Synthetic Biology: Modulating the MAP Kinase Module

In a recent study, the MAP kinase module involved in many human cancers has been reconstructed in yeast, in order to tinker with its behavior.

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Elaborate and abstruse networks abound in biology, from ecological food webs, where the nodes are predators and prey, to intracellular signaling networks, where the nodes include receptors, protein kinases and transcription factors. In recent years, many groups interested in complex networks have focused on trying to understand the function of smaller modules — such as protein kinase cascades — contained within the larger entities. However, it can be difficult to experimentally isolate a module from the surrounding network. In a recent issue of *Cell*, O'Shaughnessy *et al.* [1] studied the MAP kinase cascade signaling module by transplanting it from mammalian cells into yeast, and hence gained new insights regarding the way this cascade transforms input into output.

Anyone who examines a reasonably comprehensive map of a signaling network will conclude that it is hopelessly cryptic, with many more tiers and much more crosstalk than should be needed simply to transduce a signal across the plasma membrane, amplify it up a bit, and disseminate it to a few dozen intracellular targets [2]. Can we make sense of this complexity? A pessimistic answer to this question emerged from early work that used evolutionary algorithms to make better electronic circuits. It turned out that these evolved circuits worked well — often better, in fact, than circuits designed by human engineers. However, under the hood, the evolved solutions contained extensive cross-connections between circuit elements, which made it very hard to work out how the circuits actually achieved their tasks [3]. Indeed, when the renowned evolutionary biologist Lewis Wolpert was exposed to this work, he was moved by analogy to the opinion that developmental signaling networks are unfathomable [4].

It would be unfortunate if Wolpert were right about this, as these developmental networks are the same ones that regulate stem cells, and that are dysregulated in cancer and other diseases. Thus, there is great hope that they might be understood in enough detail to enable the design of more effective therapies. Hence, many workers are asking if (unlike evolved electronic circuits) these bewildering networks are built up from smaller modules or motifs that themselves have a comprehensible function [5,6].

The mitogen-activated protein kinase (MAPK) cascade — a series of three protein kinases acting sequentially — has been referred to as a module almost since its discovery [7,8] (Figure 1). This is because the amino-acid sequences of the kinases have been conserved throughout the eukaryotic domain, as has their strict organization into a cascade, with a MAP3 K, such as Raf, activating a MAP2 K, such as MEK, in turn activating a MAPK, such as ERK. Also conserved is a 'cascaded dual phosphorylation' mechanism of activation: Raf activates MEK by phosphorylating two residues in MEK's so-called activation loop, and MEK in turn activates ERK by phosphorylating two residues in ERK's activation loop. Also of importance is that these phosphates are added in a distributive fashion, one per enzyme–substrate encounter, so that MEK activation requires two different productive collisions with Raf, and so on. A final strongly conserved feature is docking interactions that serve to first tether the MEK to the MAPK and then tether the MAPK to various substrates [9].

While the core module is conserved in sequence, organization, activation and substrate recognition mechanisms, what is upstream and downstream of the MAPK module can vary widely from species to species and from cell to cell. The MAPK cascade is plug-and-play: it can be 'plugged in' downstream of many different types of receptors (receptor tyrosine kinases in human growth factor signaling, G-protein coupled receptors in yeast pheromone response), and upstream of many different substrates and cellular endpoints [7]. These 'weak linkages' to upstream and downstream components suggest that the module can be readily plugged into new signaling contexts during evolution [10]. In human cells, the MAPK module is frequently plugged in to signaling pathways involved in disease [7].

But what might the MAPK module do that makes it so useful? Most obviously, the module tightly regulates MAPK activity: dual phosphorylation boosts MAPK activity by over 5,000-fold. But, if a single MAPK molecule is a very tightly regulated switch, what about the population of thousands or millions of MAPK molecules per cell? This population could, in principle, respond to input in a graded, proportional manner, like a dimmer switch, or it could respond more like an on–off toggle switch. Evidence suggests that the MAPK module can do both, depending on the context. When a yeast cell is sniffing out a partner for mating, for example, a graded response that is more-or-less linearly sensitive to a wide range of doses might be useful, and has indeed been observed experimentally [8,11,12]. Alternatively, when it controls an all-or-none cell fate response, the MAPK module can respond to input in a switch-like fashion, as first shown by Jim Ferrell and colleagues using frog egg differentiation as a model system [13].

So if the MAPK module can be flexibly tuned to be either a potentiometer or a toggle, what factors control the tuning? This is the question asked by O'Shaughnessy *et al.* [1], and they did it in a bold new way. Whereas previous forays into this territory had used computational modeling (e.g., [14,15]) and/or experimental perturbation of a cascade in its endogenous context [12,16,17], O'Shaughnessy *et al.* [1] used a synthetic biology approach that involved transplanting the three-tier mammalian cascade into yeast. To do this, they expressed the mammalian Raf-1, MEK1 and ERK2 proteins in yeast, and engineered the system so
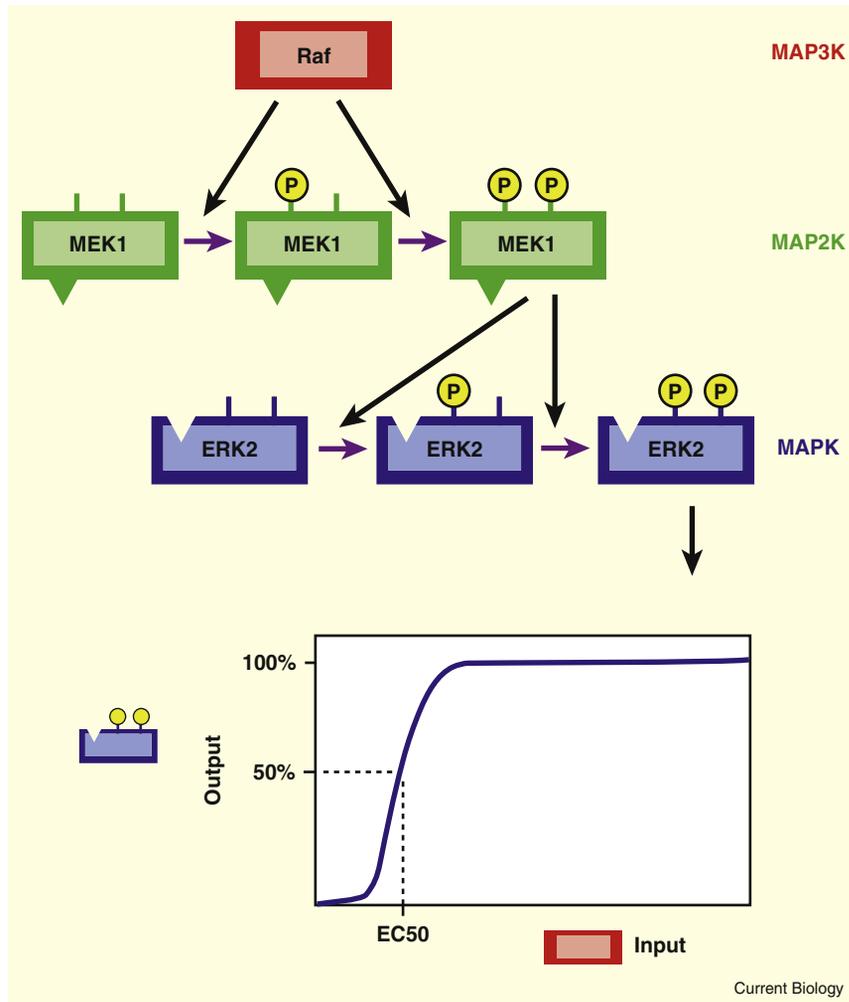
Figure 1. The MAP kinase module.

Top: The MAP kinase cascade module that O'Shaughnessy *et al.* [1] transplanted into yeast, emphasizing the cascaded dual phosphorylation mechanism. Bottom: An example of an input–output (dose–response) curve, where the input is active Raf and the output is the percentage of dually-phosphorylated (hence active) MAPK.

O'Shaughnessy *et al.* [1] first analyzed what they called the 'basic cascade'. Here, the three kinases were expressed at about 200 molecules per cell, corresponding to a cytoplasmic concentration of about 10 nM: this cascade had a threshold of about 32 nM. As noted above, the threshold is the concentration of estradiol required to activate half of the MAPK in the system, and as such is not a very useful number on its own. However, with a back-of-the-envelope (well actually, front of the spreadsheet) calculation, it can be shown that 32 nM estradiol corresponds to about 10 active Raf molecules per cell. Since this small troop of Rafs promoted the activation of about 80–100 MAPKs, the signal was amplified about 9-fold through the cascade, as measured at the EC50. The cascade was also moderately ultrasensitive, with a Hill number of 1.8, meaning that about an 11-fold increase in input was required to move from 10% to 90% maximal output; i.e., about 3 active Raf for 10% output and 33 active Raf for 90%.

MAPK cascades frequently interact with so-called scaffold proteins, which are thought to bind to two or more cascade components and tether them near each other, increasing the rate at which one activates the other. To investigate this expansion back to the core MAPK module, O'Shaughnessy *et al.* [1] co-expressed the scaffold protein paxillin along with the basic cascade. Whereas theory says that scaffolds should first boost, then inhibit signaling as their concentration is increased [15], paxillin inhibited, but did not boost. This lack of boost is perhaps not surprising, given that MEK and ERK can dock to each other directly, and given that paxillin presumably cannot protect the kinases from degradation, whereas some scaffolds may work, in part, by protecting their bound kinases from phosphatases.

One feature of the MAPK module that is *not* strongly conserved in evolution is the relative concentration of the various kinases. O'Shaughnessy *et al.* [1] wondered whether this feature could be tinkered with, by evolution or by an experimenter, to tune threshold and ultrasensitivity. Manipulating protein levels by using multicopy plasmids, stronger promoters, or a small molecule MEK inhibitor, they found that increasing the concentration of MEK and ERK reduced threshold

that the amount of active Raf produced could be controlled by titrating in the hormone estradiol. The system worked: when they added estradiol, active Raf was released, whereupon it activated MEK, which in turn activated ERK. After about half an hour, the level of active ERK reached a steady state. Perhaps surprisingly, given some previous reports [18,19], the transplanted cascade was, for the most part, insulated from endogenous yeast kinases, substrates and phosphatases. Thus, the steady state was presumed to be a consequence of protein synthesis and degradation; the authors assumed that every species in the reaction had a half-life of about twelve minutes.

The researchers concentrated their analysis on the input–output curve. To do this, they added different amounts of input (estradiol) and measured the steady-state output (active MAPK over total MAPK) corresponding to each level of input (Figure 1). They focused primarily on threshold and ultrasensitivity. Threshold refers to the amount of input required to reach some benchmark level of output. (O'Shaughnessy *et al.* [1] used 50% maximal output as their benchmark, i.e. the EC50.) Ultrasensitivity is a measure of the fold change in input required to move from a benchmark level of output (such as 10% maximal) to a higher level (such as 90% maximal). This fold change is quantified by a number designated the 'Hill coefficient'; the larger the Hill coefficient, the smaller the fold change required.

and increased the ultrasensitivity (amplification also apparently increased dramatically, although the authors did not report this metric). None of these trends is intuitively obvious, but they were qualitatively consistent with a computational model built by the authors. This model used simple ordinary differential equations to represent mass-action reactions for all the steps in the pathway, including protein synthesis and degradation. Although some parameters of the model were derived by fitting data, the model showed the ability to predict (i.e., interpolate) the results of new experiments.

Emboldened by the success of their model, the authors used it to explore parameter space more quickly, thoroughly and cheaply than could be done by experiment, and to extrapolate the effects of protein concentrations that they were simply unable to achieve in yeast. Some unusual trends were observed; for example, ultrasensitivity generally increased as MEK was increased, but, as ERK was increased, ultrasensitivity at first went up, then plummeted. Indeed, the authors found a sweet spot of ERK and MEK concentrations where the Hill coefficient was almost 4. Intriguingly, the concentrations of MEK and ERK in frog eggs suggests that they may occupy this sweet spot, consistent with the high Hill number observed experimentally for this system, which drives a switch-like response to the hormone progesterone [13]. In contrast, the yeast mating cascade occupies a region of concentration space where the Hill coefficient is less than 2, consistent with the more graded response to mating pheromone observed in yeast. In other words, natural cascades with different kinase concentrations may be innately biased toward their distinct activation profiles. This innate bias may then be reinforced by various mechanisms, e.g. sequestration to sharpen the response [20], positive feedback to make a full-out toggle switch [13], or a scaffold protein to make a dimmer switch [8].

With its combination of tight regulation, flexible tuning and plug-and-play functionality, the MAPK cascade would seem to deserve its status as the very model of a major module general.

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Auditory Neuroscience: Temporal Anticipation Enhances Cortical Processing

A recent study shows that expectation about the timing of behaviorally-relevant sounds enhances the responses of neurons in the primary auditory cortex and improves the accuracy and speed with which animals respond to those sounds.

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Scientists have traditionally viewed the auditory cortex, like other sensory cortices, as a passive detector of stimulus features. A number of studies have challenged this view, however, by showing that the responses of neurons in the primary auditory cortex (A1) can change with task demands [1] and learning [2–4], and even register behaviorally relevant non-auditory events [5]. A recent study by Jaramillo and Zador [6] builds on this growing body of evidence by showing that the responses of rat A1 neurons are modulated by the expected timing of a target sound in ways that can account for improvements in the animals' performance. Activity in the auditory cortex therefore represents not only the acoustic structure of a given sound, but also signals the cognitive functions that are carried out with it.