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# MLK3 Is Part of a Feedback Mechanism That Regulates Different Cellular Responses to Reactive Oxygen Species

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**Reactive oxygen species (ROS) influence diverse cellular processes, including proliferation and apoptosis. Both endogenous and exogenous ROS activate signaling through mitogen-activated proteins kinase (MAPK) pathways, including those involving extracellular signal–regulated kinases (ERKs) or c-Jun N-terminal kinases (JNKs). Whereas low concentrations of ROS generally stimulate proliferation, high concentrations result in cell death. We found that low concentrations of ROS induced activating phosphorylation of ERKs, whereas high concentrations of ROS induced activating phosphorylation of JNKs. Mixed lineage kinase 3 (MLK3, also known as MAP3K11) directly phosphorylates JNKs and may control activation of ERKs. Mathematical modeling of MAPK networks revealed a positive feedback loop involving MLK3 that determined the relative phosphorylation of ERKs and JNKs by ROS. Cells exposed to an MLK3 inhibitor or cells in which MLK3 was knocked down showed increased activation of ERKs and decreased activation of JNKs and were resistant to cell death when exposed to high concentrations of ROS. Thus, the data indicated that MLK3 is a critical factor controlling the activity of kinase networks that control the cellular responses to different concentrations of ROS.**

## INTRODUCTION

Reactive oxygen species (ROS) are generated as by-products of cellular metabolism and function as second messenger molecules that regulate numerous cellular processes, including proliferation, senescence, differentiation, cell cycle, and apoptosis (*1*–*4*). The intracellular concentration of ROS can determine whether cells undergo proliferation or cell cycle arrest and apoptosis; low concentrations of ROS induce proliferation, whereas high concentrations of ROS induce cell cycle arrest and apoptosis (*3*–*5*).

ROS can promote activation of extracellular signal–regulated kinases 1 and 2 (ERKs, also known as MAPK3 and MAPK1) (*6*, *7*). ERKs are members of a family of mitogen-activated protein kinases (MAPKs) that are activated by various extracellular stimuli such as growth factors, cytokines, ligands for heterotrimeric G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptors, and carcinogens (*8*). ERKs are involved in biological functions including the regulation of cell growth and anti-apoptosis (*9*). The activation of ERK2 promotes cell proliferation in part by phosphorylating and promoting degradation of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (encoded by the gene *CDKN1A*) (*10*).

ROS can also promote activation of the c-Jun N-terminal kinases 1 and 2 (JNKs, also known as MAPK8 and MAPK9) (*11*). JNKs are also members of the MAPK family, like ERKs, and are activated by stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock (*12*, *13*), thereby promoting apoptotic cell death (*13*, *14*). In contrast to ERK2, JNK1 promotes phosphorylation of $p21^{Cip1}$, leading to stabilization (*15*).

The activity of MAPKs including ERKs and JNKs is regulated through direct phosphorylation by a number of upstream kinases, including mixed lineage kinase 3 (MLK3, also known as MAP3K11). MLK3 is a ubiquitously expressed mammalian mitogen-activated protein kinase kinase kinase (MAP3K) in the JNKs pathway (*16*). MLK3 induces activation of JNKs and promotes cell death by directly phosphorylating kinases upstream of JNKs (*17*, *18*). Whether MLK3 activates or inhibits ERKs remains controversial. One group has shown that MLK3 activates ERKs in a kinase-independent manner (*19*, *20*), whereas another group has shown that MLK3 negatively regulates activation of ERKs through a transcriptional mechanism (*21*).

ROS regulates various cellular processes by influencing signaling pathways that involve complex mechanisms of feedback and crosstalk (*22*, *23*). Here, we found that different concentrations of ROS lead to reciprocal phosphorylation of ERKs and JNKs, which serves to coordinate ROS-dependent cellular responses. Mathematical modeling of MAPK networks and biochemical experiments revealed that MLK3 mediates a positive feedback loop (PFL), which balances ROS concentration–dependent signal flow between ERK and JNK pathways, leading to proliferation or cell death.

## RESULTS

### Reciprocal phosphorylation of ERKs and JNKs in response to ROS exposure

Previous studies demonstrate that cells either proliferate or die when exposed to ROS, depending on the concentration (*5*). Similarly, we found that exposing HeLa cells to a low concentration of $H_2O_2$ increased, whereas a high concentration decreased, the number of viable cells (fig. S1A). Moreover, exposing cells to a high, but not low, concentration of $H_2O_2$ increased the frequency of tetraploid and subdiploid cells (fig. S1B), suggesting that these cells were undergoing cell cycle arrest and apoptosis.

The cyclin-dependent kinase inhibitor $p21^{Cip1}$ suppresses proliferation by inhibiting cell cycle progression (*24*, *25*). Previously, we demonstrated that exposing HeLa cells to concentrations of $H_2O_2$ between 100 and 500 μM triggers the degradation of $p21^{Cip1}$, whereas exposure to concentrations greater than 500 μM does not (*26*). Here, we confirmed that exposing HeLa cells to different concentrations of $H_2O_2$ for 1 hour produced a biphasic

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response. The abundance of $p21^{Cip1}$ was strongly decreased in cells exposed to low concentrations of $H_2O_2$, but was not as affected in cells exposed to high concentrations of $H_2O_2$ (fig. S1C). In contrast, the abundance of the cyclin-dependent kinase inhibitors $p16^{Ink4a}$ (encoded by the gene *CDKN2A*) and $p27^{Kip1}$ (encoded by the gene *CDKN1B*) was unchanged in cells exposed to low or high concentrations of $H_2O_2$ for 1 hour (fig. S1C). ERKs and JNKs phosphorylate $p21^{Cip1}$ and affect its stabilization in opposite ways (*10*, *15*). We used small molecules to determine whether ERKs and JNKs affect the steady-state abundance of $p21^{Cip1}$ in HeLa cells exposed to a low concentration of $H_2O_2$. We found that U0126, which inhibits the upstream ERK-activating MEK (MAPK kinases, also known as MAP2Ks) (*27*), but not SP600125, an ATP (adenosine 5′-triphosphate)–competitive inhibitor of JNKs (*28*), reduced the ability of $H_2O_2$ to decrease the abundance of $p21^{Cip1}$ (fig. S1D). Thus, low concentrations of $H_2O_2$ activate ERK to inhibit $p21^{Cip1}$ and drive cell proliferation.

To investigate if the activity of ERKs and JNKs correlated with cellular responses to ROS, we performed Western blot analysis of phosphorylated ERKs and JNKs in lysates from HeLa cells exposed to a range of $H_2O_2$ concentrations for 1 hour. The phosphorylation of the activation loop of ERKs ($Thr^{202}$ and $Tyr^{204}$ in ERK1 and $Thr^{185}$ and $Tyr^{187}$ in ERK2) was robustly increased at the lowest concentration of $H_2O_2$ and inversely correlated with increasing concentrations of $H_2O_2$ (Fig. 1A). In contrast, the phosphorylation of JNKs ($Thr^{183}$ and $Tyr^{185}$) increased as a function of increasing concentrations of $H_2O_2$ (Fig. 1B). The reciprocal responses of ERKs and JNKs to varying concentrations of $H_2O_2$ are consistent with the hypothesis that these kinases could play a role in determining the cellular response to ROS.

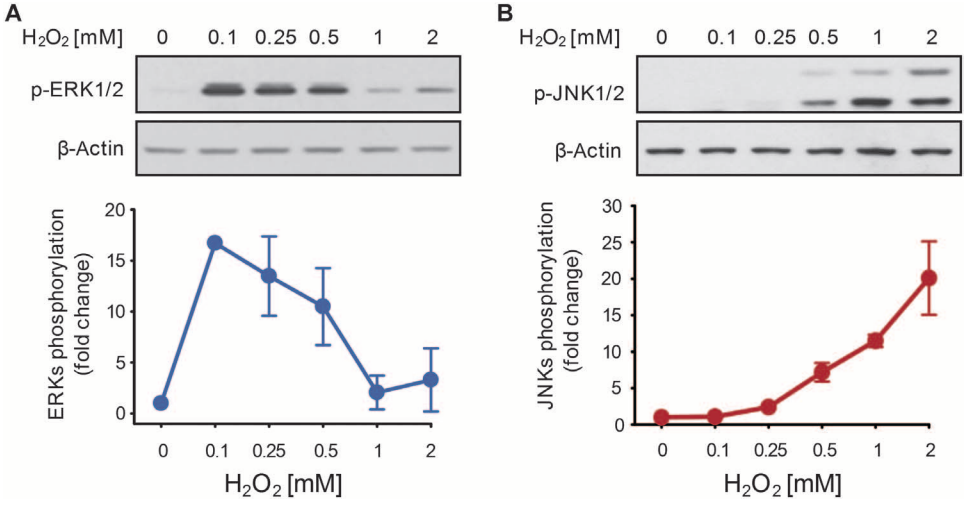


Fig. 1. Concentration-dependent differences in the phosphorylation of ERKs and JNKs in response to ROS. (A and B) Western blots for phosphorylated ERKs (A) or JNKs (B) in lysates of HeLa cells exposed to the indicated concentrations of $H_2O_2$ for 1 hour. The graphs depict the means ± SEM of three blots from biological replicates. β-Actin was used as a loading control.

## A mathematical model of the ROS signaling network with a focus on ERK and JNK signaling

To characterize the underlying mechanism of how different concentrations of ROS produce different cellular responses, we developed a mathematical model of a ROS signaling network with a focus on ERK and JNK signaling (Fig. 2A and tables S1 to S5). We

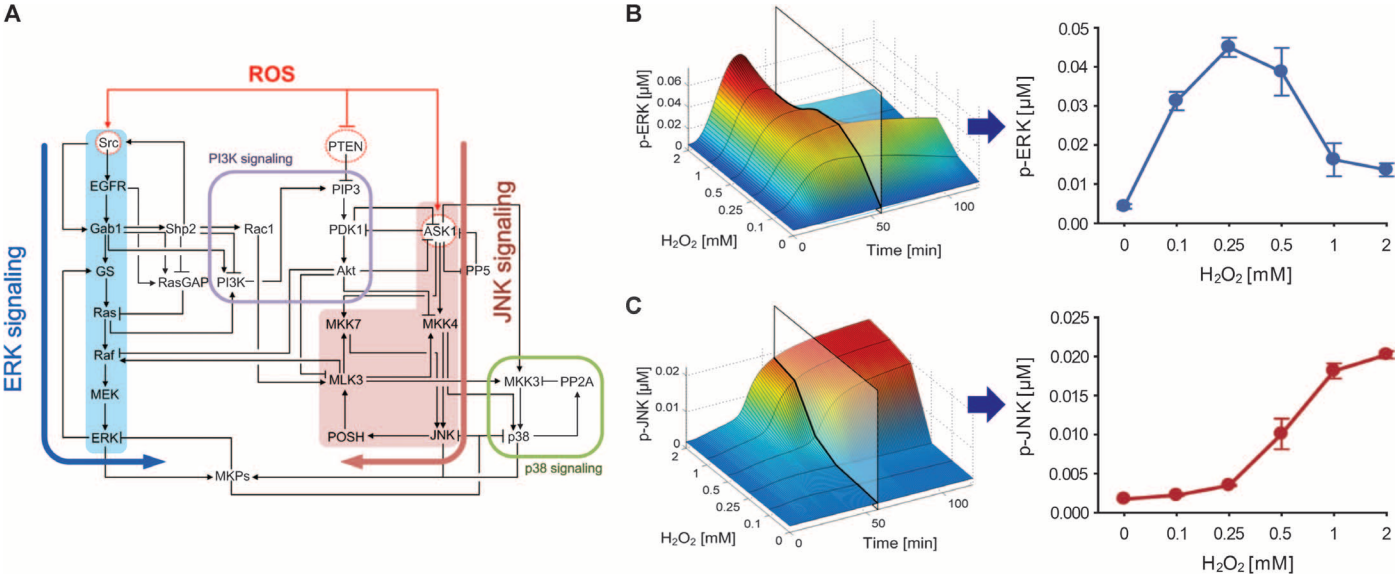


Fig. 2. Simulation of ROS-induced phosphorylation of ERKs and JNKs using a mathematical model. (A) Schematic diagram of the ROS signaling network model (see table S1). The ROS signaling network comprises four major signaling modules involving either PI3K (purple square), p38-MAPK (green square), ERK (thick blue arrow), or JNK (thick red arrow). ROS direct targets include Src, phosphatase and tensin homolog (PTEN), and apoptosis signal–regulating kinase 1 (ASK1) (dotted red circles). GS indicates the growth factor receptor–bound protein 2 (Grb2) and Son of Sevenless (SOS) complex. (B and C) Graphs showing the results of simulations using the mathematical model (see fig. S4).

reconstructed the ROS signaling network using a variety of published data (*4*, *14*, *18–20*, *29–108*). We also determined that small-molecule inhibitors of JNKs, p38-MAPKs, or MLK3 increased phosphorylation of ERKs (fig. S2) and added these data to the model. Previous studies show that MAPK phosphatases (MKPs) play a role in the crosstalk among MAPKs (*21*, *109–116*) (table S6), and thus, we also added the influences of MKPs to the model.

The model consists of four signaling modules, including PI3K (phosphoinositide 3-kinase), p38-MAPK, ERK, and JNK (Fig. 2A), comprising 57 state variables (table S3) and 238 kinetic parameters (table S4). To estimate the kinetic parameters, we used time course data of signaling molecules from previous studies (*36*, *44*, *73*, *79*, *86*, *117–121*). In addition, we measured phosphorylation of Akt ($Ser^{473}$) and p38-MAPK ($Thr^{180}$ and $Tyr^{182}$) in lysates from HeLa cells exposed to a low concentration of $H_2O_2$ from 0 to 6 hours (fig. S3) and used these data to estimate the kinetics of activation of these kinases. We used the model to simulate the time-dependent phosphorylation of ERKs and JNKs with varying concentrations of $H_2O_2$ and found that it recapitulated the experimental data (Fig. 2, B and C). In addition, the results from simulations for several other biochemical species in the ROS signaling network correlated with the experimental data (fig. S4).

## Essential feedback loops responsible for the reciprocal phosphorylation of ERKs and JNKs in response to ROS exposure

Feedback mechanisms are important in cell regulatory biology (*122*, *123*). We identified 13 feedback loops in the ROS signaling network (table S7) and selected a representative interaction (feedback link) for each (fig. S5).

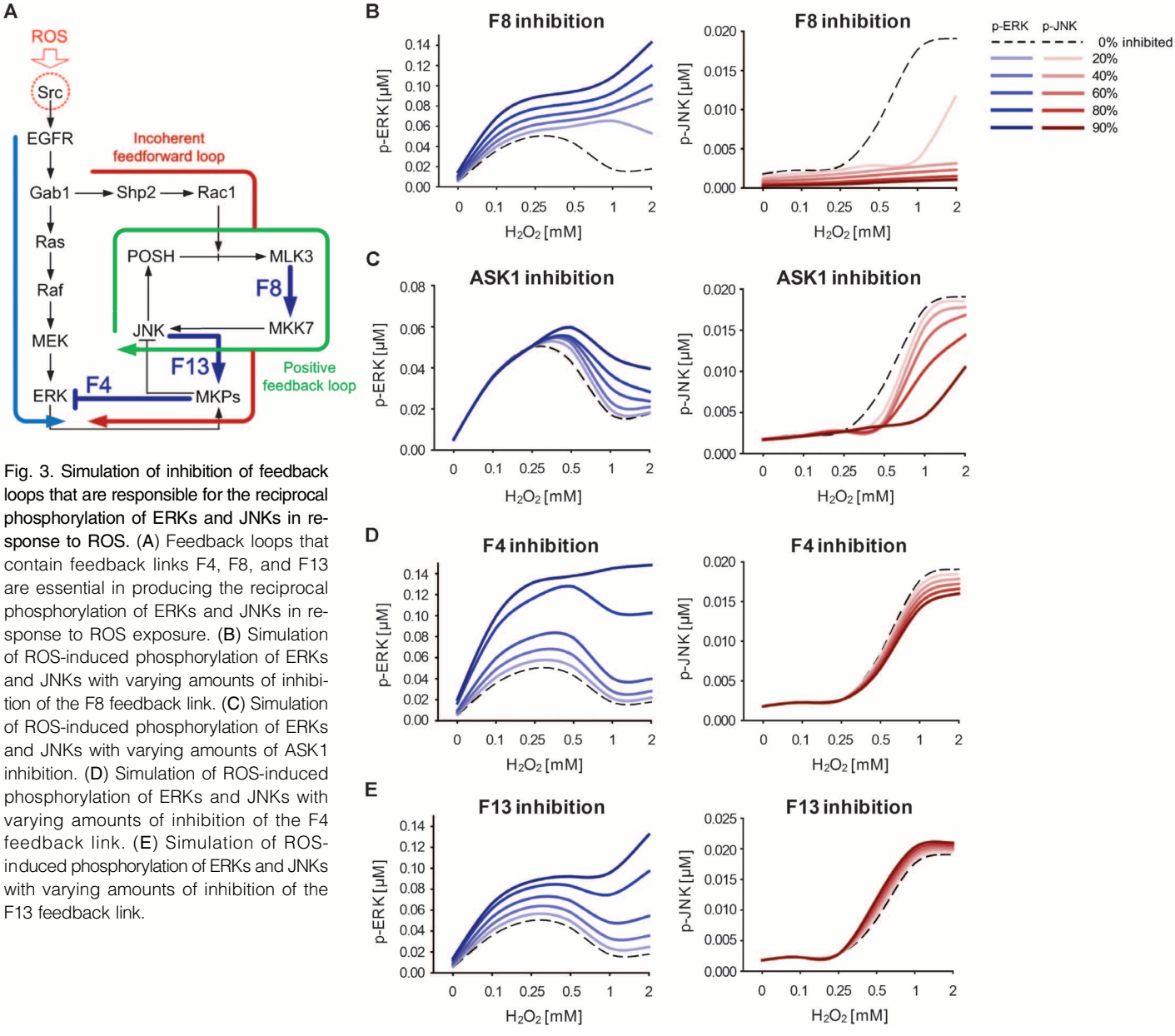


Fig. 3. Simulation of inhibition of feedback loops that are responsible for the reciprocal phosphorylation of ERKs and JNKs in response to ROS. (A) Feedback loops that contain feedback links F4, F8, and F13 are essential in producing the reciprocal phosphorylation of ERKs and JNKs in response to ROS exposure. (B) Simulation of ROS-induced phosphorylation of ERKs and JNKs with varying amounts of inhibition of the F8 feedback link. (C) Simulation of ROS-induced phosphorylation of ERKs and JNKs with varying amounts of ASK1 inhibition. (D) Simulation of ROS-induced phosphorylation of ERKs and JNKs with varying amounts of inhibition of the F4 feedback link. (E) Simulation of ROS-induced phosphorylation of ERKs and JNKs with varying amounts of inhibition of the F13 feedback link.

Next, we considered network models for all possible combinations of these 13 feedback loops, resulting in 8192 ($2^{13}$) rewired network models in which each feedback link was either connected or disconnected. We used each rewired network model to simulate the phosphorylation of ERKs and JNKs in response to different concentrations of ROS. Rewired network models that produced increased phosphorylation of ERKs at low $H_2O_2$ concentrations and increased phosphorylation of JNKs at high $H_2O_2$ concentrations commonly included three feedback links (F4, F8, and F13) (table S8). The feedback link F8 was a component of the MLK3-mediated PFL, and the feedback links F4 and F13 comprised the crosstalk between ERKs and JNKs mediated by MKPs (Fig. 3A), suggesting that these feedback loops were essential for the reciprocal phosphorylation of ERKs and JNKs to different concentrations of ROS. To investigate the role of the three essential feedback loops that contain the feedback link F4, F8, and F13 in our network model, we inhibited each essential feedback link incrementally from 20 to 90% and monitored the downstream effects. Inhibition of F8 increased phosphorylation of ERKs and suppressed phosphorylation of JNKs at high concentrations of ROS (Fig. 3B). To confirm the role of the MLK3 (F8)–containing feedback loop, we performed simulations using a network model where this loop was disconnected at a different interaction [MAPK kinase 7 (MKK7)–mediated phosphorylation of JNKs] and found that inhibition of this link increased phosphorylation of ERKs and suppressed phosphorylation of JNKs at high concentrations of ROS (fig. S6A). Moreover, inhibition of ASK1, another MAP3K in the JNKs pathway that induces apoptosis in response to ROS (*124*–*126*), also increased phosphorylation of ERKs and suppressed phosphorylation of JNKs at high concentrations of ROS (Fig. 3C), suggesting that ASK1 may be involved in the ROS-induced activation of the MLK3-mediated PFL. In contrast to the F8 inhibition, inhibition of F4 or F13 increased phosphorylation of ERKs, but did not affect phosphorylation of JNKs at high concentrations of ROS (Fig. 3, D and E). Thus, we concluded that the MLK3-mediated PFL and the MKP-mediated crosstalk between ERKs and JNKs were required for the different responses of ERKs and JNKs to ROS.

## ROS signaling balanced by the MLK3-mediated PFL

To understand how the MLK3-mediated PFL influences ROS concentration–dependent cellular responses, we used the ROS signaling network model to simulate the phosphorylation of ERKs and JNKs with varying amounts of inhibition of the F8 feedback link (MLK3). We defined the balancing point as the $H_2O_2$ concentration at which the phosphorylation of ERKs is equivalent to that of JNKs. We found that the balancing point shifted toward higher concentrations of $H_2O_2$ as we increased the percentage of inhibition of F8 (Fig. 4, A and B). Whether the balancing point reflects a critical biological factor in a cell's ability to respond to different ROS concentrations by proliferation or death is unclear because other factors beside the relative activity of ERKs and JNKs can influence these processes. Nevertheless, these observations suggest that ROS concentrations lower than the balancing point would induce proliferation due to the enhanced activity of ERKs, whereas ROS concentrations greater than the balancing point would promote apoptosis due to the enhanced activity of JNKs and that the MLK3-mediated PFL balances ROS concentration–dependent signal flow between these two kinase pathways.

## Biochemical validation of predictions from the ROS signaling network model

To validate the predictions made by the mathematical model, we used pharmacological and genetic inhibition of pathway enzymes and monitored cellular responses to ROS. Inhibition of MLK3 by exposing HeLa cells to the chemical K252a increased phosphorylation of ERKs and suppressed phosphorylation of JNKs in cells exposed to high concentrations

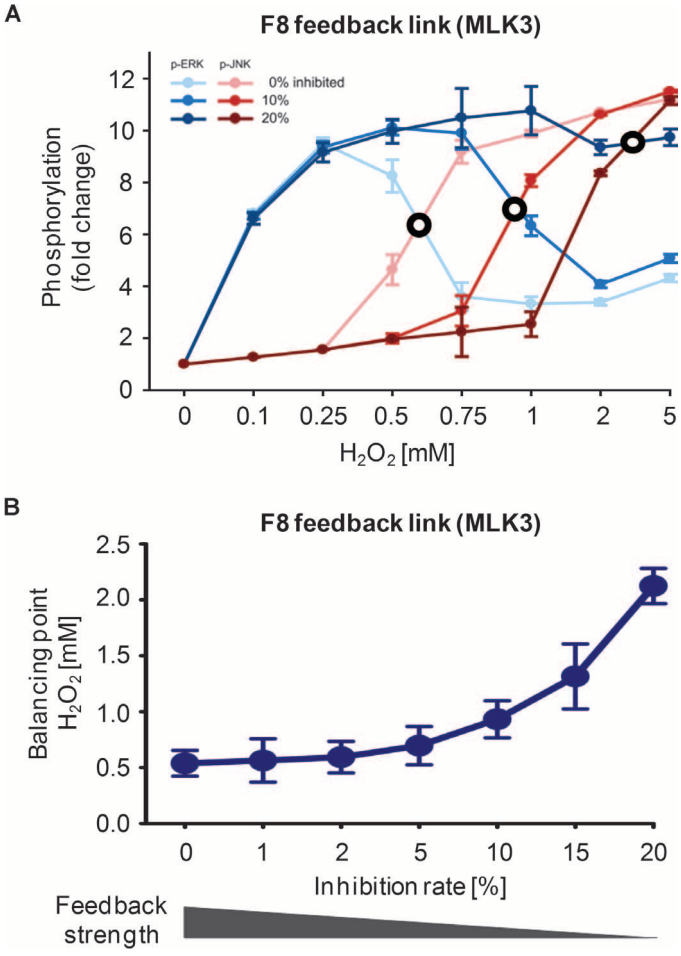


Fig. 4. ROS concentration–dependent signal flow between the ERK and JNK pathways balanced by the MLK3-mediated PFL. (A) Simulation of ROS-induced phosphorylation of ERKs and JNKs with varying amounts of inhibition of the F8 feedback link (MLK3). The black circles denote the balancing point. (B) Simulation of the shift of the balancing point with varying amounts of inhibition of the F8 feedback link (MLK3).

of $H_2O_2$ (Fig. 5, A and B). Likewise, knockdown of MLK3 (Fig. 5C) or MKK7 (fig. S6B) by infection of HeLa cells with virus encoding short hairpin RNAs (shRNAs), or inhibition of ASK1 by exposing HeLa cells to NQDI-1 (an ASK1 inhibitor) (Fig. 5D) had a similar effect as K252a on the ROS concentration–dependent phosphorylation of ERKs and JNKs. Moreover, exposing HeLa cells to NSC 95397 (an inhibitor of MKPs) increased phosphorylation of ERKs but did not significantly change the phosphorylation of JNKs in cells exposed to high concentrations of $H_2O_2$ (Fig. 5, E and F). Thus, these experimental data are consistent with the prediction from the mathematical model that an MLK3-mediated PFL is important for cellular responses to ROS.

We also examined whether these feedback loops play a role in cell viability during exposure to ROS. In HeLa cells, inhibition of MLK3 with K252a, inhibition of ASK1 with NQDI-1, or knockdown of MLK3 or MKK7 significantly increased viability at high concentrations of ROS (Fig. 6, A to C, and fig. S6C). Moreover, inhibition of MKPs by exposing cells to NSC 95397 also increased cell viability at high concentrations of ROS (Fig. 6D).

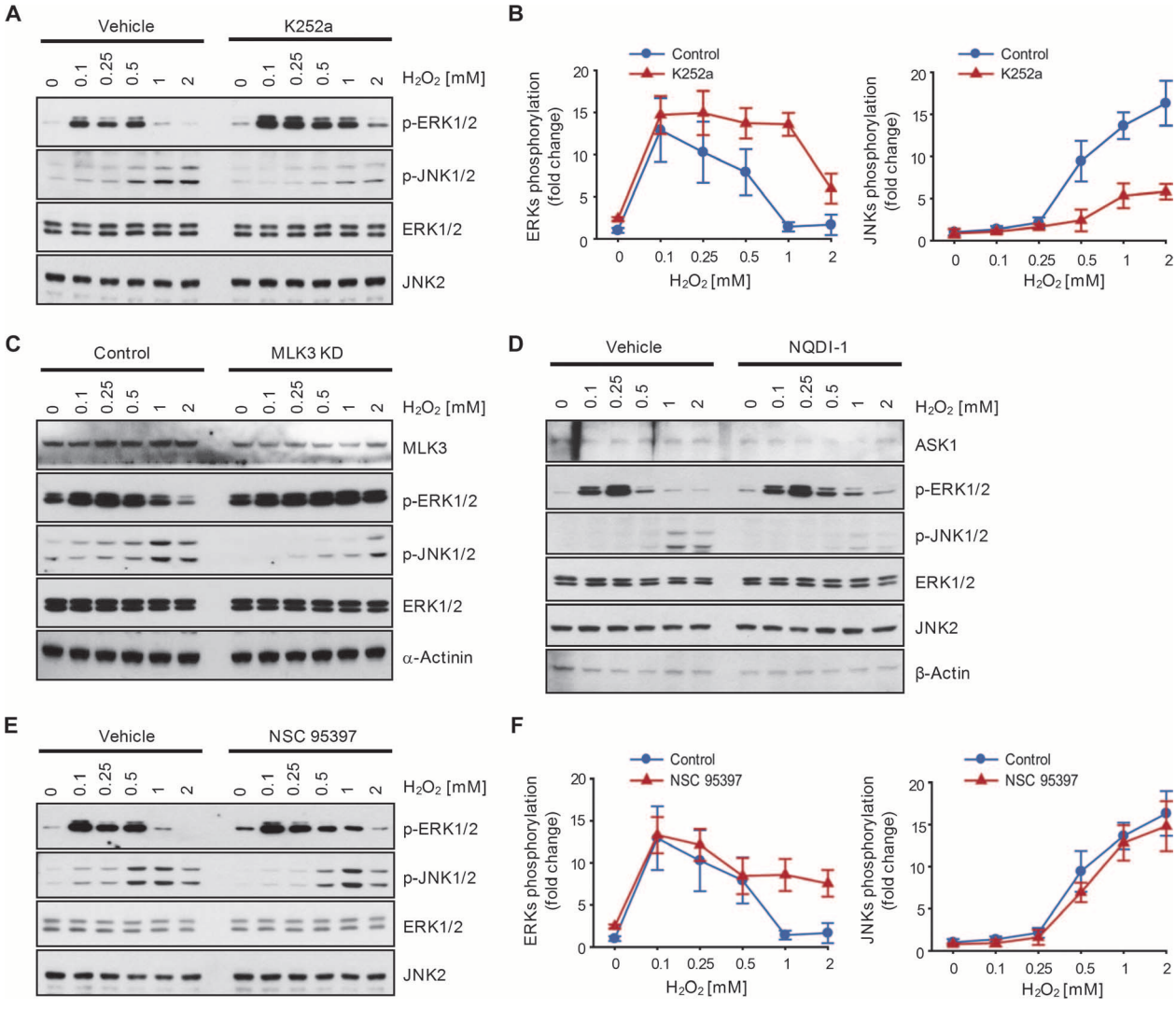


**Fig. 5. Effects of inhibitors of MLK3, ASK1, or MKPs, or knockdown of MLK3 on the phosphorylation of ERKs and JNKs.** (**A** and **C** to **E**) Western blots for phosphorylated ERKs and JNKs in lysates of HeLa cells exposed to the indicated concentrations of $H_2O_2$ for 1 hour after 1-hour preexposure to K252a [(A) 100 nM; an MLK3 inhibitor], NQDI-1 [(D) 3 μM; an ASK1 inhibitor], or NSC 95397 [(E) 20 nM; an MKP inhibitor], or HeLa cells with MLK3 knockdown exposed to the indicated concentrations of $H_2O_2$ for 1 hour (C). Antibodies against α-actinin, β-actin, ERK1/2, and JNK2 were used as loading controls as indicated. (**B** and **F**) Graphs depict the means ± SEM of phosphorylated ERKs and JNKs of three blots from biological replicates of (A) and (E).

Next, we tested whether the MLK3 feedback–dependent control of ROS-mediated cellular responses was conserved in other cancer cell types. Exposing HCT116 colorectal adenocarcinoma cells or HuH-7 hepatocarcinoma cells to K252a increased phosphorylation of ERKs and suppressed phosphorylation of JNKs when exposed to high concentrations of $H_2O_2$ (fig. S7, A and B). In contrast, exposing HCT116 cells to NSC 95397 increased phosphorylation of ERKs but did not significantly change the phosphorylation of JNKs when exposed to high concentrations of $H_2O_2$ (fig. S7A). Moreover, exposing HCT116 cells to either K252a or NSC 95397 significantly increased cell viability at high concentrations of ROS (fig. S7C). Likewise, exposing HuH-7 cells to K252a significantly increased cell viability at high concentrations of ROS (fig. S7D). Thus, feedback loops involving MLK3 and MKPs are likely a conserved requirement for ROS-dependent cellular responses in various cancer cells.

We examined whether MLK3 could control cellular responses to ROS through ERKs or JNKs or both. We tested whether MLK3 knockdown affects the cellular response to ROS exposure in the presence or absence of ERK activation. MLK3 knockdown decreased ROS-induced phosphorylation of JNKs (fig. S8A) with the increase of cell viability (fig. S8B) in the presence of U0126, suggesting that JNKs also play an ERK-independent role in the MLK3-mediated cellular response to high concentrations of ROS. Thus, both ERKs and JNKs are responsible for the phenotypic control under ROS exposure.

## DISCUSSION

ROS play a role in numerous intracellular signal transduction pathways that regulate various cellular processes (*1*–*4*) and are implicated in a variety of

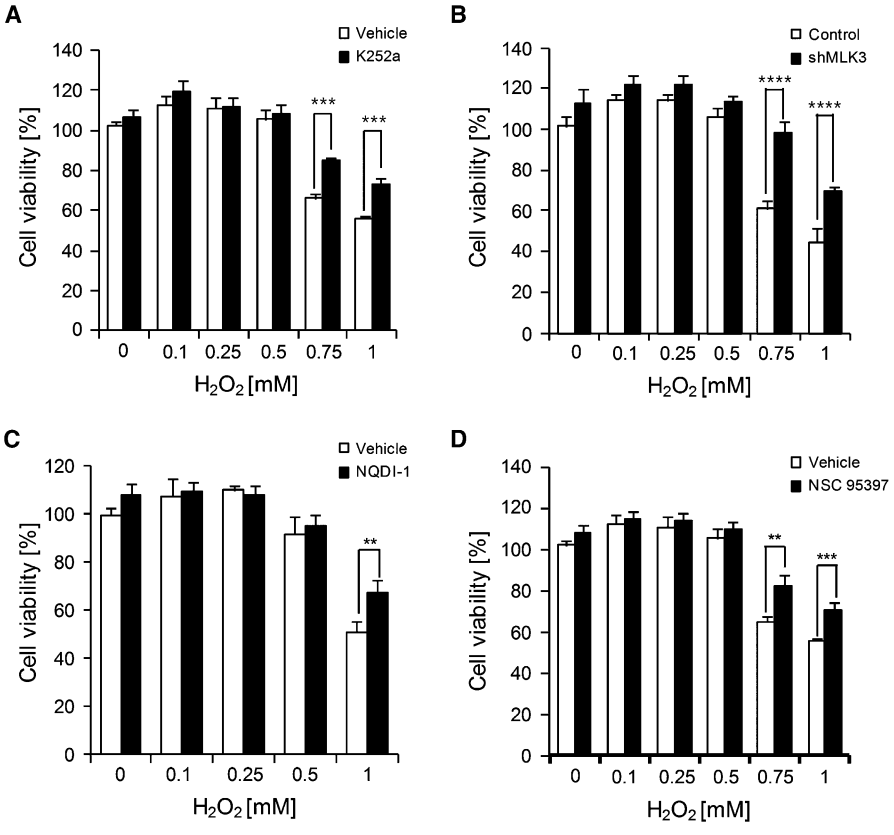


Fig. 6. Effects of inhibition of MLK3, ASK1, or MKPs, or knockdown of MLK3 on the cell viability. (A to D) Graph of the percentage of viable HeLa cells exposed to the indicated concentrations of $H_2O_2$ for 24 hours after 1-hour preexposure to K252a [(A) 100 nM; an MLK3 inhibitor], NQDI-1 [(C) 3 μM; an ASK1 inhibitor], or NSC 95397 [(D) 20 nM; an MKP inhibitor], or HeLa cells with MLK3 knockdown exposed to the indicated concentrations of $H_2O_2$ for 24 hours (B). Data represent the means + SEM of three biological replicates. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, Student's *t* test.

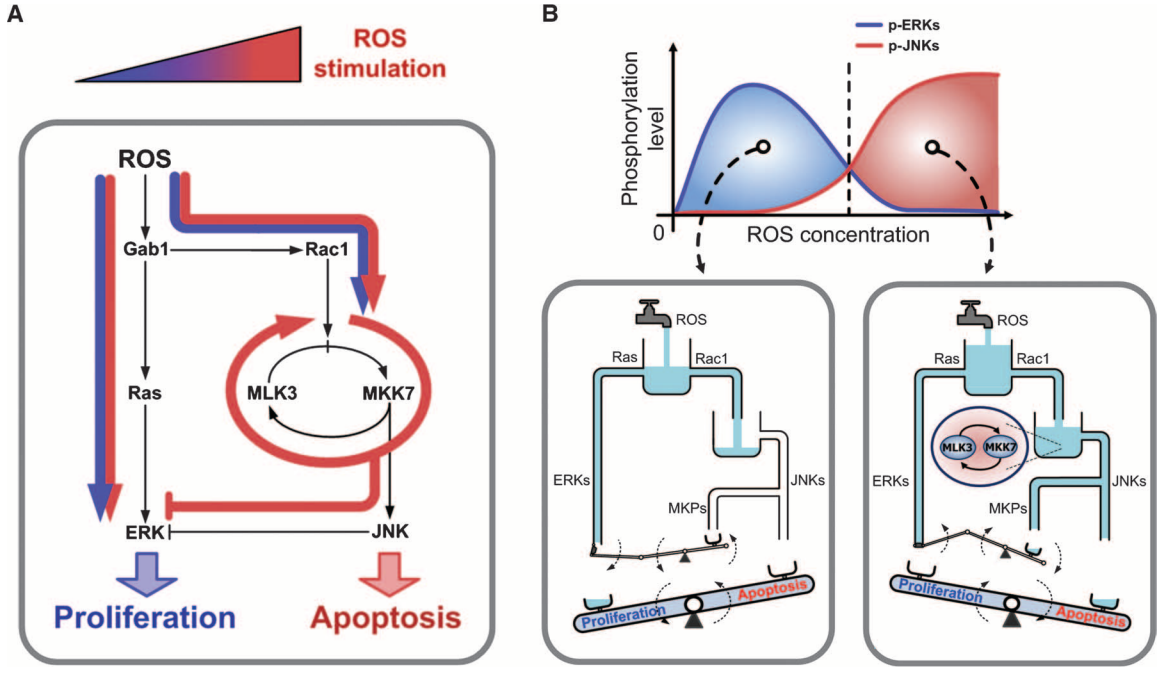


Fig. 7. The core circuit responsible for switching ROS-dependent cellular responses. (A) Core circuit is composed of the MLK3-mediated PFL and MKP-mediated crosstalk between ERKs and JNKs. The blue arrow indicates the signal flow at low concentrations of ROS, and the red arrow indicates the signal flow at high concentrations of ROS. (B) Core circuit generates the reciprocal phosphorylation of ERKs and JNKs, which leads to ROS-dependent cellular responses.

diseases (*127*–*130*). ROS influence signaling pathways connected by complicated regulatory mechanisms, including feedback and crosstalk (*23*). We used mathematical modeling to identify design principles underlying the mechanisms of ROS-mediated control of cell signaling and validated the predictions using biochemical experiments in multiple cell lines. We found that ERKs and JNKs are phosphorylated downstream of ROS in a reciprocal concentration-dependent manner balanced by an MLK3-mediated PFL. These observations are consistent with the following model (Fig. 7, A and B): When cells are exposed to low concentrations of ROS, the activity of ERKs is high and the MLK3-mediated PFL is not sufficiently activated to stimulate JNKs activity, leading to cellular proliferation (Fig. 7, A and B, lower left panel). When the cells are exposed to high concentrations of ROS, the MLK3-mediated PFL is activated, which induces JNKs activation that promotes apoptosis and suppresses proliferation through inhibition of ERKs (Fig. 7, A and B, lower right panel).

From the view of system dynamics, the core circuit responsible for the ROS-dependent cellular responses embedded in the ROS signaling network is composed of an incoherent feed-forward loop (IFFL) (*131*, *132*) and a nested PFL (*133*) (fig. S9A). The dynamics of the system is determined by integration of the positive (ROS→ERK) and the negative (ROS→JNK⊣ERK) regulatory signals, leading to biphasic responses ("→" denotes a positive regulation, and "⊣" denotes a negative regulation). In addition, the PFL nested in the negative regulatory path (ROS→JNK⊣ERK) can further balance the signal flow of two opposing signals (positive and negative regulatory signals) by activating the repressor (JNK) of ERK in an all-or-none switch-like manner. This suggests that the ROS-dependent cellular responses may arise from the combination of two distinct structural features (IFFL and PFL) with characterized functions. For instance, the biphasic response of the ERK can be enhanced or weakened depending on the strength of the PFL [compare (i) and (ii) in fig. S9B].

The identification of the core circuit that coordinates ROS-dependent cellular responses raises the question: What benefits does this mechanism confer? One possible answer is enhanced efficiency of signal transduction for multiple cellular functions. Signals from their extracellular environment tightly regulate fundamental cellular responses such as proliferation, cell cycle, and apoptosis (*134*), and cells can respond to a graded range of signals and produce discrete cellular outcomes (*135*). Our findings suggest that the MLK3-mediated PFL provides a threshold that enables cells to convert continuous stimuli into discrete cellular responses. Therefore, multiple cellular phenotypes, such as proliferation and apoptosis, can be induced efficiently by a single stimulus through the same signal transduction network.

In summary, our study provides novel insight into how an MLK3-mediated PFL regulates the relative activation of MAPKs and implies that MLK3 is a crucial positive feedback mechanism involved in the ROS-dependent cellular responses.

## MATERIALS AND METHODS

### Mathematical modeling

The ROS signaling network model was assembled from experiments presented here and from published data (table S1). The mathematical model was developed using a system of ordinary differential equations (table S2), including 57 state variables (table S3) and 238 parameters (table S4), and was solved using the MATLAB built-in function, ode15s (*136*).

To estimate the kinetic parameters of the mathematical model, we minimized the sum of squared difference between the experimental data and the simulated values using the genetic algorithm (*137*) in MATLAB Optimization Toolbox (*138*). The parameter estimates carry uncertainties due to limited amounts of experimental data and measurement noise, which may affect the reliability of the model predictions (*139*). To address this issue and confirm the robustness of the model, we carried out repetitive simulations ($n = 50$) in which we simultaneously varied all the parameters at random in a range of 30% of the initial value (fig. S4).

### Cell culture

HeLa, HCT116, HuH-7, and human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (WelGENE Inc.) with 10% fetal bovine serum and antibiotics [penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml)] (Life Technologies Corp.) at 37°C in a humidified atmosphere containing 5% $CO_2$. Cells were exposed to vehicle control, K252a (100 nM; Sigma-Aldrich), NQDI-1 (3 μM; Sigma-Aldrich), or NSC 95397 (20 nM; Sigma-Aldrich). For Western blots, small molecules were applied to the cells 1 hour prior to the exposure to $H_2O_2$ for 1 hour. For cell viability assays, small molecules were applied for 1 hour and then concurrent with $H_2O_2$ for 24 to 48 hours as indicated in the figure legends.

### Western blot analysis

Western blotting was performed as described previously (*10*). Briefly, cells were lysed in lysis buffer [20 mM Hepes (pH 7.2), 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, aprotinin (1 μg/ml), 1 μg of leupeptin, 1 mM $Na_3VO_4$, 1 mM NaF]. Antibodies against $p16^{Ink4a}$ (sc-1661), $p21^{Cip1}$ (sc-397), $p27^{Kip1}$ (sc-1641), ASK1 (sc-7931), α-actinin (sc-15335), and β-actin (sc-1616) were obtained from Santa Cruz Biotechnology Inc. Antibodies against phospho-Akt (#9271), ERK (#9102), phospho-ERK (#9101 or #9106), JNK (#9252), phospho-JNK (#9151 or #9255), phospho-p38 (#9211), MKK7 (#4172), and MLK3 (#2817) were obtained from Cell Signaling Technology Inc. Signal intensities were quantified using ImageJ (http://imagej.nih.gov/ij/) (*140*).

### Cell viability assays

Relative cell viability was measured with WST-1 as described previously (*141*). Briefly, WST-1 solution (Daeil Lab Service Co., Seoul, Korea) was added to cells grown in 96-well plates for 1 to 2 hours, and absorbance at 450 nm was measured using a VICTOR X3 Multilabel Plate Reader (PerkinElmer Inc.).

For flow cytometry, about $1 \times 10^6$ cells were trypsinized and washed twice with ice-cold phosphate-buffered saline (PBS) and then fixed overnight at −20°C in 70% ethanol. Immediately before flow cytometry, the cells were resuspended in PBS containing propidium iodide (50 μg/ml) and DNase (deoxyribonuclease)–free RNase (ribonuclease) (10 μg/ml). Flow cytometry was performed with a FACSCalibur (BD Biosciences) system.

### shRNA knockdown

Retrovirus was generated in HEK293T cells transfected with helper plasmids and pLKO.1 plasmids encoding shRNA targeting MLK3 (TRCN0000021567: CCGGAGCACAAGACCCTGAAGATCACTCGAGTGATCTTCAGGGTCTTGTGCTTTTTT) or MKK7 (TRCN0000350455: CCGGGTCAAAGACTGCCTTACTAAACTCGAGTTTAGTAAGGCAGTCTTTGACTTTTTG) (Sigma-Aldrich) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Viral supernatants were collected at 60 hours after transfection and supplemented with polybrene (4 μg/ml) and used to infect HeLa cells. Infected cells were selected with puromycin (concentration) (Sigma-Aldrich) for 14 to 21 days.

## SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/7/328/ra52/DC1

Fig. S1. Effects of the different concentrations of ROS on cellular responses.

Fig. S2. Increased phosphorylation of ERKs induced by the inhibition of MLK3, JNKs, or p38-MAPK.

Fig. S3. Time course of $H_2O_2$-dependent phosphorylation of Akt and p38-MAPK.
Fig. S4. Comparison of the simulation results to the experimental data.
Fig. S5. Thirteen identified feedback links in the ROS signaling network.
Fig. S6. Effects of simulation and knockdown of MKK7 on the phosphorylation of ERKs and JNKs and cell viability.
Fig. S7. Effects of MLK3 or MKP inhibitors on the phosphorylation of ERKs and JNKs and cell viability in HCT116 and HuH-7 cells.
Fig. S8. Effects of inhibition of ERKs on the phosphorylation of ERKs and JNKs and cell viability in cells with MLK3 knockdown.
Fig. S9. A simplified core circuit composed of an incoherent feed-forward loop and a nested PFL.
Table S1. Mathematical model reactions and processes.
Table S2. Mathematical model ordinary differential equations.
Table S3. Mathematical model initial values.
Table S4. Mathematical model kinetic parameters.
Table S5. Mathematical model constant values.
Table S6. MKP-mediated crosstalk among MAPKs.
Table S7. Feedback loops identified from the ROS signaling network.
Table S8. Rewired network models that generate the reciprocal phosphorylation of ERKs and JNKs during exposure to ROS.

## REFERENCES AND NOTES


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