$, $k_4=4.8 \times 10^3$, $k_{3M}=1$, $k_5=5 \times 10^3$, $k_{4M}=1$, $k_6=3 \times 10^2$, $k_{5M}=2 \times 10^3$,  
k_6=10$, $k_{6M}=0.1$, $k_7=1$, $k_{7M}=1 \times 10^1$.

Figure 2-17: Equations (2.12)-(2.14).  
k_1=5 \times 10^3$, $k_{1M}=1.5$, $k_2=10^3$, $k_{2M}=8 \times 10^2$, $k_3=1.5 \times 10^3$, $k_{3M}=6 \times 10^1$, $k_4=3 \times 10^3$, $k_{4M}=6 \times 10^1$, $k_5=8 \times 10^2$, $k_{5M}=3 \times 10^3$, $k_6=10^3$, $k_{6M}=1$, $k_7=5 \times 10^3$, $k_{7M}=1$.

Figure 2-19: Equations (2.15)-(2.17).  
k_1=5 \times 10^2$, $k_{1M}=1$, $k_2=10^2$, $k_{2M}=10^2$, $k_3=7.5 \times 10^4$, $k_{3M}=5 \times 10^2$, $k_4=1.9 \times 10^4$, $k_{4M}=5 \times 10^3$, $k_5=7.5 \times 10^5$, $k_{5M}=7.5 \times 10^7$, $k_6=9.6 \times 10^3$.

Figure 2-20: Equations (2.17)-(2.20).  
k_1=6 \times 10^3$, $k_{1M}=5 \times 10^2$, $k_2=2 \times 10^2$, $k_{2M}=10^3$, $k_3=10^2$, $k_{3M}=5 \times 10^2$, $k_4=4 \times 10^3$, $k_{4M}=10^2$, $k_5=4 \times 10^3$, $k_{5M}=1$, $k_6=2 \times 10^3$.

Figure 2-22: Equations (2.18)-(2.20) where used for KK. Equation (2.17) was used to model the evolution of K* with [KIP] as the upstream input and for the evolution of KI* with K* as the upstream input.  
k_1=6 \times 10^3$, $k_{1M}=5 \times 10^3$, $k_2=2 \times 10^3$, $k_{2M}=10^3$, $k_3=8 \times 10^3$, $k_{3M}=1$, $k_6=2 \times 10^3$.

For K*:  
k_1=10^3$, $k_{1M}=5 \times 10^3$, $k_2=4 \times 10^3$, $k_{2M}=10^3$ For KI*:  
k_3=2.5 \times 10^3$, $k_{3M}=10^2$, $k_4=10^4$, $k_{4M}=10^2$. 

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Figure 2-21 and Figure 2-26: Same as Figure 2-10.

Figure 2-27: Equations (2.21)-(2.23)
k_1=1, k_2=10^{-2}, k_3=80, k_4=10^{4}, k_{4M}=10^{-1}, k_5=5 \times 10^{-6}, k_{5M}=1, k_6=10, k_{6M}=10^{-1}

Figure 2-28: Equation (2.24) was used in conjunction with (2.21)-(2.23) as before.
k_{6}=10, k_{6M}=10^{-2}, k_{7}=4, k_{7M}=10^{-2}

Figure 3-2 and Figure 3-3: Equations (3.1)-(3.4).
k_1=0.5, k_{1M}=10, v_2=0.5, k_{2M}=10, k_3=0.5, k_{3M}=10, k_4=2 \times 10^{-4}, k_{4M}=1, v_5=2.5 \times 10^{-6}, k_{5M}=1, k_6=2.5 \times 10^{-2}, k_{6M}=0.01, v_7=3 \times 10^{-4}, k_{7M}=0.01, k_8=0.25, k_{8M}=2 \times 10^{-5}, k_9=1.5 \times 10^{-4}, k_{9M}=0.5, v_{10}=5 \times 10^{-5}, k_{10M}=0.5

Figure 3-5, Figure 3-6, and Figure 3-7: Equations (3.5)-(3.10) and (3.1)-(3.3) as before.
k_{20}=20, k_{20M}=10^{-2}, v_{21}=1, k_{21M}=10^{-5}, k_{22M}=10^{-4}, v_{23}=3 \times 10^{-4}, k_{23M}=10^{-2}, k_{24}=0.25, k_{24M}=2 \times 10^{-5}, k_{25}=1e-3, k_{25M}=1, v_{26}=10^{3}, k_{26M}=1, k_{30}=10^{3}, k_{31M}=1, v_{31}=2 \times 10^{3}, k_{32M}=0.25, k_{32}=10^{4}, k_{32M}=1, v_{33}=10^{5}, k_{33M}=1

Figure 4-3: Equations (4.1) and (4.2)
k_1=5.53 \times 10^{4}, k_{1M}=3.75 \times 10^{-2}, k_2=3.25 \times 10^{-2}, k_{2M}=3 \times 10^{-1}, k_3=2.55 \times 10^{-2}, k_{3M}=1, k_4=2.5 \times 10^{3}, k_{4M}=2

Input signal: S (time < pulse) and signal= S e^{-(time-pulse)/\lambda}, (time > pulse). The signal parameters for each concentration were: S=0.2,0.25,0.75,0.75, pulse= 4', 4', 6', 22', 55', and \lambda=(50, 50, 250, 300, 300) x 3600 min.

Figure 4-4: Equations (4.3)-(4.7)
F_{TOT}=8500, K_{TOT}=5500, D_{TOT}=2000, C_{TOT}=8000
k_1=4.14, k_{1M}=319, v_2=2.76, k_{2M}=2550, k_3=105, k_{3M}=5500, k_4=13.75, k_{4M}=11000, k_5=0.01, k_6=4 \times 10^{4}, k_7=10^{-5}, k_8=1.4 \times 10^{-3}, k_{9M}=100, k_9=2, k_{9M}=100, k_{10M}=10^{3}, k_{10M}=50, k_{11M}=30, k_{11M}=50

Figure 4-5: Equations (4.1) and (4.2) with the same parameters but modified input signals.
Input signal: S (time < pulse) and signal= (S-S_0) e^{-(time-pulse)/\lambda} + S_0 (time > pulse). The signal parameters for each concentration were: S=0.15, 0.22, 0.35, 0.45, 0.65, pulse=1', 1', 2', 3', 3', and \lambda=(100, 100, 500, 650, 8000) x 3600 min. S_0 = 0.0075;

Figure 4-6: Equations (4.8)-(4.13).
k_1=3.8 \times 10^{7}, k_2=4.7 \times 10^{3}, k_3=33, k_{4}=1.7 \times 10^{5}, k_{4M}=8.8 \times 10^{-3}, k_4=4 \times 10^{-7}, k_{5M}=0.23, k_6=16, k_{6M}=7.7 \times 10^{-5}, k_{10}=5.2 \times 10^{-4}, k_{10M}=3.4 \times 10^{-2}, k_{0M}=7.1 \times 10^{-4}, k_{20M}=1.41, k_{30}=3.4 \times 10^{3}, k_{30M}=9.6 \times 10^{-2}, k_{40}=8 \times 10^{-4}, k_{40M}=0.5, k_5=5.7, k_{5M}=5 \times 10^{-3}, k_9=0.6, k_{9M}=0.11

Figure 4-7, Figure 4-8, and Figure 4-9: Equations (4.14)-(4.16). The concentrations of pheromone used are indicated in the figure and we assumed 10000 rec/cell. Cell density is indicated in the text. k_1=43 rec/min, k_2=6.25 \times 10^{-2} 1/min, k_3=4.3 \times 10^{-3} 1/min, kd=4nM. The k1 profile in Figure 4-9 was: k_1(t)= [k_1 (t < tstart), k_1 (t> tstart & t < tend), k_1 (t > tend)] with tstart = 20', tend = 60', trate = 4/4

Figure 4-10: Equations (4.17)-(4.21).
k_1=7.5 \times 10^{3}, k_2=10^{-2}, k_3=500, k_4=2 \times 10^{4}, k_{4M}=10^{2}, k_5=4 \times 10^{-7}, k_{5M}=10^{-2}, k_6=8.5 \times 10^{2}, k_{6M}=10^{3}, k_6=2.8 \times 10^{-4}, k_{6M}=3.75 \times 10^{-4}, k_7=1.6 \times 10^{-4}, k_{7M}=0.3, k_8=1.2 \times 10^{-1}, k_{8M}=1, k_9=10^{-2}, k_{9M}=0.5

Figure 4-11: Equations (4.22)-(4.26).
k_1=2 \times 10^{6}, k_2=10^{-2}, k_10=67, k_{10M}=0.88, k_{11}=1.4 \times 10^{2}, k_{11M}=0.4, k_{12}=1.2 \times 10^{2}, k_{12M}=1.6 \times 10^{-3}, k_6=301 \times 10^{-5}, k_{6M}=7 \times 10^{-2}, k_{7}=8.9 \times 10^{-7}, k_{7M}=0.15, k_8=9 \times 10^{-2}, k_{8M}=4.8, k_9=2 \times 10^{3}, k_{9M}=1.1, k_4=1.6 \times 10^{3}, k_{4M}=2.5 \times 10^{-2}, k_5=1.7 \times 10^{-5}, k_{5M}=4 \times 10^{-2}

Figure 4-12: Equations (4.27)-(4.35)
The ODE systems describing the models were solved using the software Mathematica® (Wolfram Research, Champagne, Il).
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