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A systems biology approach for the study of cumulative oncogenes with applications to the MAPK signal transduction pathway

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Abstract

The Extracellular signal Regulated Kinase (ERK) pathway is one of the most well-studied signaling pathways in cell cycle regulation. Disruption in the normal functioning of this pathway is linked to many forms of cancer. In a previous study [D.K. Pant, A. Ghosh, Automated oncogene detection in complex protein networks, with applications to the MAPK signal transduction pathway, Biophys. Chem. 113 (2005) 275–288.], we developed a novel approach to predict single point mutations that are likely to cause cellular transformation in signaling transduction networks. We have extended this method to study disparate pair mutation in enzyme/protein interactions and in expression levels in signal transduction pathway and have applied it to the MAPK signaling pathway to study how *synergistic* or *cooperative* mutation within signaling networks acts in unison to cause malignant transformation. The method provides a quantitative ranking of the modifier pair of ERK activation. It is seen that the highest ranking single point mutations comprise the highest ranking pair mutations. We validate some of our results with experimental literature on multiple mutations. A second order sensitivity analysis scheme is additionally used to determine the effect of correlations among mutations at different sites in the pathways.

Keywords: MAPK signaling pathway; Cumulative mutations; Oncogenes; Sensitivity analysis; ERK activation

1. Introduction

The MAPK signal transduction pathway is an important, well-studied pathway in cell signaling which controls cell growth [2–8]. This pathway has been studied experimentally with extensive data available [9–11]. Importantly, it features several oncogenes and is deregulated in about 30% of all cancers. Thus, it is a prime target for anti-tumor therapy [6]. Drugs targeting Ras, Raf and Mek and related enzymes are in clinical trials [12–15]. However, cells are typically very robust in individual mutations and abnormal cell division occurs due to the cumulative effect of multiple mutations in oncogenes, tumor suppressor genes, DNA repair genes or in expression level changes over a period of time [16,17]. For this reason, it is useful to have a better understanding of the key mutations and their significance and the synergistic effect of multiple mutations which are responsible for neoplastic transformation.

In the normally functioning pathway, a stimulus from extracellular EGF activates ERK through a series of reactions. A simplified outline of the main central pathway may be described as follows. Extracellular EGF binds to the EGF receptor on the cell membrane. This induces receptor dimerization and autophosphorylation [18]. The bound receptor forms a docking site for the signaling molecule complex of SoS–Grb2 which then activates the G-protein Ras by stimulating the exchange of GDP by GTP. This causes a conformational change in Ras, enabling it to bind to Raf leading to Raf activation. Activated Raf phosphorylates and activates MEK which in turn activates ERK. Activated ERK can then enter the nucleus and phosphorylate transcription factors such as Elk-1 and other Ets family proteins. The central pathway is itself controlled through several feedback and feed forward loops which can act as bistable switches within the network (Fig. 1) [9,19]. This central pathway interacts with the PLC−γ-PKC pathway at two critical points. PKC activates both Raf and Ras which in turn leads to the activation of ERK in a positive feedback loop. In addition ERK activates PLA_2 which results in the activation of PKC via DAG, leading to another coupled

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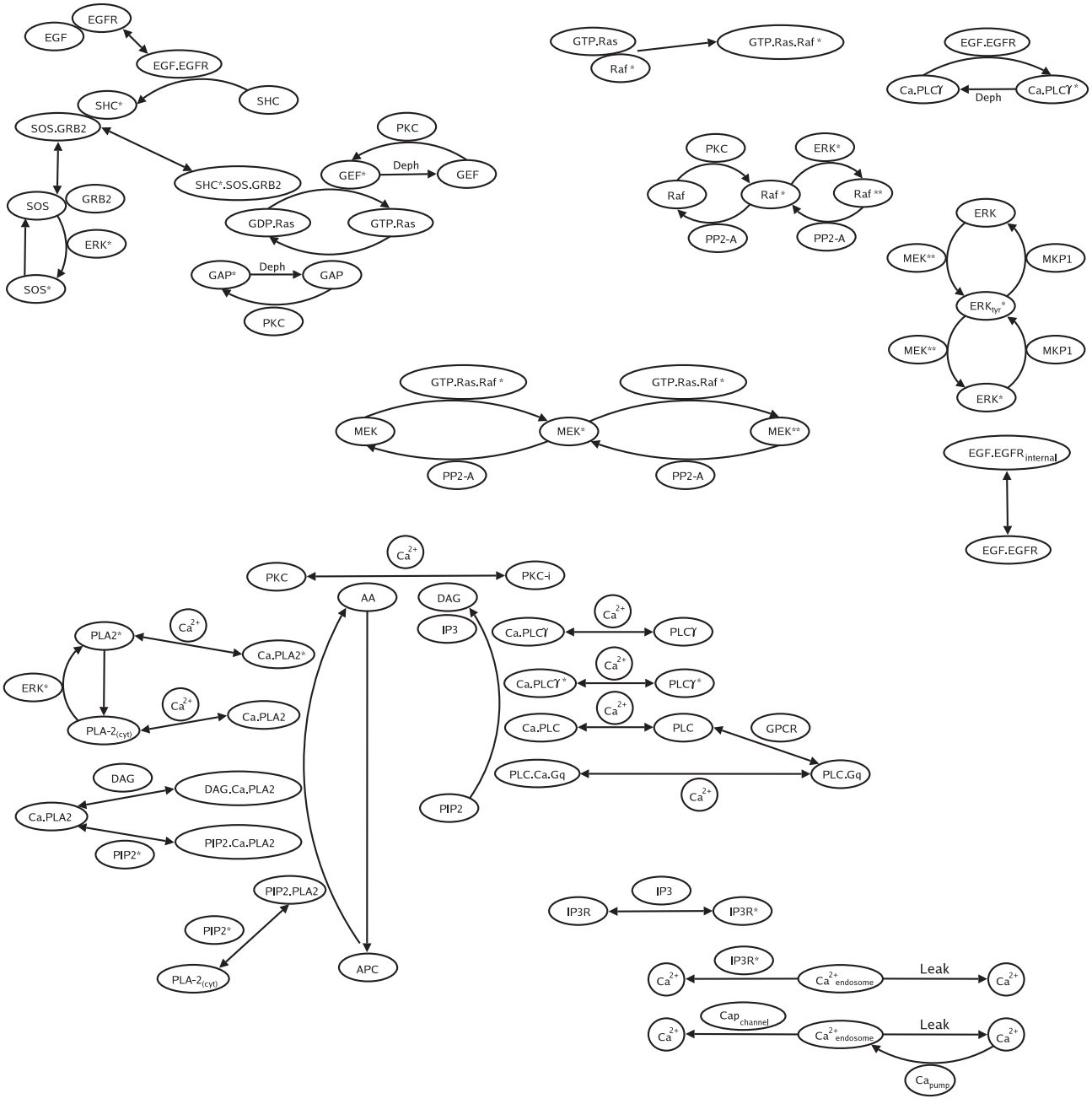

Fig. 1. The main pathway used in this model. The arcs connect species involved in enzymatic (such a phosphorylation/dephosphorylation) reactions. For example, PIP2 conversion to DAG and IP3 is carried out by four enzyme complexes Ca. PLC, Ca. Gα-GTP. PLC, Ca. PLCγ and Ca. PLCγ* of the two isoforms of PLC. The association, disassociation reactions are shown by straight arrows. In the figure, Gq≡Gα-GTP.

feedback loop. Conversely, activated ERK downregulates its own activation through the double phosphorylation of Raf in a negative feedback loop. In addition, ERK also phosphorylates SOS leading to the inactivation of signal from the receptor in another negative feed back loop. Ultimately, the careful balance of positive and negative feedback loops within these coupled pathways dictate the response [2,5]. Mutations within these cascades ultimately lead to an imbalance and manifest themselves in either uncontrolled proliferation or apoptosis.

The canonical example of this type of imbalance is found in mutations of the Ras kinase [20,3,21]. Ras is constantly in the GTP bound form even in the absence of external EGF stimulus, and consequently ERK is also in a constant state of activation. This complex set of interactions can be viewed as a circuit, with the "input" being the levels of extracellular EGF and the "output" is the activated ERK levels.

Two separate methods–a non-linear analysis using the algorithm developed previously [1] and an extended second order sensitivity analysis method–are used to study multiple mutations and their correlations. This paper is organized as follows: (1) the algorithm developed for single mutations is extended to examine multiple mutations. (2) This work is then applied to the MAPK signal transduction pathway. (3) A discussion of the results follows.

2. Methods

The model and the algorithm for the nonlinear analysis have been developed previously [1] and will not be discussed in great detail here. The network of the signal transduction pathways used is shown schematically in Fig. 1. The parameters for the model of this network of pathways are taken from the Bhalla and Iyengar [9]. Currently, the model does not have the MKP feedback loop recently described by Bhalla et al. [19]. Since this loop is not coupled to any members of the signaling pathway outside of ERK and MKP, this feedback loop is expected to have the same effect on mutations of upstream members. For this reason this new pathway has not been incorporated directly into the model.

Two methods are used for discovering how perturbations to the signaling pathway will affect signaling. As the system is inherently nonlinear, the effect of mutations may only rigorously be found by systematically integrating out the full system of the coupled ordinary differential equations (that arise from the kinetics of the enzymatic reactions involved) needed to describe the mutated signaling pathway and evaluating the response. This is the first approach, the so-called "direct" method. In this paper, two or more simultaneous mutations are examined. The flowchart in Fig. 2 outlines this approach for two simultaneous mutations.

Two types of mutations are considered. In the first mutation type, defined as an interaction mutation, the effect of the mutation is reflected in the rate constants of the protein–protein interactions. In the second type of mutation, defined as an *expression level*

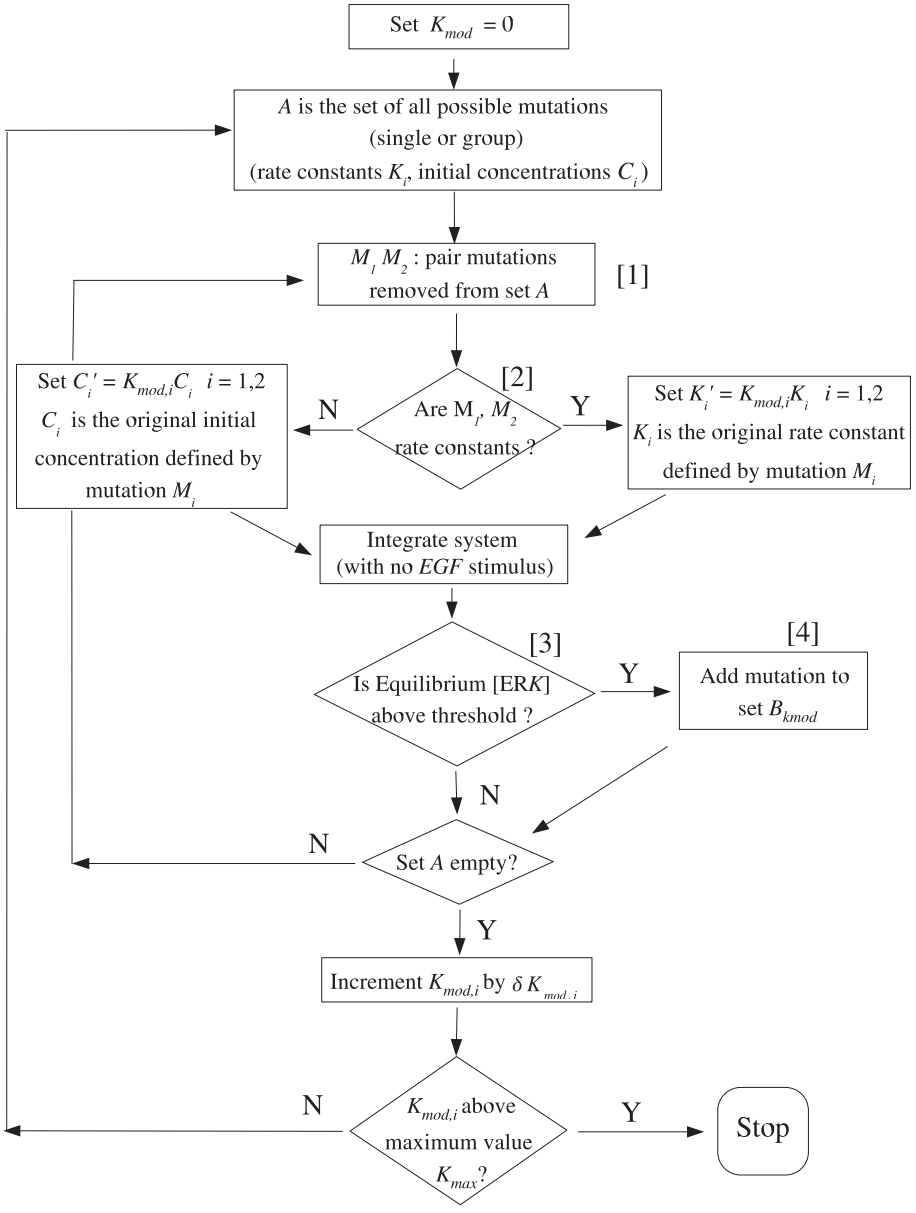

Fig. 2. This flowchart describes the logical sequence of steps to study pair mutation in the MAPK signaling pathway.

mutation, the mutation manifests itself in a change in the protein expression levels. The modified rate constant is defined as (see Ref. [1] for more detail)

$$k^i_{\mathrm{mod}} \equiv \exp\left(-\Delta E^i / k_b T\right) \tag{1}$$

$$k_i' = k^i_{\mathrm{mod}} \cdot k_i \tag{2}$$

where k_i is the rate constant in the normal pathway and $k_i'$is the mutated rate constant. Thus, k^i_{mod} is a normalized rate constant for each reaction. k_{b} is the Boltzmann's constant.

ΔE^i is the change in the barrier energy of activation for any given protein–protein reaction as given by Arrhenius theory due to the mutation [22]. A k^i_{mod} value of unity corresponds to the normally functioning pathway ($\Delta E^i = 0$). Mutations with values of k^i_{mod} less than unity arise when there is an increase in their activation energies (i. e. ΔE^i is positive, see Eq. (1)) due to the perturbation from the normal pathway, making the transition from reactants to products less probable. Such mutations are therefore called "inhibiting" mutations while those with k^i_{mod} values greater than unity (or ΔE^i negative) are analogously called "activating" mutations. Such reactions promote product formation to a greater extent than a non-mutated reaction.

From Fig. 2, set A comprises all the pair mutations. For a system of 156 reactions with 238 rate constants, this set includes approximately 28,000 possible mutations in the rate constants.

Correspondingly, a similar equation for expression level mutations has been derived (1) such that a perturbation in the expression level may be expressed as:

$$[A]'/[A] = k_{\mathrm{mod}} \tag{3}$$

where [A] is the original expression level of protein A and [A]′ is the new expression level.

To find effects between cumulative mutations, perturbation parameters k^i_{mod} are chosen from zero to some final value $k_{\max}$. A value of zero refers to an extreme case of a gene deletion event or the elimination of the particular reaction corresponding to that k^i from the enzyme reaction network.

The new reaction rate constants/initial enzyme concentrations are appropriately modified based on the particular value of k^i_{mod}. The time integration is then carried out until steady state is achieved for all reactions involved. As these mutations are considered, they are removed from set A (Fig. 2). Since the "output signal" of an abnormal pathway is a constant elevated concentration of activated ERK in the absence of EGF, those particular time evolutions which cause such high steady state levels of activated ERK are the ones chosen. Set $B_{k\mathrm{mod}}$ lists all these mutation pairs which cause abnormal signaling. This process is repeated till set A is exhausted. Then, this entire procedure is repeated for an incremented value of k^i_{mod} and so on until the final value $k_{\max}$ is reached. Having done so, a list of all the mutation pairs at various thresholds of the rate constants/concentrations is obtained which can then be ranked using the threshold value k^{mod}. Mutations which cause activation at lower threshold values are more likely to be oncogenes as they represent mutations which are activated with smaller changes to the barrier height.

For the system of 156 equations in the MAPK pathway (represented schematically in Fig. 1), the response of any "single" mutation on a system can be calculated easily on a PC workstation. We use a stiff 4th order Rosenbrock integrator to calculate the temporal evolution out to steady state and evaluate the response [23].

In addition to the approach outlined, higher order sensitivity analysis is also used. It was shown previously for single perturbations, this method is equally accurate as the "direct" method in ranking putative oncogenes [1]. Pair mutations are examined and ranked based on $d[\mathrm{ERK}\ ]/dk_{\mathrm{mod}}$. First order sensitivity analysis is identical to Metabolic Control Analysis (MCA). MCA is a well-developed technique widely used to study enzyme flux as a function of perturbations in system parameters of complex enzyme systems [24–28]. MCA relates the local response of enzymes (viz a single system considered in isolation) to their global response, viz in an entire pathway in real systems. This method has been recently applied to predict the quantitative relationship between aneuploidy and the development of cancer [29].

Besides searching for pair mutations, it is instructive to know which mutations at different nodes along a pathway (i.e. upstream or downstream) act synergistically or non-synergistically to promote abnormal cell division. To do this, we examine second order terms in the perturbations to correlations among various possible mutations. This computation can be done easily using sensitivity analysis to second order. Using MCA, however, requires the computation of higher order control coefficients which is not straightforward [30,31].

Expanding the steady state concentration of the signal in terms of two parameters k_i and k_j (with a notational change identifying k^i_{mod} with k_i and k^j_{mod} with k_j), the change in ERK levels may be expressed as:

$$d[\mathrm{ERK}]\left(k_i + dk_i, k_j + dk_j\right) = \frac{\partial[\mathrm{ERK}]}{\partial k_i} dk_i + \frac{\partial[\mathrm{ERK}]}{\partial k_j} dk_j + 0.5^* \left(\frac{\partial^2[\mathrm{ERK}]}{\partial k_i^2} dk_i^2 + \frac{\partial^2[\mathrm{ERK}]}{\partial k_j^2} dk_j^2 \right) + \frac{\partial^2[\mathrm{ERK}]}{\partial k_i \partial k_j} dk_i dk_j. \tag{4}$$

One can numerically compute the correlation term, which is the last term in the above expansion as the following:

$$\frac{\partial^2[\mathrm{ERK}]}{\partial k_i \partial k_j} = \frac{\mathrm{ERK}\left[k_i + dk_i, k_j + dk_j\right] - \mathrm{ERK}\left[k_i + dk_i, k_j\right] - \mathrm{ERK}\left[k_i, k_j + dk_j\right] + \mathrm{ERK}\left[k_i, k_j\right]}{\partial k_i \partial k_j}. \quad (5)$$

This term is a measure of the interaction between two separate mutations. Thus, if this term is large and positive, it implies a strong synergistic interaction for those two particular mutations causing an increase in the "output" signal, viz. ERK activation. A large negative term on the other hand implies an annihilating interaction. In other words, these mutations interact destructively towards the outcome of the "output" signal, in effect canceling out. A ranking based on the magnitude of this term suggests which pairs of mutations, possibly located at different is sites in the network of pathways, may act correlatively.

3. A study of cumulative mutations

We examine mutation pairs (protein–protein reaction rate constants and protein expression levels) from the set $B_{k\mathrm{mod}}$ (box labeled [4], see Fig. 2), which comprises those mutations that lead to high constant levels of activated ERK at steady state. These could be of the following three kinds. One is an inhibiting–inhibiting mutation pair, which corresponds to two individual mutations each of which is an "inhibiting" mutation, as defined in the previous section. Another is the activating–activating mutation pair, where each individual mutation is an "activating" mutation. The third is an inhibiting–activating mutation pair. The results for the highly ranked two-site mutations for each of the above three cases are presented in Tables 1, 2 and 3.

This ranking is based on which mutated pair causes a jump in ERK levels for the smallest perturbation, i.e. k^i_{mod} values closest to 1, the normal pathway.

As shown in the previous paper, with a single inhibiting mutation, only PP2-A is capable of causing ERK activation at k^i_{mod} perturbative values above 0.6. In Table 1 however, it is seen that mutations with k^i_{mod} values up to 0.88 can now cause malignant transformation of the pathway. By our analysis, the inhibition of the dephosphorylating action of PP2A is so prominent that it is featured with other abnormal reactions in all the top ranked pairs. An inhibition in the enzymatic action of PP2A is at the top of these rankings. The second rank is a PP2A coupled with activation of the Ras–Raf interaction. This is followed by suppression of the Ras–GAP interaction, activation of the Ras–GEF interaction

Table 1
This table ranks the double mutation on the basis of ERK activation at the threshold k_{mod}

k_{mod}	Reaction	Dir	Reaction	Dir
0.88	X*+PP2A⇌X*–PP2A	f	X*–PP2A→PP2A+X	f
0.85	X*+PP2A⇌X*–PP2A	f	Raf*+GTP–Ras⇌RG-Ras*	b
0.85	X*+PP2A⇌X*–PP2A	f	GTP–Ras+GAP⇌TR-GAP	f
0.85	X*+PP2A⇌X*–PP2A	f	TR-GAP→GAP+GDP–Ras	f
0.85	X*+PP2A⇌X*–PP2A	f	AA→APC	f
0.84	X*+PP2A⇌X*–PP2A	f	GDP–Ras+GEF(g)*⇌DR-GEF(g)*	b
0.84	X*+PP2A⇌X*–PP2A	f	X+RG-Ras*⇌X–RG-Ras*	b
0.84	X*+PP2A⇌X*–PP2A	f	Raf+PKC(g)⇌Raf–PKC(g)	b
0.84	X*+PP2A⇌X*–PP2A	f	DAG→PC	f
0.84	X*+PP2A⇌X*–PP2A	f	PKC–DAG–AA⇌PKC–DAG–AA*	b
0.84	X*+PP2A⇌X*–PP2A	f	Raf*–PP2A→PP2A+Raf	f
0.84	X*+PP2A⇌X*–PP2A	f	$Ca–Ca_{pump}→Ca_{pump}+Ca_{ext}$	f
0.83	X*+PP2A⇌X*–PP2A	f	X*–MKP1→MKP1+X	f
0.83	X*–PP2A→PP2A+X	f	GTP–Ras+GAP⇌TR-GAP	f
0.83	X*+PP2A⇌X*–PP2A	f	PKC_{cyt}+DAG⇌PKC–DAG	b
0.83	X*+PP2A⇌X*–PP2A	f	PKC_{cyt}+Ca⇌PKC–Ca	b
0.83	X*–PP2A→PP2A+X	f	AA→APC	f
0.83	X*+PP2A⇌X*–PP2A	f	PKC–DAG+AA⇌PKC–DAG–AA	b
0.83	X*+PP2A⇌X*–PP2A	f	GAP*→GAP	f
0.83	X*–PP2A→PP2A+X	f	Raf*+GTP–Ras⇌RG-Ras*	b
0.83	X*–PP2A→PP2A+X	f	TR-GAP→GAP+GDP–Ras	f
0.83	X*+PP2A⇌X*–PP2A	f	$Ca+Ca_{pump}⇌Ca–Ca_{pump}$	f
0.83	X*–PP2A→PP2A+X	f	Raf*+PP2A⇌Raf*–PP2A	f
0.83	X*+PP2A⇌X*–PP2A	f	MEK**–PP2A→PP2A+MEK*	f
0.82	X*+PP2A⇌X*–PP2A	f	X*+MKP1⇌X*–MKP1	f

Both mutations are "inhibiting" mutations. PP2A is one of the mutations in the entire set. Reaction with X (or X* for activated) represent enzymatic reaction with multiple species which have the same kinetics. Those with (g) represent reactions for which the various isoforms of the enzyme and its subtrate having the same kinetics. In these reactions, "f" represents the forward reaction while "b" represents the reaction in the reverse direction. The following acronyms are used: RG-Ras* for Raf–GTP–Ras*, DR-GEF(g)* for GDP–Ras–GEF(g)* and TR-GAP for GTP–Ras–GAP.

Table 2
This table ranks the double mutation on the basis of ERK activation at the threshold k_{mod}

k_{mod}	Reaction	Dir	Reaction	Dir
1.19	X*+PP2A⇌X*−PP2A	b	X+RG−Ras*⇌X−RG−Ras*	f
1.205	X*+PP2A⇌X*−PP2A	b	Raf+PKC(g)⇌Raf−PKC(g)	f
1.205	X*+PP2A⇌X*−PP2A	b	X−RG-Ras*→RG-Ras*+X*	f
1.205	X*+PP2A⇌X*−PP2A	b	GTP−Ras+GAP⇌TR-GAP	b
1.205	X*+PP2A⇌X*−PP2A	b	Raf*+GTP−Ras⇌RG-Ras*	f
1.219	X*+PP2A⇌X*−PP2A	b	Raf−PKC(g)→Raf*+PKC(g)	f
1.219	X*+PP2A⇌X*−PP2A	b	GDP−Ras+GEF(g)*⇌DR-GEF(g)*	f
1.219	X*+PP2A⇌X*−PP2A	b	DR-GEF(g)*→GTP−Ras+GEF(g)*	f
1.234	X*+PP2A⇌X*−PP2A	b	X+MEK**⇌X−MEK**	f
1.234	X*+PP2A⇌X*−PP2A	b	MEK*+RG−Ras*⇌MEK*−RG-Ras*	f
1.234	X*+PP2A⇌X*−PP2A	b	GDP−Ras+GEF*⇌GDP−Ras−GEF*	f
1.234	X*+PP2A⇌X*−PP2A	b	PKC−DAG−AA⇌PKC-DAG-AA*	f
1.234	X*+PP2A⇌X*−PP2A	b	PKC−DAG+AA⇌PKC−DAG−AA	f
1.25	X*+PP2A⇌X*−PP2A	b	GAP+PKC(g)⇌GAP−PKC(g)	f
1.25	X*+PP2A⇌X*−PP2A	b	X−MEK**→X*+MEK**	f
1.25	X*+PP2A⇍X*−PP2A	b	GEF+PKC(g)⇌GEF−PKC(g)	f
1.25	X*+PP2A⇌X*−PP2A	b	PKC_{cyt}+DAG⇌PKC−DAG	f
1.25	X*+PP2A⇌X*−PP2A	b	Ca+Ca_{pump}⇌Ca−Ca_{pump}	b
1.25	X*+PP2A⇌X*−PP2A	b	PKC−Ca⇌PKC−$Ca^*_{membrane}$	f
1.25	X*+PP2A⇌X*−PP2A	b	GDP−Ras−GEF*→GTP−Ras+GEF*	f
1.25	X*+PP2A⇌X*−PP2A	b	MEK*−RG−Ras*→MEK**+RG−Ras*	f
1.25	X*+PP2A⇌X*−PP2A	b	MEK+RG−Ras*⇌MEK−RG−Ras*	f
1.25	X*+PP2A⇌X*−PP2A	b	MEK−RG−Ras*→MEK*+RG-Ras*	f
1.25	X*+PP2A⇌X*−PP2A	b	PKC_{cyt}+Ca⇌PKC−Ca	f
1.25	X*+PP2A⇌X*−PP2A	b	Gαβγ→Gα−GTP+Gβγ	f

Both mutations are "activating" mutations. PP2A is one of the mutations in the entire set. Reactions with X (or X* for activated) represent enzymatic reaction with multiple species which have the same kinetics. Those with (g) represent reactions for which the various isoforms of the enzyme and its substrate have the same kinetics. In these reactions, "f" represents the forward reaction while "b" represents the reaction in the reverse direction. The following acronyms are used: RG-Ras* for Raf−GTP−Ras*, DR-GEF(g)* for GDP−Ras−GEF(g)* and TR-GAP for GTP−Ras−GAP.

Table 3
This table ranks the double mutation on the basis of ERK activation at the threshold k_{mod}

k_{mod}	Reaction	Dir	k_{mod}	Reaction	Dir
0.88	X*+PP2A⇌X*−PP2A	f	1.136	X*+PP2A⇌X*−PP2A	b
0.85	X*+PP2A⇌X*−PP2A	f	1.176	X+RG−Ras*⇌X−RG−Ras*	f
0.85	X*+PP2A⇌X*−PP2A	f	1.176	X−RG-Ras*→RG−Ras*+X*	f
0.85	X*+PP2A⇌X*−PP2A	f	1.176	Raf+PKC(g)⇌Raf−PKC(g)	f
0.85	X*−PP2A→PP2A+X	f	1.176	X*+PP2A⇌X*−PP2A	b
0.84	X*+PP2A⇌X*−PP2A	f	1.19	GDP−Ras+GEF(g)*⇌DR−GEF(g)*	f
0.84	X*+PP2A⇌X*−PP2A	f	1.19	DR−GEF(g)*→GTP−Ras+GEF(g)*	f
0.84	X*+PP2A⇌X*−PP2A	f	1.19	Raf−PKC(g)→PKC(g)+Raf*	f
0.84	X*−PP2A→PP2A+X	f	1.19	X+RG−Ras*⇌X−RG−Ras*	f
0.83	X*+PP2A⇌X*−PP2A	f	1.205	GAP+PKC(g)⇌GAP−PKC(g)	f
0.83	X*+PP2A⇌X*−PP2A	f	1.205	X+MEK**⇌X−MEK**	f
0.83	X*+PP2A⇌X*−PP2A	f	1.205	X−MEK**→X*+MEK**	f
0.83	X*−PP2A→PP2A+X	f	1.205	X−RG-Ras*→RG-Ras*+X*	f
0.83	X*−PP2A→PP2A+X	f	1.205	Raf+PKC(g)⇌Raf−PKC(g)	f
0.83	Raf*+GTP−Ras⇌RG−Ras*	b	1.205	X*+PP2A⇌X*−PP2A	b
0.83	Raf*+PP2A⇌Raf*−PP2A	f	1.205	X*+PP2A⇌X*−PP2A	b
0.83	GTP−Ras+GAP⇌TR-GAP	f	1.205	X*+PP2A⇌X*−PP2A	b
0.83	TR-GAP→GAP+GDP−Ras	f	1.205	X*+PP2A⇌X*−PP2A	b
0.83	AA→APC	f	1.205	X*+PP2A⇌X*−PP2A	b
0.82	Ca−Ca_{pump}→Ca_{pump}+Ca_{ext}	f	1.219	X*+PP2A⇌X*−PP2A	b
0.82	Raf*−PP2A→PP2A+Raf	f	1.219	X*+PP2A⇌X*−PP2A	b
0.82	Raf+PKC(g)⇌Raf−PKC(g)	b	1.219	X*+PP2A⇌X*−PP2A	b
0.82	X+RG-Ras*⇌X−RG-Ras*	b	1.219	X*+PP2A⇌X*−PP2A	b
0.82	X*−PP2A→PP2A+X	f	1.219	Raf−PKC(g)→PKC(g)+Raf*	f
0.82	X*−PP2A→PP2A+X	f	1.219	DR−GEF(g)*→GTP−Ras+GEF(g)*	f

The mutations described in columns 2 and 3 represent "inhibiting" mutations with k_{mod} values in column 1 while those described in columns 5 and 6 represent "activating" mutations with k_{mod} values in column 4. Reactions with X (or X* for activated) represent enzymatic reactions with multiple species which have the same kinetics. Those with (g) represent reactions for which the various isoforms of the enzyme and its substrate have the same kinetics. In these reactions, "f" represents the forward reaction while "b" represents the reaction in the reverse direction. The following acronyms are used: RG-Ras* for Raf−GTP−Ras*, DR-GEF(g)* for GDP−Ras−GEF(g)* and TR-GAP for GTP−Ras−GAP.

and of the Raf–PKC interaction. All these alterations are immediately upstream of ERK and lead to enhanced ERK activation in a direct manner. A mutation in the degradation of AA and DAG is also significant. This results in the accumulation of AA and DAG in the cytoplasm, which activates PKC leading to the same result downstream. A defect in the action of the Ca pump, which would cause excessive Ca to be present in the cytoplasm is also ranked highly. Ca is involved in the activation of PKC and PLA2, both of which are upstream effectors of ERK.

Table 2 contains the reaction pairs where both the mutations are "activating". A mutation which favors the disassociation of PP2A complexed with its substrate is involved in all the top-ranked pair mutations. This is expected, as this is equivalent to the PP2A reaction from the previous table in the reverse direction, which inhibits the complex formation of PP2A with its substrate. For bi-directional reactions, an inhibiting step in one direction is equivalent to an activating step in the other direction, and therefore such mutations would show up in both tables. For example, the mutation inhibiting the association step in the Ras–GAP interaction in Table 1 at $k^i_{\mathrm{mod}}=0.85$ has an equivalent one in Table 2 $k^i_{\mathrm{mod}}=1.205$, which promotes the disassociation of the Ras–GAP complex.

Table 3 displays those mutation pairs where one is inhibiting while the other is activating. The highest ranked is redundant in PP2A. This redundancy highlights the importance of PP2A mutations in malignant transformation. This topped-ranked mutation contains the inhibition of PP2A association and the activation of PP2A disassociation, which are equivalent. The next two ranked mutation pairs couple the inhibiting action of PP2A (at $k^i_{\mathrm{mod}}=0.88$) with an activating mutation for the enzymatic action of the activated GTP–Ras–Raf complex (at $k^i_{\mathrm{mod}}=1.176$). Activating mutations for the enzymatic action of activated GEF and activated MEK are also present. At $k^i_{\mathrm{mod}}=0.83$, a Ras–GAP inhibiting reaction is paired with a PP2A activating reaction. The important point to notice in these tables is that a smaller mutation (viz. one with k^i_{mod} value closer to unity) in the individual reactions is sufficient to cumulatively cause malignant transformation.

Sensitivity analysis is also used to predict malignant transformation for the three kinds of mutation pairs discussed above. As shown in the previous paper, the results from this method are nearly identical in ranking to the rigorous "direct" method for single mutations [1]. Table 4 displays the results for the pair inhibiting mutations, in decreasing order of the sensitivity. A comparison between Tables 1 and 4 is also very striking. Though not shown here, this is the case for the other two combinations (viz. activating–activating and inhibiting–activating) as well. This indicates that the system is nearly linear in its response to the system parameters at steady state and the correlative effects are minimal. Indeed, the top 5 predicted interactions using sensitivity analysis match exactly with the more rigorous analysis except for a few minor switches in ranking. The rankings are very similar using both

Table 4
This table ranks the double mutations from the linear response analysis

Response	Reaction	Dir	Reaction	Dir
0.0026815	X*+PP2A⇌X*–PP2A	f	X*–PP2A→PP2A+X	f
0.0021632	X*+PP2A⇌X*–PP2A	f	Raf*+GTP–Ras⇌RG-Ras*	b
0.0021172	X*+PP2A⇌X*–PP2A	f	GTP–Ras+GAP⇌TR-GAP	f
0.0021136	X*+PP2A⇌X*–PP2A	f	TR-GAP→GAP+GDP–Ras	f
0.002046	X*+PP2A⇌X*–PP2A	f	X+RG-Ras*⇌X–RG-Ras*	b
0.0019851	X*+PP2A⇌X*–PP2A	f	$Ca-Ca_{pump}\rightarrow Ca_{pump}+Ca_{ext}$	f
0.0019832	X*+PP2A⇌X*–PP2A	f	Raf*–PP2A→PP2A+Raf	f
0.0019765	X*+PP2A⇌X*–PP2A	f	Raf+PKC(g)⇌Raf–PKC(g)	b
0.0019726	X*+PP2A⇌X*–PP2A	f	AA→APC	f
0.0019288	X*+PP2A⇌X*–PP2A	f	GDP–Ras+GEF(g)*⇌DR-GEF(g)*	b
0.0019007	X*+PP2A⇌X*–PP2A	f	$Ca+Ca_{pump}\rightleftharpoons Ca-Ca_{pump}$	f
0.0018746	X*+PP2A⇌X*–PP2A	f	DAG→PC	f
0.0018716	X*+PP2A⇌X*–PP2A	f	$PKC_{cyt}+Ca\rightleftharpoons PKC-Ca$	b
0.0018652	X*–PP2A→PP2A+X	f	Raf*+GTP–Ras⇌RG-Ras*	b
0.0018309	X*+PP2A⇌X*–PP2A	f	PKC–DAG–AA⇌PKC–DAG–AA*	b
0.001827	X*+PP2A⇌X*–PP2A	f	PKC–DAG+AA⇌PKC–DAG–AA	b
0.0018215	X*–PP2A→PP2A+X	f	Raf*+PP2A⇌Raf*–PP2A	f
0.00182	X*–PP2A→PP2A+X	f	GTP–Ras+GAP⇌TR-GAP	f
0.0018165	X*–PP2A→PP2A+X	f	TR-GAP→GAP+GDP–Ras	f
0.0018088	X*+PP2A⇌X*–PP2A	f	X*+MKP1⇌X*–MKP1	f
0.0017896	X*+PP2A⇌X*–PP2A	f	$PKC-Ca\rightleftharpoons PKC-Ca^*_{membrane}$	b
0.0017781	X*+PP2A⇌X*–PP2A	f	X*–MKP1→MKP1+X	f
0.0017659	X*+PP2A⇌X*–PP2A	f	MEK**–PP2A→PP2A+MEK*	f
0.001763	X*+PP2A⇌X*–PP2A	f	X+MEK**⇌X–MEK**	b
0.0017606	X*+PP2A⇌X*–PP2A	f	MEK*+RG-Ras*⇌MEK*–RG-Ras*	b

The first column is the value of the linear response on the basis of which the double mutations are ranked in importance. Both the mutations are "inhibiting" mutations. PP2A is one of the mutation in the entire set. Reactions with X (or X* for activated) represent enzymatic reactions with multiple species which have the same kinetics. Those with (g) represent reactions for which the various isoforms of the enzyme and its substrate have the same kinetics. In these reactions, "f" represents the forward reaction while "b" represents the reaction in the reverse direction. The following acronym are used: RG-Ras* for Raf–GTP–Ras*, DR-GEF(g)* for GDP– Ras–GEF(g)* and TR-GAP for GTP–Ras–GAP.

Table 5
This table lists the highest ranked "expression" level pairs

Rank	Enzyme 1	Enzyme 2
1	PP2A (U)	PKC_{cyt} (O)
2	PP2A (U)	Raf (O)
3	PP2A (U)	Ras (O)
4	PP2A (U)	GAP (U)
5	PP2A (U)	GEF (O)
6	PKC_{cyt} (O)	Raf (O)
7	PP2A (U)	$PLA2_{cyt}$ (O)
8	PKC_{cyt} (O)	Ras (O)
9	PKC_{cyt} (O)	GAP (U)
10	PP2A (U)	MKP-1 (U)

From this table, for the pair ranked at the top, a change in the expression level of activated ERK is most sensitive to PP2A and PKC expression levels and a small change in their expression level would cause ERK to have (non-EGF stimulated) high steady state levels. The letter in the brackets indicates whether the enzyme is overexpressed (O) or underexpressed (U).

methods. This validates the use of sensitivity analysis for this system of pathways.

Expression level pair mutations have also been studied using our method. In this type of mutation, the expression levels of the proteins in the cytoplasm are different from a normally functioning cell, possibly due to mutations in the transcription process leading to excess/fewer amount of protein. Once again, the signal for such mutations is the constant elevated concentrations of activated ERK in the absence of EGF. The highly ranked ones are for those species which cause this effect is a result of the smallest change in their expression levels. Table 5 shows several such cases for the highly ranked ones. This table has been generated using just sensitivity analysis. Under-expression of PP2A coupled with the overexpression of cytosolic PKC is the highest ranked. These individual mutations were the highest ranked single point mutations (see Ref. [1]. Next in ranking is PP2A underexpression and Raf over-expression. This is followed by PP2A underexpression with Ras overexpression. Underexpressed GAP coupled with PP2A is also highly ranked, expected to cause oncogenesis. At the fifth position is PP2A and overexpression of GEF. The sixth and eighth positions have overexpressed PKC coupled with Ras and Raf. Over-expression of cytosolic PLA-2 is also paired with PP2-A as a highly ranked mutation. As can be seen, in all the top ten ranked paired mutations, altered expression levels of PP2A and PKC are present as one member of the pair. These two enzymes were predicted as the top ranked single expression level mutations and so it is reasonable to expect this pair to be a good indicator of cellular transformation [1]. It is interesting to note that while PP2-A when singly underexpressed activates ERK at a relative concentration of 0.7 and PKC activates ERK when overexpressed at 1.4, together they activate ERK when PP2-A is underexpressed at 0.9 and PKC is overexpressed at 1.2. Similarly, Raf overexpressed at 1.6 activates ERK but when both Raf and PP2A are mutated, Raf overexpression is calculated to be just 1.3 and PP2A underexpression is 0.9. If we examine the effect of three simultaneous mutations, the relative concentrations needed (from the calculation) to cause ERK activation are PP2A: 0.9, PKC: 1.1 and Raf: 1.1, which are even smaller perturbations from the unmutated pathway. In other words, smaller changes in expression levels of the enzymes when considered simultaneously are sufficient to cause synergistic activation of ERK.

4. Influence of correlations among mutations

In a network of interacting pathways, isolated mutations at different junctions in the pathways can act synergistically to influence the outcome of a signaling event, for instance, [ERK] activation. Alternatively, it is possible that separate mutations interfere destructively towards the final outcome. Thus, a mutational event expected to cause abnormal cell growth is observed to not do so experimentally, possibly because of other interacting mutations which negate the effect of the former. To understand this, we have done perturbation analysis to higher order (specifically, including second order terms) and ranked

Table 6
This table displays the magnitude of the first order terms (shown in columns 2 and 3) as compared to the second order terms (columns 4, 5 and 6) for mutation pair in the rate constants, shown in the last two columns

d[ERK]	1st order terms		2nd order terms			Reaction 1	Reaction 2
1.236	0.615	0.614	0.0015	0.0015	0.0025	craf-1*–PP2A f	GTP-Ras–GAP f
1.261	0.605	0.651	0.0009	0.0011	0.0026	GTP–Ras–GAP r	craf-1*–GTP–Ras f
1.265	0.605	0.654	0.0009	0.0017	0.0026	GTP–Ras–GAP r	craf-1*–GTP–Ras r
1.271	0.614	0.651	0.0015	0.0011	0.0027	GTP-Ras–GAP f	craf-1*–GTP–Ras f
1.272	0.615	0.651	0.0015	0.0011	0.0027	craf-1*–PP2A f	craf-1*–GTP–Ras f
1.274	0.614	0.654	0.0015	0.0017	0.0027	GTP–Ras – GAP f	craf-1*–GTP–Ras r
1.276	0.615	0.654	0.0015	0.0017	0.0027	craf-1*–PP2A f	craf-1*–GTP–Ras r
1.311	0.651	0.654	0.0011	0.0017	0.0028	craf-1*–GTP–Ras f	craf-1*–GTP–Ras r

In the columns listing the reactions, "f" represents the forward reaction while "r" represents the reverse reaction. Clearly, d[ERK] is dominated by the first order terms for all mutation pairs.

Table 7
This table displays the magnitude of the first order terms (shown in columns 2 and 3) as compared to the second order term (columns 4, 5 and 6) for "expression level" mutation pairs

d[ERK]	1st order terms		2nd order terms			Enzyme 1	Enzyme 2
1.728	1.192	0.527	0.004367	0.000649	0.004300	PKC-cyt(O)	inact-GEF(O)
1.756	0.383	1.361	0.000709	0.007023	0.003688	MKP-1(U)	PP2A(U)
1.820	1.192	0.617	0.004367	0.001577	0.004973	PKC-cyt(O)	GAP(U)
1.848	1.192	0.645	0.004367	0.001095	0.005308	PKC-cyt(O)	GDP-Ras(O)
1.857	1.361	0.483	0.007023	0.000716	0.004594	PP2A(U)	PLA2-cyt(O)
1.894	0.690	1.192	0.001302	0.004367	0.005670	craf-1(O)	PKC-cyt(O)
1.900	1.361	0.527	0.007023	0.000649	0.004951	PP2A(U)	inact-GEF(O)
1.992	1.361	0.617	0.007023	0.001577	0.005797	PP2A(U)	GAP(U)
2.020	1.361	0.645	0.007023	0.001095	0.006098	PP2A(U)	GDP-Ras(O)
2.066	0.690	1.361	0.001302	0.007023	0.006524	craf-1(O)	PP2A(U)
2.576	1.361	1.192	0.007023	0.004367	0.011170	PP2A(U)	PKC-cyt(O)

"O" represents overexpressed and "U" represents underexpressed. Like the previous table, d[ERK] is dominated by the first order term for all the mutation pairs.

the pair mutations on the magnitude of the correlation term (this is the final term in Eq. (4)). The positively correlated terms suggest that such mutations are synergistic while the negatively correlated terms suggest annihilating mutations.

Sensitivity analysis is carried out by modifying two parameters (either the reaction rate constants or the enzyme concentrations) at a time, and computing this crossterm. The first and second derivative terms in Eq. (4) are computed numerically as well. Table 6 shows each of the computed terms from Eq. (4) for the highest ranked mutation pairs. From the magnitude of these terms, it appears that the second order terms contribute almost insignificantly compared to the first order terms. This is the case for all the pairs. This implies that the single point mutations contribute virtually independently in a pair mutation event. Therefore, the highest ranked pairs are expected to be represented primarily by the highest ranked single point mutations. This is seen in Tables 1, 2 and 3. We verified this for triple mutation events as well, in that the contribution to d[ERK] is simply the sum of the three first order terms with the higher order terms contributing minimally. Table 7 shows a similar result for paired "express on level" mutations.

Therefore, underexpression of PP2-A and overexpression of PKC, which are the highest ranked single expression level mutations, are also in the top-ranked pair expression level mutations (Table 5. From this analysis, it seems unlikely that weak mutations would cumulatively couple with other weak mutations to stimulate ERK activation. The highest ranked single point mutations would be the most likely to show up in multiple mutations, whether pair, triple, quadruple mutations or higher.

5. Discussion

There is a fair amount of experimental evidence to support the results predicted by this purely computational approach. We examine a few results of Table 5. Underexpression of PP2A (a serine/threonine phosphatase) coupled with the overexpression of cytosolic PKC (a serine/threonine kinase) is ranked the highest. Evidence of these simultaneous expression mutations in multidrug resistant cells is reported in the work of Ratnasinghe et al. [32]. Their findings suggest there is a pattern consistent with the maintenance of serine and threonine residues in a phosphorylated state for drug resistant cells. The next in ranking is PP2A underexpression and Raf over-expression. Sundaram et al. have done a genetic screen on Raf mutants to identify positive regulators of Ras signaling and PP2A is among the hypomorphic alleles identified [33].

This next highest predicted ranking in our table involves PP2A and Ras over- expression. This is equivalent to the result in the paper by Baharians and Schonthal [34], in which they report that elevated levels of PP2A lessens the transformation of rat fibroblasts by the Ras oncogene. In another work [35], Hahn et al. suppress the expression of PP2A in human embryonic kidney cells expressing H-Ras and show that this leads to tumor growth. The sixth and eighth positions have overexpressed PKC coupled with Ras and Raf. While there is no direct evidence of this in the experimental literature, there is evidence that adrenomedullin-overexpressing T47D breast cancer cell lines have higher levels of proteins involved in oncogenic signal transduction pathways (such as Ras, Raf and PKC) [36].

As before, the main limitation of the current work is that not all interacting partners of these pathways are well-known. It is

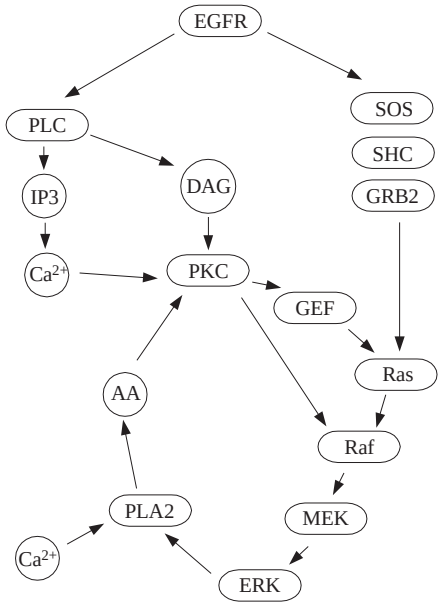

Fig. 3. A block diagram of the Ras/Raf/MEK/ERK signaling pathway, indicating the feedback loop for ERK.

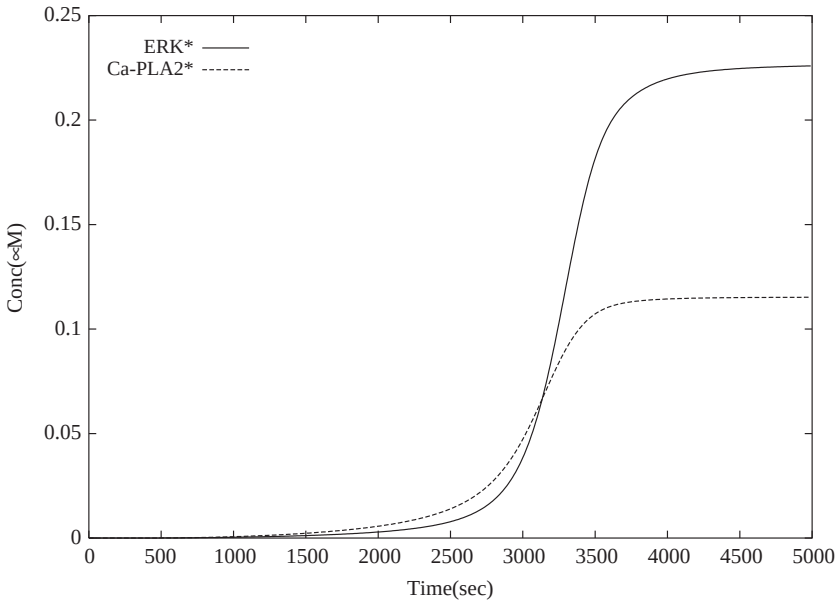

Fig. 4. Time evolution of activated ERK and the activated Ca–PLA2 complex, showing the coincident rise in concentration levels of both the species.

not expected that transformation of a cell will include only mutations in the MAPK pathway, but rather amongst the members of the MAPK pathway which are mutated in cancer lines, those ranked the highest are predicted to be mutated the most. For the most part, the major contributor to the response is additive rather than correlative. This may not necessarily be true, however, as other, parallel pathways are considered. It is of interest to note that several major cases where correlative effects existed in which two mutations that were not found to cause activation alone played a major role when coupled together. These coupled mutations represent the difficulty in pinpointing a root cause to "cancer" and highlight how distant mutations in signal pathways may act cumulatively (though in most cases additively) to cause malignant transformation.

An interesting case of synergistic coupling in the PLC pathway was found for the following pair of interactions:

$$\mathrm{PLC} + \mathrm{Gq} \rightleftharpoons \mathrm{PLC.Gq} \tag{6}$$

$$\mathrm{Ca} + \mathrm{PLC.Gq} \rightleftharpoons \mathrm{Ca.PLC.Gq}. \tag{7}$$

For the first reaction, the mutation corresponds to a complete suppression of the disassociation reaction. In the second reaction, the mutation is a complete suppression of the association reaction. Both of the above mutated reactions indicate excess formation of the complex PLC.Gq. This would imply very low concentrations of Ca.PLC and Ca.PLC.Gq which are produced, respectively, by reaction

$$\mathrm{PLC} + \mathrm{Ca} \rightleftharpoons \mathrm{Ca.PLC} \tag{8}$$

and reaction (7). These complexes of PLC are catalysts in the production of DAG from PIP2. Thus, a lower concentration of DAG would imply less activated PKC, which would eventually result in less activated ERK. However, there are actually competing effects between AA and DAG and the PLA2 feedback network involved. Reaction 6 implies complete consumption of Gq to form PLC.Gq, since the reverse reaction is mutated with a modified rate constant of zero. Gq is supplied from the following reaction

$$G - \mathrm{GDP} \rightarrow \mathrm{Gq} + G\beta\gamma. \tag{9}$$

The mutation effectively drives the above reaction to completion since Gq is being consumed as it is produced. This leads to a high concentration of $G\beta\gamma$. Since Gq is present in reaction (6) in a normal pathway, the concentration of $G\beta\gamma$ reaches a lower steady state value. $G\beta\gamma$ complexes with GEF. This complex is a catalyst for the conversion of GDP–Ras to GTP–Ras. Thus, in the mutated reaction pathway, the concentration of GTP–Ras is higher through the activation of GEF. Despite reduced DAG production, the excess GTP–Ras produced through the activation of GEF results in malignant

Table 8
This table lists the five mutated reaction pair which cause ERK activation at $k_{\mathrm{mod}}=0.2$

Reaction	Dir	Reaction	Dir
$ERK_{tyr}+MEK^{**}\rightleftharpoons ERK_{tyr}-MEK^{**}$	b	GEF+PKC-DA⇌GEF–PKC-DA	b
$ERK_{tyr}+MEK^{**}\rightleftharpoons ERK_{tyr}-MEK^{**}$	b	APC+Ca-PLA2*⇌APC–Ca-PLA2*	b
$ERK_{tyr}+MEK^{**}\rightleftharpoons ERK_{tyr}-MEK^{**}$	b	$PLA2_{cyt}+ERK^{*}\rightleftharpoons PLA2_{cyt}-ERK^{*}$	b
GEF+PKC-DA⇌GEF–PKC-DA	b	$PLA2_{cyt}+ERK^{*}\rightleftharpoons PLA2_{cyt}-ERK^{*}$	b
APC+Ca-PLA2*⇌APC–Ca-PLA2*	b	$PLA2_{cyt}+ERK^{*}\rightleftharpoons PLA2_{cyt}-ERK^{*}$	b

Individually, mutations in these reactions are insufficient for ERK activation, even at $k_{\mathrm{mod}}=0$, but when these mutation occur synergistically, non-EGF stimulated ERK activation occurs. This how the cumulative effect of the weakest of interaction mutations. The acronym PKC-DA stands for PKC–DAG–AA*.

transformation of the pathway through the PLA2 activated feedback network as explained below (Fig. 3). The activated ERK produced catalyzes the activation of cytosolic PLA2. In the normal pathway, the concentration of activated ERK is insufficient to generate a significant concentration of activated PLA2, whereas in the mutated pathway, the higher concentration of activated ERK is sufficient to produce a large amount of activated PLA2. Since cytosolic PLA2 is consumed in this reaction, its concentration and therefore the concentration of Ca complexed with PLA2 is lower in the mutated pathway. An increased production of activated PLA2 leads to a much greater concentration of calcium bound PLA2* than observed in the normal pathway. Ca-PLA2* is in turn a catalyst for AA production which in turn activates PKC leading back to the activation of more ERK. The activation of the feedback loop is reflected in the evolution of PLA2* and activated ERK concentrations shown in Fig. 4. The sharp rise in the ERK concentration is coincident with the rise in PLA2* (and Ca-PLA2*).

In the normal pathway, since the Ca-PLA2* concentration is very small, AA production from this catalysis is low. AA is also produced from APC with other catalysts, namely DAG–Ca-PLA2, Ca-PLA2, PIP2–Ca-PLA2 and PIP2–PLA2. However, the concentrations of these species are only about five times larger in the normal pathway than in the mutated pathway, whereas Ca-PLA2* concentration in a pathway with this particular double site mutation is about three hundred times that of the normal signaling pathway.

Cumulative mutations may sometimes activate ERK when individually neither of these mutations lead to activation (even for the extreme case of complete inhibition when the perturbation parameter k^i_{mod} is set to 0). Investigation of such pairs leads to the finding that 110 reaction pairs exist which activate ERK at $k^i_{\mathrm{mod}}=0$ and five pairs at $k^i_{\mathrm{mod}}=0.2$. These five pairs are listed in Table 8. The small value for k^i_{mod} (i.e. 0.2) implies that the interaction mutations are quite strongly perturbed before the effects of this mutation can manifest. One mutation which is featured prominently in this set is the promotion of the first phosphorylation of ERK via doubly phosphorylated MEK by suppressing ERK–MEK disassociation. This mutation is coupled with interactions from the PLA2 pathway as well as the PKC pathway. The last reaction pair in the table is entirely from the PLA-2 pathway. This mutation can be explained as follows: Suppression of the PLA–2–ERK disassociation generates more activated PLA-2, which leads to more Ca–PLA-2. This is an enzyme for the production of arachidonic acid (AA) from APC. The other mutation in this pair suppresses disassociation of the APC-enzyme complex, which also produces more AA. Thus, both mutations cumulatively generate more AA, which activates PKC leading eventually to higher levels of activated ERK. The mutational points here belong to the same pathway while the MEK–ERK mutation pairs are from two completely separate pathways.

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