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Review

Cell signaling: What is the signal and what information does it carry?

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ABSTRACT

This paper reviews key findings from quantitative study of the yeast pheromone response system. Most come from single cell experiments that quantify molecular events the system uses to operate. After induction, signal propagation is relatively slow; peak activity takes minutes to reach the nucleus. At each measurement point along the transmission chain, signal rises, overshoots, peaks, and declines toward steady state. At at least one measurement point, this decline depends on negative feedback. The system senses and relays percent receptor occupancy, and one effect of the feedback is to maximize precision of this transmitted information. Over time, the system constantly adjusts quantitative behaviors to convey extracellular ligand concentration faithfully. These behaviors and mechanisms that control them are likely to be general for metazoan signaling systems.

1. Introduction

One central distinction between biological systems and complex dynamic non-living systems (from asteroid belts to hurricanes) is the centrality of information to the living state. Cells operate upon information about extracellular conditions, about internal processes, and upon information stored in their genome, to make decisions that determine their future actions. The continuing triumphal progress of the molecular biological agenda (begun in the 1930s [1]) had by the 1990s revealed much of the machinery that carries out these functions. One important next step is to understand more formally the relationship between these molecular components and the flows of information on which they operate. In order to find a relatively accessible entry point to this complex general issue, my coworkers and I have studied the quantitative function of a particular cell signaling system (here, a system that senses an extracellular condition, and transmits that information deeper into the cell).

Like all cell signaling systems, the yeast pheromone response system operates via chains of molecular events. The system operates in *Saccharomyces cerevisiae*, a model eukaryote. *S. cerevisiae* is a particularly tractable experimental organism; in particular, it features facile directed genetic manipulation, it is well suited for forward genetic experimentation, and it is supported by well-developed genomic and proteomic resources. Both specific molecular components of this system (e.g. seven helix G protein coupled receptors) and general themes (scaffold proteins, protein kinase cascades) are widely conserved throughout higher eukaryotic signaling systems.

Fig. 1 depicts some of the molecular details of the system and its operation. Pheromone binding to the Ste2 receptor causes dissociation of the single yeast heterotrimeric G-protein into a monomeric GTPase, Gpa1, and a dimer, Ste4–Ste18. Upon dissociation of the G-protein, Ste4 recruits the mitogen-activated protein kinase scaffold, Ste5 to the membrane. (Reassociation of Gpa1 with Ste4–Ste18 is promoted by the GTP-activating protein (GAP) function of the regulator of G-protein signaling (RGS-protein) Sst2 (not shown).) Ste5 recruitment leads to activation of the MAP kinase cascade, in which each of the protein kinases Ste20, Ste11, Ste7, and the MAP kinase Fus3 sequentially phosphorylates the next. Phosphorylated Fus3 translocates to the nucleus and phosphorylates Dig1 and Ste12, eliminating Dig1 repression of Ste12, a transcriptional activator. Ste12 activates transcription of pheromone responsive genes (shown in figure as PRGs, here gene derivative that fuses a pheromone-inducible promoter to YFP). Ribosomes translate mRNAs into proteins. In general, this signaling system is well understood, and its operation is well reviewed [2].

2. Single cell and other methods

When possible, cells are derived from a single reference strain, ACL 379 [3], which is in turn a descendent of the lab strain W303a. In particular, the strain carries a loss of function mutation in the *bar1* gene, so that it does not produce the secreted protease that degrades extracellular pheromone. In addition, most derivatives carry an inhibitor-sensitive allelic variant of the Cdc28 cell cycle

Abbreviations: FRET, fluorescence resonance energy transfer; RFP, red fluorescent protein; YFP, yellow fluorescent protein; MAP kinase, mitogen-activated protein kinase; DoRA, dose-response alignment; DoRR, dose-response relationship.

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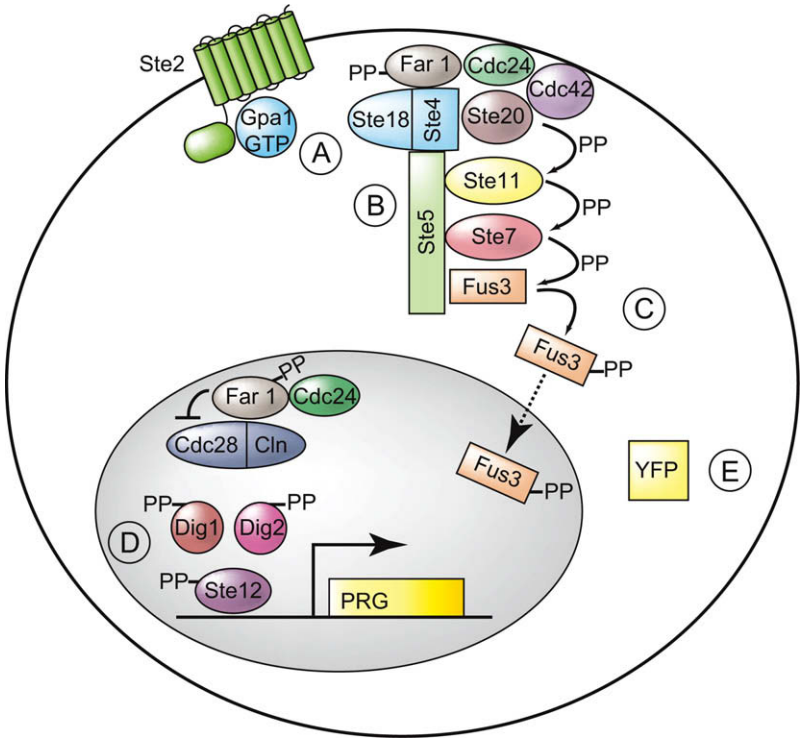

Fig. 1. Operation of the yeast pheromone response system. Proteins are indicated by labeled ovals, translocation by dotted lines, protein activation by arrows, inhibition by T-bar arrows, and protein association by double-headed dashed arrows, and measurement points by capital letters. Pheromone binding to the Ste2 receptor causes dissociation of the single yeast heterotrimeric G-protein into a monomeric GTPase, Gpa1, and a dimer, Ste4–Ste18 (measurement point A in figure). Reassociation of Gpa1 with Ste4–Ste18 is promoted by the GTP-activating protein (GAP) function of the regulator of G-protein signaling (RGS-protein) Sst2 (not shown). Upon dissociation of the G-protein, Ste4 recruits the MAP kinase scaffold, Ste5 to the membrane (point B in figure). Ste5 recruitment leads to activation of a protein kinase cascade, in which the proteins Ste20, Ste11, Ste7, and the MAP kinase Fus3 (and Kss1, not shown) sequentially phosphorylate one another. Phosphorylated Fus3 (point C in figure) translocates to the nucleus and phosphorylates Dig1 and Ste12, causing dissociation or conformational change in relationship between Dig1 and Ste12 (point D in figure), derepressing activation by Ste12, a transcriptional activator. Ste12 activates transcription of pheromone responsive genes (here, PRG); here, figure shows a gene derivative which fuses a pheromone-inducible promoter from the PRM1 gene to YFP. Transcribed mRNA is translated into protein by ribosomes (not shown) into yellow fluorescent protein (YFP, measurement point E).

protein kinase, so that investigators can disable cell cycle progression, and eliminate cell cycle dependent variation from any measurements.

Descendent strains also carry reporter constructs to enable quantification of different molecular events within the signaling pathway. By doctrine, when possible reporter constructions replace the native chromosomal gene copies with derivatives (such as fluorescent protein derivatives) expressed from the native promoters. By doctrine, we typically verify that the expression of fluorescent protein derivatives is equivalent to that of the native proteins by Western gels, comparing the amounts of expressed proteins to those of wild-type proteins determined from careful Western gel and fluorescent protein quantification [4]. Construction of most of the strains is described in the main text and supplemental materials of three papers, Colman-Lerner et al. [5], Gordon et al. [4], and Yu et al. [6].

These strains enabled single cell measurements of different molecular events the system uses to operate. We refer to molecular events for which we can measure system activity as "measurement points" (Fig. 1). These include the recruitment of yellow fluorescent protein- (YFP-) tagged Ste5 scaffold to the membrane [6] (Colman-Lerner et al., unpublished) (point B in figure), de-repression of YFP-tagged Ste12 and a cyan fluorescent protein- (CFP-) tagged derivative of the transcription suppressor Dig1 (measured by loss of fluorescence resonance energy transfer (FRET) between these chromophores) [6]) (D in figure), and expression of pheromone-inducible YFP and (red fluorescent protein) RFP reporter genes [4–6] (E in figure). We also used unrelated strains, W303 derivatives [7], that carried CFP and YFP G protein reporter constructs enabling single cell measurements of G protein dissociation (A in figure) by loss of FRET.

We induce system activity by single-step additions of pheromone to minimal medium [5,6]. We perform cell cytometry and quantification with epifluorescence microscopy, the open source software package Cell-ID [4], and analyze by canned routines written in PAW and R [4,8]. In cell populations, we measure the amount of phosphorylated and total Fus3 protein kinase by careful quantification using Western gels probed with antibodies that register total protein and antibodies that detect activated protein [6], and second antibodies conjugated to an infrared fluorophore. We measure the amount of pheromone-induced FUS1 mRNA using a specific DNA probe and nuclease protection [6].

3. Signal propagation

Fig. 2a shows the most typical system input regime. At the start of an experiment, we expose genetically identical cells to a given external concentration of mating pheromone. The extracellular medium contains casein to block adsorption of the pheromone to the inside of the plastic vessel, so that extracellular pheromone concentration and total system input remain constant thereafter.

Fig. 2b shows propagation of the signal in the first few minutes after system induction [6]. It graphs, on the *Y*-axis, the total percentage activity at four different measurement points, against time on the *X*-axis. The utility of this graphic representation is that it immediately suggests operational ways to define and quantify signal propagation and the signal. For example, we can define peak signal as 100% of the maximum activity at a given measurement

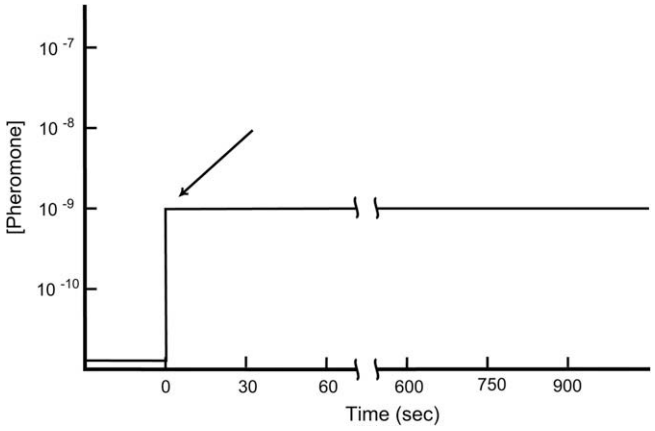

Fig. 2a. The typical system input regime. At the start of an experiment, we expose genetically identical cells to a given external concentration of mating pheromone. The extracellular medium contains casein to block adsorption of the pheromone to the inside of the plastic vessel, so that extracellular pheromone concentration and total system input remains constant during the course of the experiment, which can span hours.

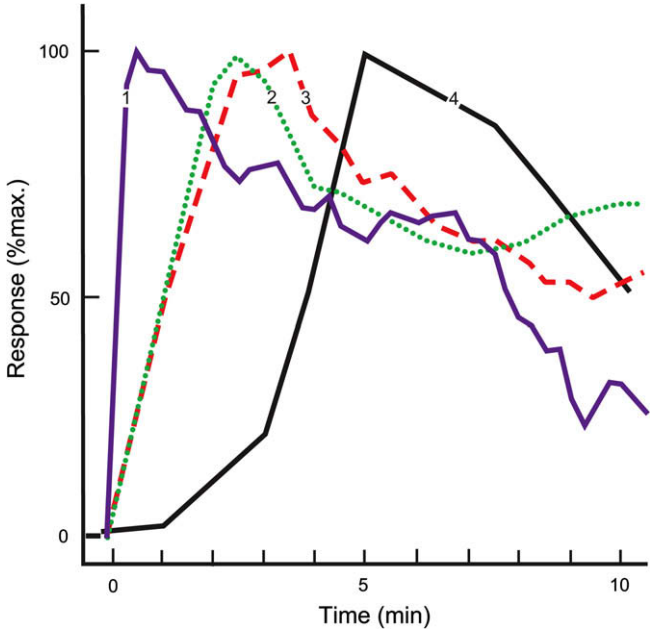

Fig. 2b. Propagation of signal, quantified from membrane, cytoplasmic, and nuclear events. Figure (redrawn from Yu et al. (2008)) graphs, on the *Y*-axis, the total percentage activity at four different measurement points, against time on the *X*-axis. Curve 1 (solid purple line) shows recruitment of Ste5 scaffold to membrane. Curve 2 (dotted green line) shows phosphorylation of the MAP kinase Fus3. Curve 3 (dashed red line) shows de-suppression of Ste12 (by loss of FRET between tagged Ste12 and Dig1). Curve 4 (solid black line) shows 5′ end of the pheromone-inducible Fus1 mRNA transcript.

point, and thus define signal propagation time from membrane to nucleus as time between peak recruitment of scaffold protein (line 1 in figure) to membrane and time to dissociation of Ste12 and Dig1 and depression of the Ste12 transcription activator (line 3). From such experiments, it becomes sensible to state that propagation of peak signal to the nucleus takes minutes.

4. Signal dynamics

A second conclusion from plots like those in Fig. 2b is that there is a common temporal pattern of activation of whatever molecular events one quantifies at each measurement point. At each measurement point, activity rises, peaks, and then declines to (or in some cases toward) a steady state. Armed with this information, it becomes sensible to use the terminology that this common dynamic behavior reflects common signal dynamics.

5. The signal carries information about invariant extracellular ligand concentration

Insights into system function also come from observation of quantitative behavior in steady state. One set of observations comes from quantification of cumulative expression of pheromone-responsive reporter gene, by measuring accumulation of a yellow or red fluorescent pheromone-inducible reporter protein ([5] and Pesce et al., unpublished). At a given extracellular pheromone concentration, reporter accumulates over time, and the rate of the accumulation is constant. This observation means that system output is constant over time. At increased concentrations of extracellular pheromone, the rate of reporter protein accumulation is faster, but remains constant over time. Another way of stating these observations is that the system senses information about external pheromone concentration and transmits that information, through its relay of molecular steps, all the way to the reporter gene downstream.

6. The signal carries updated information about changes in extracellular ligand concentration

The information the signaling system carries about external pheromone concentration is updated continually. As measured by expression of a pheromone responsive reporter gene, when the extracellular concentration of pheromone is increased after prolonged operation at lower pheromone input, system output increases to a higher, steady state rate (Pesce et al., unpublished). This adjustment is rapid. This is apparent from measurement of system activity at an intermediate measurement point, the amount of phosphorylated Fus3 MAP kinase. Within minutes after an increase in extracellular pheromone input, the percent of Fus3 that is phosphorylated (or active) also increases (Yu et al., unpublished). This behavior after increased extracellular pheromone concentration is also mirrored in the rapid increase to new steady state of membrane-recruited scaffold protein at the Ste5 measurement point (Colman-Lerner, Gordon, et al., unpublished).

7. Dose-response alignment (DoRA)

Fig. 3 shows a remarkable property of the system, which is that, at different measurement points, dose (or external ligand concentration), and response (as measured system output) are aligned. That is, the amount of system activity at a given measurement point (figure shows two measurement points, G protein dissociation and reporter gene output) matches closely the calculated percentage of the receptor (Ste2) bound by ligand. We term this relationship between system input and system output "dose-response alignment", or DoRA.

The existence of DoRA in this signaling system is a simple result, but one that leads to some strong conclusions. Again, the output of the system at different measurement points mirrors the percentage of occupied surface receptor. Restated, this fact indicates at least one kind of information the signal carries is the external ligand concentration. Moreover, this fact suggests that the system measures external pheromone concentration by the percentage of ligand bound surface receptor.

It thus becomes reasonable to view the set of receptors on the surface of the cell as a sensor for a condition in the extracellular universe (ligand concentration) and to view this sensor as a device

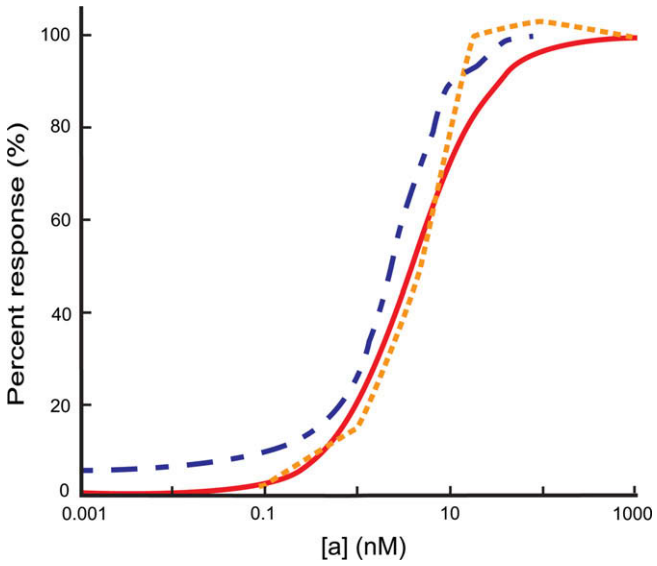

Fig. 3. Dose-response alignment in the yeast pheromone response system. Figure (redrawn from Andrews et al., submitted) shows the response curve (system activity) at the upstream G protein dissociation measurement point (dotted orange line) and the downstream Prm1-YFP pheromone-inducible-reporter gene output measurement point (dashed blue line; whose activity depends on a minimum of 12 intervening chemical reactions) matches closely the calculated percentage of the receptor (solid red line) occupied by extracellular pheromone.

that converts a condition in the external universe into information. In this view, the molecular events downstream of the sensor relay this information about the extracellular universe deeper into the cell, and the cell then operates on this information to make decisions.

One can consider alignment (DoRA) to be a particular form of a dose-response relationship (DoRR). It is important to note that this particular relationship, DoRA, is not new. Numerous vertebrate cell signaling systems, including the insulin, acetylcholine, thyroid stimulating hormone, angiotensin II, and epidermal growth factor response systems, exhibit alignment [9–13]. Frequent alignment between posited receptor occupancy and cell response was essential to the work of Clark [14] who built on the by-then-accepted idea that cell responses to drugs depended on the binding of drug to particular "receptive substances" [15]. In fact, for Clark (and physiologists of his generation) the receptor, whose identity and nature was of course otherwise unknown, was *defined* as the molecule to which the drug bound to exert its biological effects. Given this assumption, the idea that the percent of maximum cellular response might equal the percent bound receptor was simply what one expected. This idea was so strongly entrenched that, during the initial era of molecular cloning of receptors (1980s to middle 1990s), binding of drug molecules to particular cloned proteins at concentrations so that half maximal binding equaled half maximal cellular response was frequently adduced as evidence that the protein of interest was in fact the receptor [16–18].

In fact, it is only after the elucidation of different cell signaling pathways and the realization that they usually involve relays of molecular events that the widespread existence of dose-response alignment becomes a puzzle. My coworkers and I came to realize the difficulty of preserving dose-response alignment through chains of biochemical reactions without much knowledge of the relevant scientific history. However, the relationships between percent activity in different steps of multi-reaction systems had been well treated in the early 1980s by Black and Leff [19]. These researchers showed that without extremely careful attention to parameters, it is difficult, even in models, to come up with chains of reactions that preserve dose-response alignment and give high dynamic range for output. Rather, in any relay of biochemical reactions, a more normal behavior is for the activity curve at each successive downstream step to steepen, and to shift to the left. Restated, the more normal behavior for any chain of reactions is for the response to external stimulus to become more "switchlike" at each downstream step, and to become triggered at lower amounts of external stimulus.

This property of multistep chemical reaction systems was to some extent re-discovered during the 1990s – see, for example, Huang and Ferrell [20] – who showed, by model and experiment in *Xenopus laevis* ooctyes, that the response of downstream steps of protein kinase cascades become progressively more sensitive (and, again, as the response curve steepened, more "switchlike").

Consideration of other previous work also suggested one means by which multi-component systems can adjust their behavior to attain linear input/output relationships, even given nonlinear interactions among internal components. That is of course the use of negative feedback, as first analyzed by Maxwell [21] and worked out in electrical circuits by Harold Black at Bell Labs (1934, Fig. 4) [22]. In fact, we have shown that in the pheromone response system, good dose-response alignment at the MAPK measurement point requires protein kinase activity of Fus3, acting upstream to suppress a previously-unidentified positive function of the RGS protein Sst2 [6].

However, even given this identified instance of negative feedback, the preservation of dose-response alignment over many hours of system induction is nothing short of remarkable. During system operation, there are wholesale changes in cell morphology and in levels of system proteins [2]. It seems likely that additional control mechanisms must operate during this time to maintain alignment (see Section 8).

One consequence of dose-response alignment is to increase the precision of the transmitted signal and the cellular response [6]. Much of the reasoning and experimentation that establish this point is now largely unpublished, and a detailed account of all the different ways that downstream alignment increases signal information content is thus beyond the scope of this review.

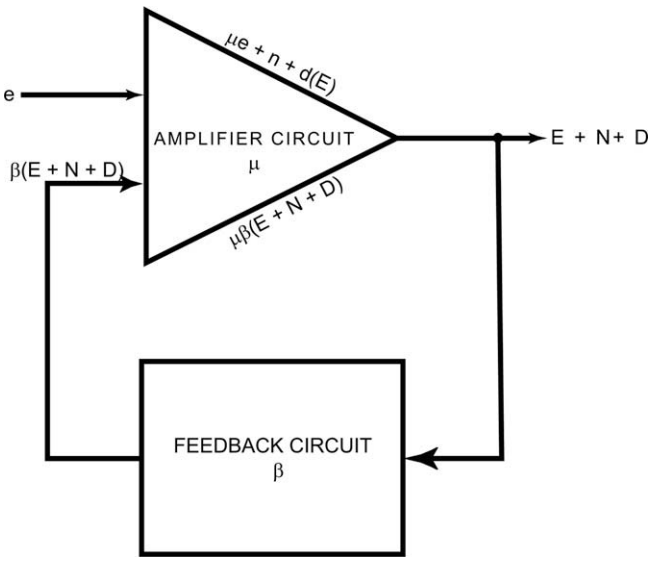

Fig. 4. Negative feedback in an electrical circuit. Figure redrawn from "Stabilized feedback amplifiers", by Black [22]. Black's cartoon omits all internal workings of the amplifier system by depicting it as a triangle with defined inputs and outputs. This representation emphasizes the fact that negative feedback can assure a linear input/output relationship even given variable and nonlinear relationships among [whatever] components relay and amplify the signal inside the triangle.

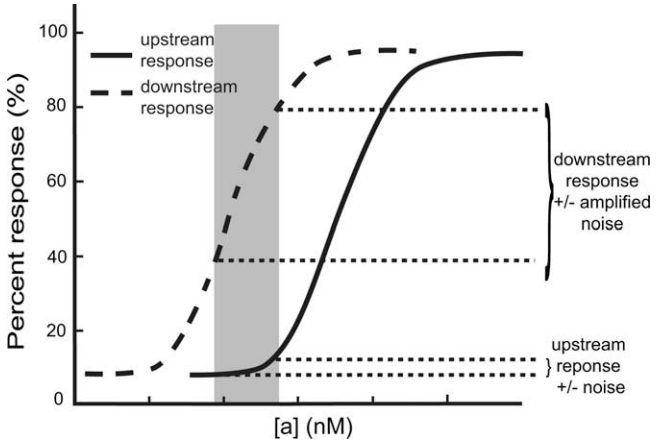

Fig. 5. Misalignment of upstream and downstream response can decrease the fidelity of transmitted information about extracellular ligand concentration. Figure shows one means by which this occurs. Figure shows activity at a downstream measurement point for a system in which the dose response is shifted to become more sensitive. Solid black line shows upstream response, dashed line, downstream response. Gray box shows a range of input ligand concentrations in which small differences in upstream response (bottom brackets, due for example to stochastic noise in signal transmission farther upstream) are amplified into larger differences in downstream response (top brackets).

Fig. 5, however, illustrates one such means. The figure shows activity at a downstream measurement point for a system in which the dose response is shifted to become more sensitive. One consequence of this shift is to make the downstream response more sensitive to, for example, stochastic noise due to random molecular collisions during signal transmission.

8. Discussion and avenues for future work

So far, perhaps the most important accomplishment from the previous years of painstaking work on the system has simply been to identify questions that should reward future study. Although there has yet been comparatively little work detailing the quantitative behaviors of cell signaling systems per se, there are a great many pertinent observations from 20th century pharmacology that one must now interpret through the knowledge of the signaling systems responsible for the measured pharmacological effects. Taken together, both lines of research establish the point that certain quantitative signaling system behaviors, including dynamic behaviors such as signal overshoot and decline toward equilibrium by negative feedback, and certain steady state behaviors, such as dose-response alignment and its consequent increase in the information carrying capacity of the signaling channel, are very widely conserved.

There are numerous important questions about mechanisms and system behaviors. For mechanisms, among the most important questions concern the molecular means by which different levels of different cell signaling systems encode information about extracellular conditions. In 1997, Kholodenko and coworkers published a theoretical treatment that posited that each level of a signaling relay consisted of an active and inactive form of a protein, and that signal was carried by the amount of active protein [23]. For lack of a better term, one can call coding schemes like that implicit in this work "amplitude modulation". Our finding of dose-response alignment at many levels of system operation [6] is consistent with amplitude encoding schemes. That is, our findings are consistent with the idea that at each step in the chain information about external ligand concentration is encoded in percent active form of some particular system intermediate. But, taken literally, the previous statement would suggest that the system might know the possible percent maximum activity (which seems unlikely) or alternatively that it counts, at different steps, ratios of active and inactive states (which seems possible). Moreover, nothing in our results is inconsistent with the possibility that the dose information (a kind of "control information", see below) might be encoded by different molecules than those the system uses for its operation. It thus remains possible that at some levels in the yeast system and other signaling systems, dose information might be transmitted by as-yet-unknown molecular events.

In the yeast pheromone response system, one postulated amplitude encoding scheme is worth mentioning. Dohlman, Elston, and coworkers [24,25] quantified MAP kinase activity during pheromone signaling. They did so using different yeast strains, different immunological methods, and longer time intervals than my coworkers and me. Dohlman, Elston and coworkers find that at low system inputs the total amount of activated MAP kinase peaks, then declines. These observations led the authors to suggest that the total system activation is a function of the extent and time over which the MAP kinases remain active, an idea they term "dose-to-duration" encoding. We have not yet sought to reconcile their findings of transient MAP kinase activation with our findings of steady state activation at many steps at earlier times, and for very long times at more downstream steps. Whatever the explanation, their results are consistent with their proposed amplitude encoding scheme. Moreover, their results are also consistent with schemes in which dose information at some steps of system operation is carried by now-unidentified molecular events.

Finally, there is evidence consistent with a different encoding scheme for certain intracellular signaling events. This comes from elegant work by Elowitz and coworkers [26 and this volume]. In individual yeast cells, the cellular complement of the Calcium stimulated transcription activator Crz1 localizes stochastically and transiently to the nucleus. The frequency of these localization bursts depends on (extracellular and intracellular) calcium concentration. These results are nicely accounted for if the level of expression of calcium-responsive genes depends on the frequency of localization bursts, or on the aggregate residence time of Crz1 in the nucleus. In this case, at the level of system operation, information about [intracellular] calcium concentration may be encoded in the frequency with which Crz1 localizes to the nucleus.

Of course, there are important questions about other mechanisms than those that represent the dose information. For example, it seems possible that system output at the far-downstream pheromone-inducible-reporter measurement point (E in Fig. 2b) must stay in alignment with occupied receptor because of additional feedbacks originating further downstream than the single one we have now identified at Fus3 MAP Kinase. If such downstream feedbacks exist, it will be possible to find them and figure out how they work. Similarly, if, as now seems to be the case, this and other signaling systems sense and relay extracellular ligand concentration by measuring percent receptor occupancy, that suggests that cell signaling systems might have one or more mechanisms that count total surface receptors or un-occupied receptors. The molecular means by which such counting mechanisms (if these exist) function, are now wholly unknown. Going forward, one can expect that forward genetic studies will identify additional possible quantitative regulatory mechanisms. I am optimistic that existing and newly devised genetic, biochemical, and single cell physiological tools will be able to establish how these function in the yeast system, and that these insights will be relevant to conserved function in higher eukaryotes.

Although it may be more difficult to identify good "systems level" questions about cell signaling systems, this task also seems possible. For example, it now appears likely that the system maintains one of its quantitative behaviors, dose-response alignment,

by continual feedback adjustment in response to changing numbers of system proteins, and that a consequence of this adjustment is to maximize the information carrying capacity of the signaling channel. This assertion is an example of a possible unifying principle or "design property" of cell signaling systems. That is, it is an example of a simplifying abstraction that provides explanations for a number of otherwise disparate observations.

Other "systems-level" observations (and perhaps simplifying answers) might come from consideration of the origins and architecture of cell signaling systems. In particular, it is said to be axiomatic in control theory that all dynamic systems with a predictable relationship between input and output separate operation from control [27]. In fact, it appears possible at this point (Resnekov, Maxwell et al., unpublished) that some sites of phosphorylation on system proteins that change after system induction are needed for *system operation*, while other sites are required for *system control*, that is, regulation of quantitative behavior. But one difference between the systems studied by control theory and cell signaling systems is that the latter systems were not designed by thinking engineers. For example, when compared with von Neumann computers, biological systems (including cell signaling systems) lack crisp distinction between processing functions and "output" [28]. Rather, in biological systems, "output" is best defined a "biological function" or "phenotype" upon which selection operates. Selection can operate simultaneously at many levels within cells and organisms, not all of these are known, and not all of which may be knowable [28]. If cell signaling systems sometimes transmit control information (such as ligand dose) without separating operation from control, and we can learn how they do so, then those findings might suggest paths for human engineers to design controlled dynamical systems different from those today.

Finally, additional insight into quantitative behaviors of cell signaling systems should of course provide insight into normal and disease function from humans and other multicellular organisms. The reason is that signaling systems sense and relay information cells use to make decisions pertinent to proper development and (cancer free) maintenance of the adult soma. It seems likely that dysfunctions in the proteins that control quantitative function of cell signaling systems, and the genes that encode them, may lead to aberrant development and adult disease, and that some of the proteins that control these mechanisms (such as protein kinases) might thus become targets for small molecule pharmacological intervention.

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