Supplementary results

**Role of receptor internalization.** Comparing the kinetics of activation of the different signaling molecules, it can be seen that the majority of the signal results from the receptors at the plasma membrane at the EGF concentration of 50 ng/ml. The phosphorylation and association of phosphorylated Shc to receptors, which are still at the surface, is so fast that Ras-GTP activation has already peaked and then decayed to <50% before phosphorylated endocytosed receptors accumulate in the endosomes to a significant extent. The internalized receptors could also contribute to Ras activation, in the range of <10% of the total signal. This implies that all the subsequent steps of the MAP kinase cascade exhibit kinetics very similar to the activation of plasma membrane receptors. Thus, the high initial velocity of (EGF-EGFR)2* phosphorylation seems to be sufficient to dominate ERK activation (Fig.4 in the main text).

However, at low (sub-$K_D$) concentrations of EGF, the model predicts a distinct contribution of the plasma membrane and the internalized receptors to ERK activation. Similar calculations as performed in Figure 4 in the main text, but at an EGF concentration of 0.125 ng/ml, predict that at this low EGF concentration, EGF receptor autophosphorylation becomes slow and only 10% of the total receptors are activated (Figure 5 in the main text). Now, this activation kinetics is similar to the internalization rate of receptors (the ratio of internalized to externally exposed receptors after 7 min is ~1:1). This implies that the relative signal contribution of the internalized receptors throughout the cascade is considerably higher at low EGF concentrations than at saturating concentrations. It must be recalled, however, that the total signal conveyed by internalized receptors (for example, the calculated number of activated Ras-GTP), is not different for the two simulations. This indicates that the strength of the cellular response is dominated by a plasma membrane–induced signal.
**Sensitivity analysis.** As we established our computational model and collected experimental data on the EGF receptor cascade, a great variance in the determined parameters became apparent, with reported differences of one order of magnitude or more. Part of this variation may be accounted for by cell type–specific differences in expression levels of signaling molecules. However, the model suggests that the EGF receptor–induced MAP kinase network is extremely stable toward apparent variations in signaling components. Within a rather wide range, neither the number of intracellular signaling proteins per cell nor the rate constants of association and dissociation seem to influence the final biological outcome (ERK-1/2 activation). Moreover, the experimental data obtained for ERK-1/2 and c-fos activation fully support the predictions made, suggesting that intracellular concentrations of signaling molecules are typically not response-limiting. Experimental overexpression of all major components of this signal pathway in cells (including Grb2 or Sos, as well as Ras, Raf, MEK, or ERK, in their natural forms) did not result in an increased transforming potential, with the exception of Shc. Only the corresponding constitutively active compounds led to transformation of the cells^{1–6}.

To assess sensitivity to variation in individual parameters, we took parameter sets developed for the model and varied one parameter while holding all others fixed. As shown in Supplementary Figure 6, the model tolerates 10- to 1,000-fold variation in the value of many parameters. Most solutions are highly robust to variation in individual parameter values. We determined quantitatively the extent to which we could vary the association and dissociation rates selectively while maintaining the same initial conditions, without changing the principal behavior of the output signal. This means that the results were in a range that always fitted to the experimental
data of ERK activation and allowed the same conclusions as presented in the paper. For example, maximal ERK activation over a wide range of EGF concentrations showed strong correlation with delayed peak maxima, receptor internalization, and receptor overexpression.

There are, however, some parameters (especially those concerning the MAP kinase cascade), which are very sensitive to parameter variations. The system's behavior is strongly influenced by those sensitive parameters (Supplementary Fig. 6).

We evaluated the model's sensitivity to initial conditions and discovered that a rather stable pattern arises for a variety of changes. First, we used the set of initial conditions and varied one initial condition while holding the others fixed. The variability is shown in Supplementary Table 2, and comprises in most cases a factor of 3–5. Then a whole set of initial conditions were drastically underestimated (Supplementary Fig. 7) with the goal of obtaining the same solution while at the same time keeping as many parameters as constant as possible. Parameters that were especially sensitive, as determined by sensitivity analysis, were re-estimated by evolutionary optimization. Despite these extreme assumptions, only very few parameters (out of a total of 60) had to be changed to obtain the "same solution". From these results it may be concluded that the model has only a few absolute demands on initial conditions, and many solutions may be found that describe the system. Thus the model emerged with unexpected robustness to variation in parameters and initial conditions.


