Mathematical modeling and analysis of mitochondrial retrograde signaling dynamics

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Highlights
Differential equation-based model of mitochondrial retrograde signaling is proposed
The model contains an activation layer and a modulation layer
Retrograde signal was switched on by a input signal through a Boolean decision model
A low-pass filter maintained the robustness of the retrograde signaling pathway

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SUMMARY
Mitochondria, semi-autonomous eukaryotic organelles, participate in energy production and metabolism, making mitochondrial quality control crucial. As most mitochondrial proteins are encoded by nuclear genes, maintaining mitochondrial function and quality depends on proper mitochondria-nucleus communication and designated mitochondrial retrograde signaling. Early studies focused on retrograde signaling participants and specific gene knockouts. However, mitochondrial signal modulation remains elusive. A mathematical model based on ordinary differential equations was proposed to simulate signal propagation to nucleus following mitochondrial damage in yeast. Mitochondrial retrograde signaling decisions were described using a Boolean model. Dynamics of retrograde signaling were analyzed and extended to evaluate the model response to noisy damage signals. Simulation revealed localized protein concentration dynamics, including waveforms, frequency response, and robustness under noise. Retrograde signaling is bistable with localized steady states, and increased damage compromises robustness. We elucidated mitochondrial retrograde signaling, thus providing a basis for drug design against yeast and fungi.

INTRODUCTION
Mitochondria serve as the cells’ powerhouse, utilizing the established proton motive force to generate the high-energy molecule, ATP, thus facilitating various cellular processes (Osellame et al., 2012). The analogy of a simple electrical circuit model is a convenient way to describe the interactions between ion dynamics and energetics in the complex mitochondrial system (Nicholls and Budd, 2000; Friedman and Nunnari, 2014). Mitochondria regulate various cellular mechanisms under normal physiological conditions, as well as in disease, including ATP generation, regulation of signal transduction, immune responses, and cell apoptosis (Osellame et al., 2012). Therefore, mitochondria serve as an essential regulatory metabolic hub (Mottis et al., 2019).

However, their extensive role within the cell renders mitochondria prone to damage (Liu and Butow, 2006; Zdralević et al., 2012). Mitochondrial quality control is therefore essential for cell viability, which relies on mitochondria-driven energy production (Osellame et al., 2012), lipid synthesis (Aon et al., 2014), and amino acid metabolism (King, 2007). However, energy production via the electron transport chain results in an oxidative stress burden (Knorre et al., 2016). Unlike other membrane-bound organelles, which rely solely on protein products from the nuclear genome, mitochondria possess their own genome, encoding dedicated enzymes and repair systems; however, they remain semi-autonomous organelles, as most mitochondria protein-encoding genes have been translocated into the nucleus (Gabaldón and Huynen, 2004). In fact, in budding yeast, only 35 of the 1,000 mitochondrial genes are located in the mitochondrial genome, representing a minor fraction of the metabolic system (Malina et al., 2018). Therefore, mitochondria require various protein products from nucleus genome to maintain functionality. The interaction between mitochondria and the nucleus is a bidirectional process (da Cunha et al., 2015; Mottis et al., 2019). Once mitochondria are damaged, their status is reported to the nucleus via signaling factors or metabolic pathways, enabling the cell to coordinate mitochondrial biogenesis. Furthermore, mitochondrial quality control depends on mitochondrial feedback, referred to as mitochondrial retrograde (RTG) signaling (Butow and Avadhani, 2004; Cagin and Enriquez, 2015; da Cunha et al., 2015; Liu and Butow, 2006).
The most well-characterized mitochondrial RTG signaling cascade is that in budding yeast (Chelstowska et al., 1999; da Cunha et al., 2015; Jazwinski and Kriete, 2012; Liao and Butow, 1993; Sekito et al., 2000). The RTG signaling is triggered during compromised mitochondrial respiratory function (Epstein et al., 2001), causing a shift in carbohydrate and nitrogen metabolism (Liu and Butow, 1999). CIT2, one of the regulated genes, encodes a peroxisomal isoform of citrate synthase involved in the glyoxylate cycle, thereby bypassing the TCA cycle. Thus, the upregulation of CIT2 expression allows for more efficient usage of carbon sources when mitochondria are damaged. This RTG pathway was first characterized by investigating various yeast gene deletion strains. For instance, in a study by Sekito et al. (2000), RTG genes were independently knocked out, and green fluorescent protein (GFP) was employed to localize the RTG proteins, revealing details of the mitochondria-to-nucleus communication system. Furthermore, the localization of RTG proteins was compared among deletion strains, as well as strains with dysfunctional mitochondria, such as rho cells, which lack a mitochondrial genome.

The RTG pathway is initiated by the activation of Rtg2p (Retrograde regulation protein 2), a cytoplasmic sensor of mitochondrial damage with an ATP-binding domain (Rios-Anjos et al., 2017). Rtg2p is activated by the loss of mitochondrial membrane potential or the ATP/ADP ratio (da Cunha et al., 2015; Ferreira et al., 2005; Liu and Butow, 1999), which results in the dissociation of Bmhp/Mksp heterodimer that inhibits the Rtg3p (Retrograde regulation protein 3) activation (Ferreira et al., 2005; Liao and Butow, 1993). Rtg1p/3p are basic helix-loop-helix-leucine zipper (bHLH/Zip) transcription factors with nucleus localization signal (NLS) that induce the retrograde response via nucleus translocation and binding to the promoter R box region of RTG response genes to initiate transcription (Jazwinski, 2013).

Without Rtg2p sensing mitochondrial status, Rtg3p and Rtg1p localization occurs independently of mitochondrial damage because of constant inhibition of Mksp. Rtg3p and Rtg1p remain cytoplasmic in wild type and dysfunctional mitochondria (rho strain) (Sekito et al., 2000). In addition, Rtg1p regulates the translocation of Rtg3p. Although Rtg1p participates in the activation of the retrograde response, it retains Rtg3p in the cytoplasm in the absence of mitochondrial damage. In contrast, Rtg3p accumulates in the nucleus regardless of mitochondrial status in Rtg1p knockout mutants, whereas the opposite is not true. Initiation of retrograde response signaling requires nuclear accumulation of both Rtg3p and Rtg1p like the AND logic gate (Hashim et al., 2014; Rothermel et al., 1997; Sekito et al., 2000). Therefore, Rtg1p acts as a positive regulator of retrograde response genes, as well as a negative regulator that retains Rtg3p in the cytoplasm in the absence of mitochondrial damage.

Cells usually harbor a number of mitochondria that share the same retrograde signaling pathway, resulting in a multiplexing issue first described in telecommunications (Cover and Thomas, 2005). Sharing signaling channels may lead to unwanted crosstalk. Furthermore, biochemical pathway noise restricts the cells’ ability to receive information from their organelles. In eukaryotic cells, such as budding yeast, mitochondria interact with the nucleus via the same biochemical channels, and the consequent multiplexing issue represents a barrier to assessing the health state of each mitochondrion. How biological systems manage multiplex communication networks during the mitochondrial RTG response remains unclear.

To investigate the communication properties of mitochondrial retrograde signaling, a differential equation-based model was developed to simulate the process. The mitochondrial status and deleted genes are defined as input, whereas translocation of Rtg1p/Rtg3p is the defined output. These interactions were previously observed using immunofluorescence microscopy (Sekito et al., 2000, 2002), and subsequently summarized in a Boolean table. The parameters were fitted with the Boolean relationship using Monte Carlo simulation. A parameter set was verified by solving the steady states under each observed genotype. Collectively, our mathematical model interprets the dynamics of mitochondrial retrograde signaling. Furthermore, the proposed mathematical model also has potential applications in research on antifungal drugs and aging.

RESULTS
An ordinary differential equation-based model of mitochondrial retrograde signaling
As a channel of communication between mitochondria and the nucleus, RTG signaling is well regulated and fine-tuned for a proper response to mitochondrial damage signals. The dynamics and interactions of Rtg1p, Rtg2p, Rtg3p, Mksp, and Bmhp are described by ordinary differential equations (ODEs) and biochemical reaction network. The first part of RTG signaling is the activation layer, during which the mitochondrial damage
signal activates Rtg2p. Then, the signal is transmitted to the modulation layer where Rtg1p/Rtg3p complexes translocate into the nucleus and initiate transcription (Figure 1A). The interactions among retrograde proteins and the translocation of Rtg1p/Rtg3p are assumed to obey the law of mass action (Voit et al., 2015; Reyes et al., 2022; King et al., 2021), and the total concentration of each protein was regarded as conserved. To connect retrograde proteins with mitochondrial damage, the activation of Rtg2p was modeled using the Hill equation with the sum of mitochondrial damage (Frank, 2018). The purpose of applying Hill equation is to summarize the cascading effect on sensing mitochondrial damage, such as ROS-induced ROS release (Chowdhury et al., 2020). Furthermore, proteins with activation states or locations are defined separately, leading to a total of 17 protein species and 24 kinetic coefficients (Table S1).

To identify the parameter sets that would make the model compatible with the experimental observations (Ghaemmaghami et al., 2003; Sekito et al., 2000,2002), a Monte Carlo simulation method was used to explore the parameter sets. First, to leverage the microscopic data (Sekito et al., 2000,2002), the translocation of Rtg1p/Rtg3p and mitochondrial states were summarized in a Boolean model with 17 conditions, including the combination of RTG gene knockouts (Table 1). Second, the relative protein concentrations of Bmhp and Rtg1/2/3p were determined based on the gene expression (Gomar-Alba et al., 2015) and quantitative western blot analyses (Ghaemmaghami et al., 2003) (Figure S1). Third, a random search method was applied to explore the parameter sets. Finally, the steady states of the model were compared to the Boolean model (Figure 2). A parameter set was regarded as valid when the steady states fulfilled the observations of all 17 knockout conditions (Figure S2).

**The ultrasensitivity of Bmhp/Mksp dissociation causes a switch-like response of Rtg1/3p translocation to mitochondrial damage**

Nuclear accumulation of Rtg1p and Rtg3p activates the retrograde response. To understand the mechanism by which information is propagated from the upstream Bmhp/Mksp inhibitor to Rtg1/3p translocation, a sigmoid signal of mitochondrial damage was applied to simulate the transition of RTG proteins. The step response revealed sophisticated details on RTG protein dynamics induced by the fast sigmoid transition of the damage signal (Figure 1B). The dissociation of Bmhp/Mksp indicated an ultrasensitive response to the input signal. However, the translocation of Rtg1p and Rtg3p was relatively smoother and delayed than Bmhp/Mksp dissociation. The delayed stimulation of Rtg1p and Rtg3p may be caused by the slow Rtg3p autoactivation rate. Besides, the step responses overshoot pattern was identified in the particular forms of Rtg1p and Rtg3p, whereas summation canceled this effect (Figure S3A).

Two forces control the nuclear translocation of Rtg3p, namely the autoactivation of the NLS and dissociation of the cytoplasmic Rtg1/3p heterodimer, releasing extraRtg3p monomers that permeate the nuclear membrane. When the mitochondrial damage signal dissociates Bmhp/Mksp complex via activated Rtg2p, the autoactivation of Rtg3p turns on the NLS and increases the influx kinetic coefficient. Furthermore, the overshoot of activated Rtg3p concentration causes the decline observed in the early response (Figure S3A). The decreased cytoplasmic Rtg3p concentration causes further dissociation of the Rtg1p/Rtg3p heterodimer, releasing more free Rtg3p monomers. Autoactivation of the Rtg3p NLS contributes to the sharp increase in Rtg3p nuclear concentration shortly after the damage signal. Once autoactivation reaches a steady-state, free Rtg3p is conserved and translocated to the nucleus by simple diffusion. Hence, the transition of Rtg3p becomes smoother in the second stage, leading to a capacity-charging curve at the end (Figure S3B). In addition to Rtg3p, the translocation of Rtg1p also results from dissociation of the cytoplasmic Rtg1p/Rtg3p heterodimer and simple diffusion. When Rtg1p is absent or negligible, simple diffusion causes Rtg3p to accumulate in the nucleus independently of the damage signal. The role of Rtg1p is to retain Rtg3p in the cytoplasm, however, the retention force is insufficient to retain activated Rtg3p (Sekito et al., 2000). Decreasing the pool of Bmhp or Mksp to simulate protein knockout caused the response curve of Rtg3p to shift upper-left, making the system more sensitive to mitochondrial damage signals (Figures S4 and S5). The current simulation supports previous observations in knock-out strains (Komeili et al., 2000; Dilova et al., 2002) and provides details regarding the ultrasensitivity of Bmhp/Mksp dissociation, the two-staged transition of Rtg3p, and the capacity-charging curve of Rtg1p in response to mitochondrial damage signals.

**Competitive binding to Mksp between Bmhp and Rtg2p contributes to the ultrasensitivity of Bmhp/Mksp dissociation**

The ultrasensitivity of Bmhp/Mksp dissociation is indicated by the phase plot with mitochondrial damage as input (Figure 3). Although the Hill function models this process with the dissociation constant of input
Figure 1. Mitochondrial retrograde signaling pathway model
(A) The mitochondria-to-nucleus communication circuit. Retrograde signaling is divided into the activation layer and modulation layer. Retrograde proteins (Bmh, Mksp, and Rtg1/2/3p) are classified into an active (-act) and inactive state (-ina). Cellular compartments are divided based on the nucleus (-nuc) and cytosol (-cyt). The arrow represents the binding of two proteins, transformation of active states, or translocation between nucleus and cytosol, with the kinetic coefficient labeled besides it. The input is defined as the summation of mitochondrial damage signals. Rtg2p is activated once the mitochondrion loses its membrane potential, or the ATP concentration decreases. The active form of Rtg2p suppresses the formation of the Mksp/Bmh complex by competitive binding with Mksp, resulting in dephosphorylation of Rtg3p and translocates to the nucleus accompanied by Rtg1p. The Rtg1p-Rtg3p complex eventually triggers the retrograde response and upregulates CIT2 expression.

(B) Step responses of Bmh/Mksp, Rtg1p, and Rtg3p showing switch-like responses of Bmh/Mksp, capacity-charging curve with a time delay for Rtg1p, and delayed two-staged rise with a sharp transition at the beginning, for Rtg3p.

(C) The sinusoidal inputs of 1.6×10⁻³ Hz response reveals RTG signaling pathway as a low-pass filter. The mitochondrial damage signal (thin gray line), labeled as the input with quantities shown on the right y axis. The concentration of RTG proteins (thick blue line), regarded as outputs with quantities labeled on the left y axis. To normalize the response, the mass fraction is defined as the ratio of the concentration of a given protein set to its total concentration. Nucleus accumulation is described by the ratio of nucleus concentration to total protein concentration.
the phase plot of Rtg2p reveals a two-staged sigmoid transition with two thresholds significantly below \( k_{sd} \), and with higher stiffness on the first one. The dissociation of Bmhp/Mksp occurs at the first transition, wherein the threshold for sensing mitochondrial damage is lower than that of the input Hill function. Therefore, the ultrasensitivity of Bmhp/Mksp is independent of the Hill input model (Figure S6) and is caused by the competitive binding between activated Rtg2p and Bmhp.

To quantify the sensitivity of Bmhp/Mksp dissociation, the relative amplification method (Legewie et al., 2005) was applied to estimate the Hill coefficient with baseline effect. As shown in Figure 3C, the response of inactive Rtg2p (Rtg2p\(^{inact}\)) is with lower Hill coefficients for both transitions compared to the input locally and globally (Figure 3C, left). Meanwhile, the dissociation of the Bmhp/Mksp heterodimer occurs at the first transition of inactive Rtg2p, with a higher Hill coefficient, which was lower than that of the input (Figure 3C, right). Despite knowing the quantity of the Hill coefficient, the transition of Bmhp/Mksp occurred far below the dissociation constant \( k_{sd} \), which is unlikely to be caused by the input Hill function, leading to questions regarding the source of its ultrasensitivity.

To elucidate the underlying mechanism, we analyzed the Rtg2p-Mksp-Bmhp motif by solving the steady states algebraically. The exact analytical solution revealed that the transitions of inactivated Rtg2p stem from the molecular titration (Buchler and Louis, 2008) (Figure 3D). Furthermore, the competitive binding between Rtg2p and Bmhp with Mksp forms a binary switch that could convert the analog input signal into digital information (Figure 3E). Another interesting observation is that there is minimum concentration of Rtg2p needed for detecting mitochondrial damage. Rtg2p is only functional when its concentration is beyond a critical amount, and there is no effect on changing threshold of RTG response when Rtg2p is beyond the critical value (Figure 3F). Therefore, the molecular titration provides a simple switch for detecting mitochondrial damage that can be used for denoising mitochondrial damage signal under fluctuated environment.

| Rtg1p | Rtg2p | Rtg3p | Mito-damage | Mksp | GFP-labelled protein | Nucleus Accumulation |
|-------|-------|-------|-------------|------|----------------------|---------------------|
| 0     | 0     | 1     | 0           | 1    | Rtg3-GFP             | N/A                 |
| 0     | 0     | 1     | 1           | 1    | Rtg3-GFP             | 1                   |
| 0     | 1     | 1     | 0           | 1    | Rtg3-GFP             | 1                   |
| 0     | 1     | 1     | 1           | 1    | Rtg3-GFP             | 1                   |
| 1     | 0     | 1     | 0           | 1    | Rtg3-GFP             | 0                   |
| 0     | 0     | 1     | 1           | 1    | Rtg3-GFP             | 0                   |
| 0     | 1     | 1     | 0           | 1    | Rtg3-GFP             | 0                   |
| 1     | 0     | 1     | 1           | 1    | Rtg3-GFP             | 1                   |
| 1     | 0     | 0     | 0           | 1    | Rtg1-GFP             | N/A                 |
| 0     | 0     | 1     | 1           | 1    | Rtg1-GFP             | N/A                 |
| 1     | 0     | 0     | 0           | 1    | Rtg1-GFP             | 0                   |
| 1     | 1     | 0     | 1           | 1    | Rtg1-GFP             | 0                   |
| 0     | 0     | 1     | 0           | 1    | Rtg1-GFP             | 0                   |
| 1     | 0     | 1     | 1           | 1    | Rtg1-GFP             | 0                   |
| 1     | 1     | 0     | 1           | 1    | Rtg1-GFP             | 1                   |
| 1     | 0     | 1     | 0           | 0    | Rtg3-GFP             | 1                   |
| 1     | 1     | 0     | 0           | 0    | Rtg1-GFP             | 1                   |

Table 1. The Boolean model of mitochondrial retrograde signaling

The existence of RTG proteins and mitochondrial damage signal is described in binary quantity based on the experimental observations of the sub-cellular localization of GFP-tagged-RTG proteins in wild type and rtg\( ^{\Delta} \) mutant derivatives of \( \rho_{+} \) and \( \rho_{0} \) cells (Sekito et al., 2000). The 0 represents deleted protein or absence of mitochondrial damage, and 1 represents the opposite phenomena. Untested conditions are designated as N/A.
Frequency modulation of mitochondrial retrograde signaling

In addition to the dose response, we further investigated the frequency modulation of the RTG signaling. Bmhp/Mksp heterodimer, and nuclear Rtg1p, and Rtg3p in response to a sinusoidal damage signal of multiple frequencies exhibited low-pass filter behavior (Figure 4 and S7). The recovery rate of Bmhp/Mksp association was slower than its dissociation, leading to a sign-sensitive delay that keeps the switch on after damage is detected (Alon, 2019), whereas Bmhp/Mksp can be slowly synthesized for a long period of approximately 3000 s compared to the transition of less than 10 s. Although the concentration of Bmhp/Mksp recovers slowly after stimulation, Rtg1p and Rtg3p are still able to convey input signal dynamics. At a low frequency, both reach high and low saturation states in a sinusoidal input cycle and produce a square wave pattern. As the frequency becomes high, the amplitude of output diminishes with drifting, indicating that mitochondrial damage signal is low-pass filtered by retrograde signaling. Moreover, the delay of RTG translocation may contribute to the instability and compromise robustness. To investigate the feedback instability of mitochondrial retrograde signaling, we further derived a Bode plot of the retrograde response in the stationary stage (Figure S7). In the frequency response of Bmhp/Mksp, the gain decreased in two stages separated by 10^−0.5 Hz. The gain of the high-frequency stage decreased more...
Figure 3. Ultrasensitivity of the Bmh/Mksp dissociation results from molecular titration

(A) Scheme of the competitive binding between Rtg2p and Bmh with Mksp. The forward reactions are labeled with thick arrows, and reverse reactions are labeled with dashed arrows.

(B) Phase plots. The input signal is defined within the interval between 0 and 1, and the corresponding steady states are plotted separately with the parameters:

- $n = 7$, $k_{2in} = 0.96$, $k_{1} = 11.67$, $k_{2in} = 4.94$, $k_{2} = 0.043$, $k_{2in} = 1604.15$, $k_{2} = 2.413$, $k_{2in} = 0.059$ in arbitrary unit.

(C) Relative amplification approach of Rtg2p and Bmh/Mksp in response to mitochondrial damage. The activated fraction is converted from the input signal normalized by its range; the response coefficient is the normalized sensitivity.
rapidly than in low frequency stage. On the other hand, though, the phase delay increased significantly in the high-frequency region. The cutoff frequency of nuclear Rtg1p and Rtg3p was approximately $10^{-2}$ Hz as low-pass filters. For nuclear Rtg3p, there was a second cutoff frequency at approximately $10^{-0.5}$ Hz with a more rigid downhill slope. Finally, the Nyquist stability theorem was applied to identify the closed-loop stability of mitochondrial retrograde signaling (Iglesias and Ingalls, 2009). Despite the phase delay, retrograde signaling remained stable in response to the damage signal. The phase and gain margin derived from the Bode plots can further indicate the stability tolerance to delay caused by the molecular communication like protein translocation. The low-pass filtering can remove the perturbation that is usually in high frequency (Figure 4A) (Tsimring, 2014).

The intensity of the mitochondrial damage signal decreases the robustness of Rtg1/3p translocation

Although the dose-response curve describes the communication between mitochondria and nucleus in a deterministic point of view, the molecular noise causes uncertainty and further limits the information conveyed to the nucleus (McAdams and Arkin, 1997; Bowsher and Swain, 2014). To understand how noise influences robustness with the damage signal, the ODE model was further extended to the chemical Langevin equations (Gillespie, 2000). The proposed deterministic model is incorporated with the random chemical reaction, and the potential landscape of nuclear Rtg1/Rtg3p was derived by screening the damage input with ensemble simulations (Figure 5). In addition to the damage signal, the sigmoid curve of the potential landscape (Figure 5B) was consistent with the deterministic results of Rtg1/Rtg3p (Figure 5A). A steady state represents the Rtg3p distribution that is in high probability. In response to a constant damage, three conjugated steady states were identified by the peak of the probability, which represented the high likelihood of the Rtg1/Rtg3p concentration in a given input. The conjugated steady states located within the bandwidth of the probability density function (PDF) became distanced as the input signal increased, indicating that noise was amplified and caused an increased bandwidth, leading to a compromised signal-to-noise ratio. Notably, three conjugated steady states were further investigated by measuring the output distribution for a given input (Figure SC). This result suggested that mitochondrial retrograde signaling was not only bistable but also a toggle-switch with multiple locally conjugated stable steady states. In addition, the uncertainty of the output was worsened by a high input signal, which might compromise information relayed between mitochondria and the nucleus when the former continues to send high-intensity messages.

DISCUSSION

The RTG retrograde signaling pathway is an important mechanism for mitochondrial quality control because it serves as both a sensor and a reporter of the mitochondrial health status (Butow and Avadhani, 2004). Quantitative and systemic analysis of this major communication channel will help to elucidate the crosstalk between mitochondria and the nucleus, which maintains cellular function under physiological and pathological conditions. Herein, a novel mathematical model of mitochondrial retrograde signaling in yeast was constructed based on microscopic data of the RTG pathway knockout experiments with gene and protein expression data for model validation. The ordinary differential equation (ODE)-based model provides a comprehensive approach for studying the dynamic response of mitochondrial retrograde signaling, which has been experimentally challenging because of the lack of means for precise mitochondrial quality manipulation in real time. Retrograde signaling dynamics were observed after introducing the input signal with step and sinusoidal waveforms. Moreover, an analytical solution identified the source of ultrasensitivity in the dissociation of the Bmh/Mksp heterodimer.

**Figure 3. Continued**

of output with respect to the input signal. The transformed responses of Rtg2p and Bmh/Mksp (thick blue line) are compared via the Michaelis-Menten reaction with a Hill coefficient equal to one, described as a linear function (dashed line). $n_{\text{global}}$ represents global Hill coefficient; $n_1$ and $n_2$ are local Hill coefficients in the first and second transition of Rtg2p.

(D) Analytical solution of molecular titration with Hill activation.

(E) The influence of mitochondrial damage on RTG protein concentration. The dash line in Bmhp plot denotes the IC50 value of mitochondrial damage for activating Rtg translocation.

(F) The effect of expression level on triggering mitochondrial retrograde response. The threshold is defined as the amount of mitochondrial damage ($s$) that achieves the concentration of Bmhp equal to the $K_M$ of inhibition ($k_{13}^{-1}$).
The switch-like behavior of the yeast RTG pathway was previously reported (da Cunha et al., 2015), and this switch-like behavior is validated through mathematical formulation and numerical simulation in this study. The switch-like behavior was resulted from the competitive binding between active Rtg2p and Bmhp. Besides, the effect of molecular titration (Buchler and Louis, 2008) triggered a two-staged transition in inactive Rtg2p. Ultrasensitivity is typical in cellular communication, allowing cells to remove noise and amplify input signals (Haney et al., 2010; Thattai and van Oudenaarden, 2002). Rtg2p senses mitochondrial quality through the modulation of Bmhp and Mksp, facilitating detection via competitive binding. The “all-or-none” response can further filter the fluctuation and adjust to the unpredictable environment. Gene knockouts would alter the response curve. For example, knocking out Rtg1p, Mksp, or Bmhp made Rtg3p translocate to the nucleus regardless the damage signal, whereas knocking out Rtg2p did the opposite and the system was unresponsive to the damage signal (Figure S8).

The current study further elaborates on the properties of signal modulation via Rtg1/3p translocation following static and sinusoidal damage signals. Rtg1p acts as both a positive and negative regulator (Sekito et al., 2000), retaining the transcription factor Rtg3p in the cytoplasm. This considerably constrains the valid parameter set. Further mechanistic details were identified by the proposed model: the two-staged transition of Rtg3p translocation in step response and the sign-sensitive delay of Rtg3p with a short ON delay in oscillation signal. Without NLS modification, Rtg1p is required to retain Rtg3p in the cytoplasm when a mitochondrial damage signal is absent, which is one of the criteria of our model. Rtg1p exerts an anchoring effect on Rtg3p by forming a heterodimer, which may not permeate through the nuclear membrane. The
translocation of Rtg1p is not affected by the sign of the input change and reveals a simple diffusion process with a capacity-charging pattern. The frequency response further revealed the low-pass filtering of the RTG pathway, which can reject high-frequency noisy fluctuations and retain the overall information of the input signal for requesting nuclear supply.

In the current study, we demonstrated feedback stability of RTG signaling using Bode and Nyquist diagrams (Iglesias and Ingalls, 2009); the communication between mitochondria and the nucleus is a two-way process (Butow, 2002). The phase and gain margin in the Bode plot determined the stable region when cascading with these functional units under mitochondrial oscillation.

Our model also elucidates how the RTG pathway processes the input signal as a low-pass filter, reporting status to the nuclear genome, which may, in turn, support the synchronization of mitochondrial membrane potential in yeast (Lloyd et al., 2002,2003; Murray et al., 2001) and insulin secretion from pancreatic cells (Heart et al., 2007). Moreover, mitochondria undergo energization cycles driven by the ultradian clock, contributing to respiratory oscillations of mitochondria (Lloyd et al., 2002,2003; Murray et al., 2001). These oscillations may be a source of reactive oxygen species generated by mitochondria (Lloyd et al., 2003), and further interfere the retrograde signaling with sinusoidal input.

Figure 5. Robustness of Rtg1/3p translocation in response to mitochondrial damage signal
(A) Stochastic Langevin model to screen the damage input with ensemble simulations.
(B) The potential landscape of the nuclear Rtg1/Rtg3p response to mitochondrial damage signals. The damage signal was sampled in 100 grids and assigned to the chemical Langevin model to simulate the stochastic process. Every combination of parameters was applied with 100-time length with automatic time step. The y axis was limited within the range of 0 and the maximum concentration of Rtg3p.
(C) The probability density function (PDF) with respect to Rtg1/Rtg3p influenced by damage signal. The distribution is simulated by setting the input signal as 0 (blue, minimal input), 0.45 (orange, intermediate input), and 1 (green, maximum input). The PDF is measured by time series simulation and estimates the probability via kernel density estimation.
The mitochondrion acts as a biochemical signaling hub in eukaryotic cells (Chen et al., 2016). Apart from energy production, this semi-autonomous organelle also participates in various metabolic reactions related to aging (Christian and Shadel, 2014; Miceli et al., 2012; Nystrom, 2013), cellular communication (Heart et al., 2007; Mottis et al., 2019) and cell viability (Fatima et al., 2020; Moye-Rowley, 2005).

The RTG response facilitates the adaptation of eukaryotic organisms to unfavorable factors (Fatima et al., 2020; Trendeleva and Zvyagilskaya, 2018). In yeast, the key role of RTG signaling is the regulation of mitochondrial function and metabolic reprogramming, including the maintenance of intracellular glutamate supplies (Liu et al., 2002; Trendeleva and Zvyagilskaya, 2018). Studies have reported the association between the retrograde response and drug resistance in yeast (Hans et al., 2019; Moye-Rowley, 2005; Trendeleva and Zvyagilskaya, 2018). For example, Rtg3p has been reported as essential for antifungal drug tolerance associated with the formation of the fungal plasma membrane, which contains ergosterol (Yan et al., 2014). The Δrtg3 mutant of pathogenic yeast, Candida albicans, exhibits compromised biofilm formation and epithelial cell adherence, resulting in reduced infectivity of nematode model Caenorhabditis elegans (Hans et al., 2019). Moreover, in Saccharomyces cerevisiae, pleiotropic drug resistance is activated by RTG signaling, inducing the transcription of pleiotropic drug resistance genes (Trendeleva and Zvyagilskaya, 2018). RTG signaling is also associated with aging, highlighting yeast as a model organism for the study of mitochondrial quality control and its influences on lifespan (Jazwinski, 2013).

Retrograde signaling is connected to glutamate synthesis, mTOR signaling, and other feedback circuits (Butow and Avadhani, 2004; Liu and Butow, 2006; Whelan and Zuckerbraun, 2013). The RTG pathway is a major factor in cellular adaptation, serving a cytoprotective role via metabolic reprogramming (Trendeleva and Zvyagilskaya, 2018; Ždralevič et al., 2012). Our simulations showed that the addition of glutamate or rapamycin stabilizes the Rtg2/Mks complex (Liu et al., 2003) and makes the system more sensitive to the mitochondrial damage signal (Figure S8). The robustness of retrograde signaling is essential for adjusting to unfavorable conditions (Fatima et al., 2020). For instance, RTG signaling contributes to drug resistance in pathogenic yeast, such as C. albicans, which is responsible for approximately 80% of major systemic fungal infections (Trendeleva and Zvyagilskaya, 2018; Badiee and Hashemizadeh, 2014). Various antifungal drugs target mitochondria-related amino acid and ergosterol metabolism (Jastrzebowska and Gabriel, 2015; Pasko et al., 1990), and the RTG pathway enables pathogenic yeast to overcome therapeutic agents by sensing the loss of mitochondrial functionality and subsequently reprogramming their metabolism (Singh et al., 2018; Trendeleva and Zvyagilskaya, 2018; Yan et al., 2014). Hence, the yeast retrograde signaling system represents a target for the development of novel antifungal drugs (Trendeleva and Zvyagilskaya, 2018). However, how yeast senses mitochondrial stress via the RTG pathway in a dynamic environment remains unclear.

In this study, we applied mathematical modeling to unravel the dynamics of RTG signaling from a control system point of view. We further identified the threshold of the RTG response, which can be used for determining the optimal dosages for the elimination of pathogenic yeast without triggering RTG signaling and the minimum expression of Rtg2p that is needed for the detection of mitochondrial damage. Furthermore, the frequency response reveals how yeast manages to resist fluctuation via low-pass filtering of the RTG pathway and the hysteresis effect, which maintains retrograde signaling as the damage signal fluctuates, indicating that persistent treatment with a concentration slightly below the RTG response threshold is an optimal strategy against pathogenic yeast. Therefore, comprehensively understanding the qualitative properties of retrograde signaling may shed light on signaling mechanisms and provide potential therapeutic strategies.

Limitations of the study
The long-term effects of mitochondrial damage are related to more complex mechanism including mTOR and aging, which was not addressed in the proposed model. The assumptions and predictions made in this study also need further experimental validations.

STAR Methods
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
METHOD DETAILS

- Boolean model of mitochondrial retrograde signaling
- From a Boolean model to an ordinary differential equation-based model
- The differential equation-based model of RTG signaling
- Activation layer
- Modulation layer
- Parameter searching with the qualitative data of protein translocation
- The analytical solution of the activation layer
- The competitive binding of Bmhp and Rtg2p with Mksp is the source of the ultrasensitivity
- The modified competitive binding model for Rtg2 system
- Analysis ultrasensitivity with relative amplification approach
- Frequency response of RTG signaling
- Stochastic simulation of Rtg1/3p translocation in response to mitochondrial damage

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105502.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.C. and A.W.; software, S.C.; formal analysis, S.C., W.T., and A.W.; writing—original draft, S.C. and A.W.; writing—review and editing, S.C., W.T., and A.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Alon, U. (2019). An Introduction to Systems Biology: Design Principles of Biological Circuits (CRC Press), pp. 37–54. https://doi.org/10.1201/9780429283321.

Aon, M.A., Bhatt, N., and Cortassa, S.C. (2014). Mitochondrial and cellular mechanisms for managing lipid excess. Front. Physiol. 5, 282. https://doi.org/10.3389/fphys.2014.00282.

Badiee, P., and Hashemizadeh, Z. (2014). Opportunistic invasive fungal infections: diagnosis & clinical management. Indian J. Med. Res. 139, 195–204.

Bezanson, J., Edelman, A., Karpinski, S., and Shah, V.B. (2017). Julia: a fresh approach to numerical computing. SIAM Rev. Soc. Ind. Appl. Math. 59, 65–98. https://doi.org/10.1137/141000671.

Bowsher, C.G., and Swain, P.S. (2014). Environmental sensing, information transfer, and cellular decision-making. Curr. Opin. Biotechnol. 28, 149–155. https://doi.org/10.1016/j.copbio.2014.04.010.

Buchler, N.E., and Louis, M. (2008). Molecular titration and ultrasensitivity in regulatory networks. J. Mol. Biol. 384, 1106–1119. https://doi.org/10.1016/j.jmb.2008.09.079.

Butow, R.A. (2002). Cellular responses to mitochondrial dysfunction: it’s not always downhill. Cell Death Differ. 9, 1043–1045. https://doi.org/10.1038/sj.cdd.4401083.

Butow, R.A., and Avadhani, N.G. (2004). Mitochondrial signaling: the retrograde response. Mol. Cell 14, 1–15. https://doi.org/10.1016/S1097-2765(04)00179-0.

Cagin, U., and Enriquez, J.A. (2015). The complex crosstalk between mitochondria and the nucleus: what goes in between? Int. J. Biochem. Cell Biol. 63, 10–15. https://doi.org/10.1016/j.biocel.2015.01.026.
Chelstowska, A., Liu, Z., Jia, Y., Amberger, D., and Butow, R.A. (1999). Signalling between mitochondria and the nucleus regulates the expression of a new D-lactate dehydrogenase activity in yeast. Yeast 15, 1377–1391. https://doi.org/10.1007/s12225-001-0999-0.

Frank, S.A. (2018). A biochemical logarithmic sensor with broad dynamic range. PLoS Res. 7, e2000108. https://doi.org/10.1371/journal.plocr.0010108.

Friedman, J.R., and Nunari, J. (2014). Mitochondrial form and function. Nature 505, 335–343. https://doi.org/10.1038/nature12985.

Gabaldón, T., and Huynen, M.A. (2004). Shaping the mitochondrial proteome. Biochem. Biophys. Acta 1659, 212–220. https://doi.org/10.1016/j.bbaco.2004.07.011.

Ghaemmaghami, S., Huh, W.-K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O’Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. Nature 427, 737–741.

Gillespie, D.T. (2000). Chemical Langevin equation. J. Chem. Phys. 113, 297–306. https://doi.org/10.1063/1.1361611.

Haney, S., Bardwell, L., and Nie, Q. (2010). Ultrafast signaling and specificity in cell signaling. BMC Syst. Biol. 4, 119. https://doi.org/10.1186/1752-0509-4-119.

Hans, S., Fatima, Z., and Hameed, S. (2019). Retrograde signaling disruption influences ABC superfamily transporter, ergosterol and chitin levels along with biofilm formation in Candida albicans. J. Mycol. Med. 29, 210–218. https://doi.org/10.1016/j.jymed.2019.07.003.

Hao, N., Budnik, B.A., Gunawardena, J., and O’Shea, E.K. (2013). Tunable signal processing through modular control of transcription factor translocation. Science 339, 460–464. https://doi.org/10.1126/science.1232799.

Hashim, Z., Mukai, Y., Bamba, T., and Fukusaki, E. (2014). Metabolic profiling of retrograde pathway transcription factors rtg1 and rtg3 knockout yeast. Metabolites 4, 580–598. https://doi.org/10.3390/metabo4020249.

Harris, C.R., Millman, K.J., van der Walt, S.J., Gommers, R., Virtanen, P., Cournapeau, D., Wieser, E., Taylor, J., Berg, S., Smith, N.J., et al. (2020). Array programming with NumPy. Nature 585, 357–362. https://doi.org/10.1038/s41586-020-2649-2.

Hashim, Z., Mukai, Y., Bamba, T., and Fukusaki, E. (2014). Metabolic profiling of retrograde pathway transcription factors rtg1 and rtg3 knockout yeast. Metabolites 4, 580–598. https://doi.org/10.3390/metabo4010008.

Hosea, M., and Shampine, L. (1996). Analysis and implementation of tr-bdf2. Appl. Numer. Math. 20, 21–37.

Hunter, J.D. (2007). Matplotlib: a 2D graphics environment. Comput. Sci. Eng. 9, 90–95. https://doi.org/10.1109/MCSE.2007.55.

Iglesias, P.A., and Ingalls, B.P. (2009). Control Theory and Systems Biology (MIT Press).

Jastrzębska, K., and Gabriel, J. (2015). Inhibitors of amino acids biosynthesis as antifungal agents. Amino Acids 47, 227–249. https://doi.org/10.1007/s00726-014-1873-1.

Jazwinski, S.M. (2013). The retrograde response: when mitochondrial quality control is not enough. Biochim. Biophys. Acta 1833, 400–409. https://doi.org/10.1016/j.bbamer.2012.02.010.

Jazwinski, S.M., and Kriet, A. (2012). The yeast retrograde response as a model of intracellular signaling of mitochondrial dysfunction. Front. Physiol. 3, 139. https://doi.org/10.3389/fphys.2012.00139.

Kim, S., Ji, W., Deng, S., Ma, Y., and Rackauckas, C. (2016). Stiff neural ordinary differential equations. Chaos 31, 093122. https://doi.org/10.1063/1.496694.

King, J., Gisgelbrecht, S., Truckenmuller, R., and Carlier, A. (2021). Mechanistic computational models of epithelial cell transporters—the adored heroes of pharmacokineticians. Front. Pharmacol. 12, 780620. https://doi.org/10.3389/fphar.2021.780620.

King, N. (2007). Amino acids and the mitochondria. In Mitochondria, S.W. Schaffer and M.S. Suleiman, eds. (Springer), pp. 151–166.

Knorre, D.A., Sokolov, S.S., Zyrina, A.N., and Severin, F.F. (2016). How do yeast sense mitochondrial dysfunction? Microb. Cell 3, 532–539. https://doi.org/10.1007/s41586-016-0015-7.

Komell, A., Wedaman, K.P., O’Shea, E.K., and Powers, T. (2000). Mechanism of metabolic control. target of rapamycin signaling links nitrogen quality to the activity of the trg1 and rtg3 transcription factors. J. Cell Biol. 151, 863–878. https://doi.org/10.1083/jcb.151.4.863.

Legewie, S., Bützgen, N., and Herzl, H. (2005). Quantitative analysis of ultrafast responses. FEBS J. 272, 4071–4079. https://doi.org/10.1111/j.1742-4658.2005.04816.x.

Liao, X., and Butow, R.A. (1993). RTG1 and RTG2: two yeast genes required for a novel path of communication from mitochondria to the nucleus. Cell 72, 61–71. https://doi.org/10.1016/0092-8674(93)90050-Z.

Liu, Z., and Butow, R.A. (1999). A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction in respiratory function. Mol. Cell. Biol. 19, 6720–6728. https://doi.org/10.1128/MCB.19.10.6720.

Liu, Z., and Butow, R.A. (2006). Mitochondrial retrograde signaling. Annu. Rev. Genet. 40, 159–185. https://doi.org/10.1146/annurev.genet.40.110405.090613.

Liu, Z., Sekito, T., Epstein, C.B., and Butow, R.A. (2002). RTG-dependent mitochondria to nucleus signaling is negatively regulated by the seven WD-repeat protein Lst8p. EMBO J. 20, 7209–7219. https://doi.org/10.1093/emboj/20.24.7209.

Liu, Z., Sekito, T., Spierek, M., Thornton, J., and Butow, R.A. (2003). Retrograde signaling is
regulated by the dynamic interaction between rgt2p and mks1p. Mol. Cell. Biol. 23, 1037–1039. https://doi.org/10.1128/mcb.2013.10.049.

Nystöm, T. (2013). Aging: filtering out bad mitochondria. Curr. Biol. 23, 1037–1039. https://doi.org/10.1128/mcb.2013.10.049.

Singh, S., Fatima, Z., Ahmad, K., and Hameed, S. (2018). Fungicidal action of geraniol against candida albicans is potentiated by abrogated cad3p drug efflux and fluconazole synergism. PLoS One 13, e0203079. https://doi.org/10.1371/journal.pone.0203079.

Thattai, M., and van Oudenaarden, A. (2002). Attenuation of noise in ultrasensitive signaling cascades. Biophys. J. 82, 2943–2950. https://doi.org/10.1016/S0006-3495(02)75635-X.

Trendeleva, T.A., and Zvyagilskaya, R.A. (2018). Retrograde signaling as a mechanism of yeast adaptation to unfavorable factors. Biochemistry. 83, 98–106. https://doi.org/10.1134/S0006297918020025.

Tsirimg, L.S. (2014). Noise in biology. Rep. Prog. Phys. 77, 026601.

Van Rossum, G., and Drake, F.L. (2014). Python 3 Reference Manual (CreateSpace).

Virtanen, P., Gommers, R., Oliphant, T.E., Haberland, M., Reddy, T., Cournapeau, D., Burovski, E., Peterson, P., Weckesser, W., Bright, J., et al. (2020). SciPy 1.0: fundamental algorithms for scientific computing in Python. Nat. Methods 17, 261–272. https://doi.org/10.1038/s41592-019-0666-2.

Voit, E.O., Martens, H.A., and Omholt, S.W. (2015). 150 years of the mass action law. PLoS Comput. Biol. 11, e1004012. https://doi.org/10.1371/journal.pcbi.1004012.

Wang, Z.X. (1995). An exact mathematical expression for describing competitive binding of two different ligands to a protein molecule. FEBS Lett. 360, 111–114. https://doi.org/10.1101/2014-579395000662-E.

Whelan, S.P., and Zuckerbraun, B.S. (2013). Mitochondrial signaling: forwards, backwards, and in between. Oxid. Med. Cell. Longev. 2013, 351613. https://doi.org/10.1155/2013/351613.

Yan, H., Zhao, Y., and Jiang, L. (2014). The putative transcription factor cartg3 is involved in tolerance to cations and antifungal drugs as well as serum-induced filamentation in candida albicans. FEMS Yeast Res. 14, 614–623. https://doi.org/10.1093/fmy/fmy030.

Zdralievic, M., Guaragnella, N., Antonacci, L., Marra, E., and Giannattasio, S. (2012). Yeast as a tool to study signaling pathways in mitochondrial stress response and cytoprotection. Sci. World J. 2012, 1–10. https://doi.org/10.1101/2012/912147.
STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Deposited data      |        |            |
| RTG Microarray Data | NCBI Gene Expression Omnibus | GSE59659 |
| RTG Microscopic Data| (Sekito et al., 2000) and (Sekito et al., 2002) | https://pubmed.ncbi.nlm.nih.gov/10848632/; https://pubmed.ncbi.nlm.nih.gov/11907262/ |
| Yeast GFP Database and Quantitative Western Blot | Yeast GFP Fusion Localization Database | https://yeastgfp.yeastgenome.org/ |

| Software and algorithms |        |            |
|-------------------------|--------|------------|
| Python (v3.7)           | (Van Rossum and Drake, 2009) | https://www.python.org/ |
| Matplotlib (v3.1.1)     | (Hunter, 2007) | https://matplotlib.org/ |
| Scipy                   | (Virtanen et al., 2020) | https://www.scipy.org/ |
| Numpy (v1.19.0)         | (Harris et al., 2020) | https://numpy.org/ |
| Sympy (v1.6)            | (Meurer et al., 2017) | https://www.sympy.org/ |
| PyJulia (v0.5.6)        | N/A    | https://github.com/JuliaPy/pyjulia |
| Pandas (v1.0.5)         | https://doi.org/10.5281/zenodo.3509134 | https://pandas.pydata.org/ |
| Julia (v1.5.3)          | (Bezanson et al., 2017) | https://julialang.org/ |
| DifferentialEquations.jl(v6.15.0) | (Rackauckas and Nie, 2017) | https://github.com/SciML/DifferentialEquations.jl |
| Julia codes             | This paper | https://github.com/NTUMitoLab/MitoRetroDynamics |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, An-Chi Wei (acwei86@ntu.edu.tw).

Materials availability
This study did not generate new materials.

Data and code availability
The source code is available at https://github.com/NTUMitoLab/MitoRetroDynamics. All data produced in this study are included in the published article, its supplemental information, or are available from the lead contact upon request. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

METHOD DETAILS

Boolean model of mitochondrial retrograde signaling
To summarize the systematic behavior of mitochondrial retrograde signaling, a Boolean model was established to discretize the mechanism of protein translocation associated with mitochondrial damage. In this model, the population of $N$ mitochondria is indexed into a vector $\mathbf{MT}$ (Equation 1).

$$\mathbf{MT} = [MT_1, ..., MT_N]$$ (Equation 1)

Though it is known that damaged mitochondria activate Rtg2p and promote the dephosphorylation of Rtg3p by decreasing the Bmhp/Mksp heterodimer (Jazwinski and Kriete, 2012; Sekito et al., 2000), it is unclear how Rtg2p senses mitochondrial dysfunction. Therefore, we assume mitochondria activates Rtg2p simultaneously under Michaelis-Menten kinetics, and the activation rate of Rtg2p is influenced by the weighted linear summation of mitochondrial damage signals (s) with their volume (Equation 2).
In the Boolean model, the signal \( s \) is set to 0 for the healthy mitochondrial population and to 1 when mitochondria are damaged, as in \( \rho \) yeast cells which have no mitochondrial DNA. These genetically damaged mitochondria have no respiratory functionality and constitutively activate retrograde signaling, which causes the translocation of Rtg1p and Rtg3p, when cells contain intact RTG components (Table S3). The translocation of Rtg1p and Rtg3p can switch on the retrograde response, including the upregulation of CIT2 (Jazwinski and Kriete, 2012).

\[
\text{Sekito et al. (2000) determined the nuclear concentration of Rtg1p, Rtg2p and Rtg3p relative to cytoplasmic levels via GFP labeling, RTG knockouts, and the comparison between } \rho^+ \text{ and } \rho^0 \text{ cells. Therefore, the translocation event, existence of RTG components, and mitochondrial functionality can be summarized into a Boolean table, describing the system behavior of the RTG signaling pathway (Table 1) (Sekito et al., 2000). The Boolean relation between mitochondrial damage (input) and the translocation of Rtg1p or Rtg3p can be further simplified by the Karnaugh map (Figure 2A).}
\]

\[
\text{From a Boolean model to an ordinary differential equation-based model}
\]

To investigate the dynamics of mitochondria-nucleus communication, an ordinary differential equation-based model with two compartments (cytosol and nucleus) was developed to simulate RTG activation and translocation (Figure 1). Reactions of mitochondrial retrograde response were derived based on the law of mass action and Michaelis-Menten kinetics. There are total of 17 differential equations and 24 kinetic coefficients (Table S1) to describe the protein dynamics with different activation states or locations in the RTG signaling pathway.

The activation layer consists of Rtg2p, Mksp and Bmhp. This layer is constantly suppressed by Bmhp-Mksp heterodimer, and conveys the mitochondrial damage signal via Rtg2p activation. Later on, Rtg1p and Rtg3p form the modulation layer. Rtg3p contains nuclear localization sequence (NLS) and is polyphosphorylated when the RTG pathway is inactive. In contrast, upon mitochondrial damage, Rtg3p is less phosphorylated and tends to translocate into the nucleus. The process of transcription factor translocation was modeled with simplified mass kinetics (Equation 3) (Hao et al., 2013).

\[
\frac{TF_c - k_{out}}{k_{in}} \rightarrow TF_n
\]

where TF represents transcription factor, and the subscripts describes the location with n representing nucleus and c the cytosol. The compartmental model is further performed with the following equations:

\[
\begin{bmatrix}
\text{Rtg1}_c^\text{Total} \\
\text{Rtg1}_n^\text{Total} \\
\text{Rtg3}_c^\text{Total} \\
\text{Rtg3}_n^\text{Total}
\end{bmatrix}
= 
\begin{bmatrix}
1 & 0 & 0 & 0 & 0 & 1 & 1 & 0 & 0 \\
0 & 1 & 0 & 0 & 0 & 0 & 0 & 1 & 1 \\
0 & 0 & 1 & 1 & 0 & 1 & 0 & 1 & 0 \\
0 & 0 & 0 & 1 & 0 & 1 & 0 & 0 & 1
\end{bmatrix}
\begin{bmatrix}
\text{Rtg1}_c \\
\text{Rtg1}_n \\
\text{Rtg3}_c^a \\
\text{Rtg3}_n^a \\
\text{Rtg1}/3_c^a \\
\text{Rtg1}/3_n^a \\
\text{Rtg1}/3_c^i \\
\text{Rtg1}/3_n^i
\end{bmatrix}
\]

(Equation 4)

where \([\text{Rtg1}_c^\text{Total}]\) is defined as the total cytoplasmic concentration of Rtg1p and heterodimers containing Rtg1p. The localization is labeled with cytosol (c) and nucleus (n) in subscript. a and i in superscript represent partial phosphorylation and hyper-phosphorylation forms of Rtg3p. The dephosphorylation process turns on the nucleus localization sequence (NLS) in Rtg3p (Sekito et al., 2000).
The differential equation-based model of RTG signaling

The RTG pathway model was based on differential equations to explore the system dynamics. The activation layer conveys the mitochondrial damage signal via Rtg2p activation. The modulation layer translocates Rtg3p and Rtg1p to nucleus to initiate gene expressions.

1. Activation layer: Rtg2p and mitochondrial damage signal \((s)\).

\[
Rtg2_{c}^{\text{act}} \xrightarrow{\text{Hill}(s, k_{av}, k_{sd}, n)} \frac{k_{av} s^n}{k_{av} s^n + k_{sd}} Rtg2_{c}^{\text{act}}
\]

(Equation 5)

where \(\text{Hill}(s, k_{av}, k_{sd}, n) = \frac{k_{av} s^n}{k_{av} s^n + k_{sd}}\)

Competitive interaction between Rtg2p and Bmhp on Mksp.

\[
Rtg2_{c}^{\text{act}} + Mks \xrightleftharpoons[k_{mn}]{k_{cc}} Rtg2Mks
\]

(Equation 6)

\[
Bmh + Mks \xrightleftharpoons[k_{mn}]{k_{cc}} BmhMks
\]

(Equation 7)

2. Modulation layer: Translocation of Rtg3p, Rtg1p and their derivatives (Sekito et al., 2000; Dilova et al., 2004).

The constant inhibition of Bmh/Mksp is described by

\[
Rtg13_{c}^{\text{a}} \xrightarrow{k_{13} + \text{MM}\left[\frac{[\text{BmhMks}]}{k_{13}^{(\text{b})}} \frac{k_{13}^{(\text{b})}}{k_{13}^{(\text{b})}} \frac{k_{13}^{(\text{b})}}{k_{13}^{(\text{b})}} \frac{k_{13}^{(\text{b})}}{k_{13}^{(\text{b})}} \right]} Rtg13_{c}^{\text{a}}
\]

(Equation 8)

\[
Rtg3_{c}^{\text{a}} \xrightarrow{k_{3}^{(\text{a})}} Rtg3_{c}^{\text{a}}
\]

(Equation 9)

\[
Rtg3_{n}^{\text{a}} \xrightarrow{k_{3}^{(\text{a})}} Rtg3_{n}^{\text{a}}
\]

(Equation 10)

where \(\text{MM}[\text{BmhMks}, k_{13}^{(\text{b})}, k_{13}^{(\text{b})}] = k_{13}^{(\text{b})} \frac{[\text{BmhMks}]}{k_{13}^{(\text{b})} + k_{13}^{(\text{b})}}\)

The formation of Rtg1/3

\[
Rtg1_{c} + Rtg3_{c}^{\text{a}} \xrightarrow{k_{13}^{(\text{a})}} Rtg13_{c}^{\text{a}}
\]

(Equation 11)

\[
Rtg1_{c} + Rtg3_{c}^{\text{a}} \xrightarrow{k_{13}^{(\text{a})}} Rtg13_{c}^{\text{a}}
\]

(Equation 12)

\[
Rtg1_{n} + Rtg3_{n}^{\text{a}} \xrightarrow{k_{13}^{(\text{a})}} Rtg13_{n}^{\text{a}}
\]

(Equation 13)

\[
Rtg1_{n} + Rtg3_{n}^{\text{a}} \xrightarrow{k_{13}^{(\text{a})}} Rtg13_{n}^{\text{a}}
\]

(Equation 14)

Translocation of Rtg1/3p

\[
Rtg1_{c} \xrightarrow{k_{1}^{(\text{a})}} Rtg1_{n}
\]

(Equation 15)

\[
Rtg3_{c}^{\text{a}} \xrightarrow{k_{3}^{(\text{a})}} Rtg3_{n}^{\text{a}}
\]

(Equation 16)
To determine the relative concentrations of proteins in the RTG pathway, the expression level of RTG genes under normal and osmotic stress conditions (GEO access number: GSE59659) (Gomar-Alba et al., 2015), and protein expression level obtained from S. cerevisiae fusion library (Ghaemmaghami et al., 2003) were analyzed and used for determining the total concentration of RTG proteins (Figure S1). The full set of differential equations is given by:

**Activation layer**

\[
\frac{ds(t)}{dt} = 0 \quad \text{(Equation 18)}
\]

\[
\frac{d\text{Rtg}_2(t)}{dt} = - \frac{k_v(s(t))^3}{k_{sd}^2 + (s(t))^2}\text{Rtg}_2(t) + k_1\text{Rtg}_2(t)
\]

\[
\frac{d\text{Rtg}_2(t)}{dt} = \frac{k_v(s(t))^3}{k_{sd}^2 + (s(t))^2}\text{Rtg}_2(t) - k_1\text{Rtg}_2(t) - k_m\text{Rtg}_2(t)\text{Mks}(t) + k_m\text{Rtg}_2Mk_{s}(t)
\]

\[
\frac{d\text{Mks}(t)}{dt} = - k_m\text{Rtg}_2(t)\text{Mks}(t) + k_m\text{Rtg}_2Mk_{s}(t) - k_m\text{Bmh}(t)\text{Mks}(t) + k_m\text{BmhMks}(t)
\]

\[
\frac{d\text{Bmh}(t)}{dt} = - k_m\text{Bmh}(t)\text{Mks}(t) + k_m\text{BmhMks}(t)
\]

\[
\frac{d\text{BmhMks}(t)}{dt} = k_m\text{Bmh}(t)\text{Mks}(t)
\]

**Modulation layer**

\[
\frac{d\text{Rtg}_{13}(t)}{dt} = - \left( k_1^3 + \frac{\text{Rtg}_2(t)^2}{k_{13}^2} \text{BmhMks}(t) \right) \text{Rtg}_{13}(t)
\]

\[
\frac{d\text{Rtg}_{13}(t)}{dt} = \left( k_1^3 + \frac{\text{Rtg}_2(t)^2}{k_{13}^2} \text{BmhMks}(t) \right) \text{Rtg}_{13}(t)
\]

\[
\frac{d\text{Rtg}_{13}(t)}{dt} = - k_1^3\text{Rtg}_3(t) + k_1^3\text{Rtg}_3(t)
\]

\[
\frac{d\text{Rtg}_{13}(t)}{dt} = - k_1^3\text{Rtg}_3(t) + k_1^3\text{Rtg}_3(t)
\]
Parameter searching with the qualitative data of protein translocation

The parameters determined the steady-state behavior with respect to mitochondrial damage. A valid parameter set should make the model fulfill all conditions observed in the experiments (Sekito et al., 2000; Liu et al., 2002) (Table 1). The parameter set was sampled in log-uniform with a fixed domain, and the relative amount of translocation kinetic coefficients is considered. To elaborate, the inward coefficient of nuclear Rtg3p with an active \( k_{in}^{a} \) was higher than the inactive one \( k_{in}^{i} \). Conversely, \( k_{out}^{a} \) was higher than \( k_{out}^{i} \) (Hao et al., 2013). The algorithm below was used to find the valid parameter sets (Figure S2).

**Algorithm : Framework of finding valid parameter sets of Boolean satisfiability**

Data: Boolean relation of deletions and mitochondrial status (test conditions in Figure 2).

Result: Dictionary of parameter set with Boolean satisfiability on each conditions.

while number of trials ≤ Maximum iteration do

Sample a parameter array with elements in the range of table;

Shuffle the order of test conditions;

Summing up each protein species and redistributed;

for \( i = 0 \) to number of conditions do

if condition[\( i \)] is missing then

\[
\frac{d}{dt}Rtg3^a(t) = k_{in}^a Rtg3^a(t) - k_{in}^i Rtg3^i(t) - k_{out}^a Rtg1^a(t) Rtg3^a(t) + k_{out}^i Rtg1^i(t) Rtg3^i(t) \\
+ k_{out}^a Rtg3^a(t)
\]

(Equation 28)

\[
\frac{d}{dt}Rtg3^i(t) = - k_{in}^a Rtg3^a(t) - k_{in}^i Rtg1^i(t) Rtg3^i(t) \\
+ k_{out}^a Rtg1^a(t) Rtg3^a(t) + k_{out}^i Rtg3^i(t) - k_{out}^a Rtg3^a(t)
\]

(Equation 29)

\[
\frac{d}{dt}Rtg1^a(t) = - k_{out}^a Rtg1^a(t) Rtg3^a(t) \\
+ k_{out}^i Rtg1^i(t) Rtg3^i(t) - k_{out}^a Rtg1^a(t) + k_{out}^i Rtg1^i(t)
\]

(Equation 30)

\[
\frac{d}{dt}Rtg1^i(t) = - k_{out}^a Rtg1^a(t) Rtg3^a(t) \\
+ k_{out}^i Rtg1^i(t) Rtg3^i(t) - k_{out}^a Rtg1^a(t) Rtg3^a(t) + k_{out}^i Rtg1^i(t) - k_{out}^a Rtg1^a(t)
\]

(Equation 31)

\[
\frac{d}{dt}Rtg1^i(t) = - k_{out}^a Rtg1^a(t) Rtg3^a(t) \\
+ k_{out}^i Rtg1^i(t) Rtg3^i(t) + k_{out}^i Rtg1^i(t) Rtg3^i(t) - k_{out}^a Rtg1^a(t) - k_{out}^i Rtg1^i(t)
\]

(Equation 32)

\[
\frac{d}{dt}Rtg1^i(t) = k_{out}^a Rtg1^a(t) Rtg3^a(t) - k_{out}^i Rtg1^i(t) Rtg3^i(t)
\]

(Equation 33)

\[
\frac{d}{dt}Rtg1^i(t) = k_{out}^a Rtg1^a(t) Rtg3^a(t) - k_{out}^i Rtg1^i(t) Rtg3^i(t)
\]

(Equation 34)
The initial values of each component were sampled based on the RNA expression levels. The relative amount of protein under both normal and stress conditions was considered. The Boolean relations were tested by setting deleted proteins near zero, including modifications and complexes. The unstable solutions were discarded if the iteration exceeded $10^6$ with a deviation of derivatives beyond $10^{-8}$ in total. Further, the ratio of the nuclear concentration to cytoplasmic one was used to determine protein translocation. A nucleus-to-cytosol ratio larger than 1.5 was defined as a translocation event and vice versa. The translocation process under each deletion condition was compared with the Boolean model.

The differential equations were solved by DifferentialEquations.jl (https://diffeq.sciml.ai/stable/), an open-source package for scientific computing implemented in the Julia programming language (https://julialang.org/). The parameter searching was performed on one machine with 30 Intel Xeon Processors in TWCC cloud service (https://www.twcc.ai/) for $10^9$ iterations.

The analytical solution of the activation layer

The activation layer contains three cytoplasmic proteins: Rtg2p, Bmh1/2p and Mks1p (Ferreira et al., 2005; Jazwinski and Kriete, 2012; Butow and Avadhani, 2004). A decline in mitochondrial membrane potential ($\Delta \Psi_{mt}$) leads to activation of Rtg2p, and interferences with the formation of Mks1p-Bmh1/2p complex, thereby impeding the nucleus accumulation of Rtg3p by inhibiting its partial dephosphorylation (Jazwinski and Kriete, 2012).

The reaction is summarized in the form of competitive binding with three components. Rtg2p and Bmhp (including Bmh1p and Bmh2p) are two ligands that compete for the Mks1p. The input of the activation layer is the mitochondrial damage (s) which can be the decline of its $\Delta \Psi_{mt}$ and further defined in the domain between 0 and 1 which represents the continuous state from healthy to the damaged. Further, the activation of Rtg2p induced by mitochondrial damage is modeled by a Hill equation. In the proposed model, the kinetic coefficients and total concentration of each protein are regarded as constants.

With the verified parameter set, the two-staged transition of Rtg2p activation induced by the mitochondrial damage signal was identified. The first stage of transition is a sigmoidal curve with the following steady region as the damage signal turns high, revealing the behavior of ultrasensitivity. After a certain value, the active form of Rtg2p increases in a convex trend with a slower slope compared to the first transition.
Furthermore, by screening possible Hill coefficient of Rtg2p activation (n), we found that the threshold of the first transition, rather than the stiffness of the curve, becoming larger when n increases.

To further understand these phenomena, it is necessary to derive the analytical solution of steady states to understand the input-output relation of mitochondrial damage and the total concentration of Bmh/Mksp heterodimer.

**The competitive binding of Bmhp and Rtg2p with Mksp is the source of the ultrasensitivity**

**Derivation of competitive binding with Hill activation**

Consider a competitive binding model (Wang, 1995) with Hill activation.

\[ A' \xrightleftharpoons{\text{Hill}(k_v, k_d, n)} k_v A \]  
(Equation 35)

\[ P + A \rightleftharpoons PA \]  
(Equation 36)

\[ P + B \rightleftharpoons PB \]  
(Equation 37)

The kinetic coefficients are defined as:

\[ K_A = \frac{|P|/|A|}{|PA|} \]  
(Equation 38)

\[ K_B = \frac{|P|/|B|}{|PB|} \]  
(Equation 39)

\[ \text{Hill}(s, k_v, k_d, n) = k_v s^n / (k_d s^n + s^n) \]  
(Equation 40)

where \(| \cdot |\) represents the steady state concentration. Noted that \(| \cdot | \geq 0\). Besides, \(k_r\) is the reaction rate of the deactivation of A. The kinetic coefficient of A activation is modulated by input signal s. Thus, activation reaction is the function of input \(s \in (0, 1)\) (Equation 41).

\[ \frac{|A|}{|A'|} = \frac{k_v}{k_v} \frac{s^n}{k_d s^n + s^n} = K_A \]  
(Equation 41)

The conservation rule is applied in rapid response. Therefore, the total concentrations are constants in the system.

\[ |A|_0 = |A| + |PA| + |A'| \]  
(Equation 42)

\[ |B|_0 = |B| + |PB| \]  
(Equation 43)

\[ |P|_0 = |P| + |PA| + |PB| \]  
(Equation 44)

where \(|A|_0\), \(|B|_0\) and \(|P|_0\) are total concentrations.

Combining Equations 39 and 43, we get

\[ |PB| = \frac{|P|/|B|_0}{K_A + |P|} \]  
(Equation 45)

Besides, with Equations 38, 41, and 42, the steady state of \(|PA|\) is a function of s.

\[ |PA| = \frac{|A|_0 |P|}{K_s (1 + K_s) + |P| K_s} \]  
(Equation 46)

\[ = \frac{|A|_0 |P|}{K_s (1 + K_s) + |P|} \]  
(Equation 47)

where \(K_s\) is a constant with fixed input (s) and Hill coefficient (Equation 41). Noted that \(K_s \neq 0\) when \(s \neq 0\).
To derive \([P]\), the equation can be derived with algebraic substitution of \([PA]\) (Equation 47) and \([PB]\) (Equation 48).

\[
[P]_0 = [P] + [PA] + [PB] \quad \text{(Equation 48)}
\]

\[
= [P] + \frac{[P][A]_0}{K_A(1 + \frac{c}{P})} + \frac{[P][B]_0}{K_B + [P]} \quad \text{(Equation 49)}
\]

To simplify Equation 49, let \(K'_A = K_A(1 + \frac{c}{P})\).

Finally,

\[
[P]_0 = [P] + \frac{[P][A]_0}{K'_A + [P]} + \frac{[P][B]_0}{K_B + [P]} \quad \text{(Equation 50)}
\]

Then we can use the cubic polynomial equation to drive the analytical solution of \([P]\) (Wang, 1995).

\[
|P|^3 + a|P|^2 + b|P| + c = 0 \quad \text{(Equation 51)}
\]

where

\[
a = K'_A + K_B + |A|_0 + |B|_0 - |P|_0 \quad \text{(Equation 52)}
\]

\[
b = K_B(|A|_0 - |P|_0) + K_A(|A|_0 - |P|_0) + K'_AK_B \quad \text{(Equation 53)}
\]

\[
c = -K'_AK_B|P|_0 \quad \text{(Equation 54)}
\]

The solution of Equation 51 is

\[
|P| = -\frac{a}{3} + \frac{2}{3}\sqrt{\frac{a^2 - 3b}{a^2 - 3b}\cos^3\frac{\theta}{3}} \quad \text{(Equation 55)}
\]

\[
[PA] = \frac{|A|_0\left(2\sqrt{a^2 - 3b}\cos(\theta/3) - a\right)}{3K_A + 2\sqrt{(a^2 - 3b)\cos(\theta/3) - a}} \quad \text{(Equation 56)}
\]

\[
[PB] = \frac{|B|_0\left(2\sqrt{a^2 - 3b}\cos(\theta/3) - a\right)}{3K_B + 2\sqrt{(a^2 - 3b)\cos(\theta/3) - a}} \quad \text{(Equation 57)}
\]

\[
[B] = |B|_0 - [PB] \quad \text{(Equation 58)}
\]

\[
[A] = \frac{|A|_0 - |PA|}{1 + K_S} \quad \text{(Equation 59)}
\]

\[
[A'] = \frac{|A|_0 - |PA|}{1 + K_S} \quad \text{(Equation 60)}
\]

where \(\theta = \cos^{-1}\left(\frac{-a^2 + 9ab - 27b^2}{2\sqrt{(a^2 - 3b)^3}}\right)\)

The modified competitive binding model for Rtg2 system

Consider the activation layer of the RTG pathway and plug in the Rtg2 system to the competitive binding model derived previously. The RTG proteins are denoted by: Mks as \(P\), Rtg2\(_{acet}\) as \(A\), Rtg2\(_{na}\) as \(A'\), and Bmh as \(B\). We used \([\text{Rtg2}_{acet}]\), \([\text{Rtg2}_{na}]\), \([\text{Bmh}]\) and \([\text{Mks}]\) to represent their steady-state concentrations.

\[
\frac{k_A}{k'_A} \frac{\text{Rtg2}_{acet}}{\text{Rtg2}_{na}} = \frac{\text{Rtg2}_{acet}}{\text{Rtg2}_{na}} \quad \text{(Equation 61)}
\]
\[ \text{Mks} + \text{Rtg2}^{\text{act}} \frac{k_{\text{Ms}}}{k_{\text{M}}^*} \text{Rtg2Mks} \quad (\text{Equation 62}) \]

\[ \text{Mks} + \text{Bmh} \frac{k_{\text{Mbs}}}{k_{\text{bs}}} \text{BmhMks} \quad (\text{Equation 63}) \]

The dissociation coefficients are defined as

\[ K_D = \frac{[\text{Rtg2}^{\text{act}}] \cdot [\text{Mks}]}{[\text{Rtg2Mks}]} = \frac{k_{\text{ms}}}{k_{\text{M}}^*} \quad (\text{Equation 64}) \]

\[ K_B = \frac{[\text{Bmh}] \cdot [\text{Mks}]}{[\text{BmhMks}]} = \frac{k_{\text{bs}}}{k_{\text{bs}}^*} \quad (\text{Equation 65}) \]

\[ K_T = \frac{[\text{Rtg2}^{\text{act}}]}{[\text{Rtg2}]} = \frac{k_{\text{r}}}{k_{\text{r}}^* + [\text{Mks}]} \quad (\text{Equation 66}) \]

where \( K_D \) and \( K_B \) are the dissociation coefficients for the Mksp’s binding of Rtg2p^{act} and Bmhp. For the Rtg2p activation, we assume the input influences the activation of Rtg2p^{ina} in the manner of Hill formula \((\text{Equation 61} \text{ and Equation 66})\). To avoid zero division, we set the domain of input as \( s \in (0, 1] \).

The conservation rule requires that

\[ [\text{Rtg2}]_0 = [\text{Rtg2}^{\text{ina}}] + [\text{Rtg2}^{\text{act}}] \quad (\text{Equation 67}) \]

\[ [\text{Bmh}]_0 = [\text{Bmh}] + [\text{BmhMks}] \quad (\text{Equation 68}) \]

\[ [\text{Mks}]_0 = [\text{Mks}] + [\text{Rtg2}^{\text{act}} \cdot \text{Mks}] + [\text{BmhMks}] \quad (\text{Equation 69}) \]

where \([\text{Rtg2}]_0\), \([\text{Bmh}]_0\) and \([\text{Mks}]_0\) represent total concentration, and are assumed constants. Therefore, we can make \([\text{Bmh}]\) and \([\text{Rtg2}^{\text{act}} \cdot \text{Mks}]\) into the dependent variables of \([\text{Mks}]\) and their total concentrations.

\[ [\text{BmhMks}] = \frac{[\text{Mks}] [\text{Bmh}]_0}{K_B + [\text{Mks}]} \quad (\text{Equation 70}) \]

\[ [\text{Rtg2}^{\text{act}} \cdot \text{Mks}] = \frac{[\text{Rtg2}]_0 [\text{Mks}]}{K_T + [\text{Mks}]} \quad (\text{Equation 71}) \]

To derive the polynomial function of \([\text{Mks}]\), put \text{Equation 70} and \text{Equation 71} together:

\[ [\text{Mks}]_0 = [\text{Mks}] + [\text{Rtg2}^{\text{act}} \cdot \text{Mks}] + [\text{BmhMks}] \]

\[ = [\text{Mks}] + \frac{[\text{Mks}] [\text{Rtg2}]_0}{K_D (1 + \frac{1}{K_T})} + [\text{Mks}] [\text{Bmh}]_0 \]

\[ = [\text{Mks}] + \frac{[\text{Mks}] [\text{Rtg2}]_0}{K_T^* + [\text{Mks}]} + [\text{Mks}] [\text{Bmh}]_0 \]

where \( K_T^* = K_T (1 + \frac{1}{K_T}) \) is the dissociation coefficient influenced by the input coefficient \( K_T \) \((\text{Equation 66})\).

Finally, we can get the cubic polynomial equation of \([\text{Mks}]\) from \text{Equation 72}:

\[ [\text{Mks}]^3 + a[\text{Mks}]^2 + b[\text{Mks}] + c = 0 \quad (\text{Equation 73}) \]
where
\[ a = K_T + K_a + |Rtg2|_0 + |Bmh|_0 - |Mks|_0 \]
\[ b = K_a(|Rtg2|_0 - |Mks|_0) + K_T(|Bmh|_0 - |Mks|_0) + K_K a \]
\[ c = -K_a K_b[Mks|_0 \]

The polynomial equation of [Mks] can be solved by applying trigonometry. The analytical solution of competitive ligand binding is
\[ [Mks] = -\frac{a}{3} + \frac{2}{3} \sqrt{(a^2 - 3b)\cos^2 \theta} \quad \text{(Equation 74)} \]
\[ [Rtg2Mks] = \frac{|Rtg2|_0 \left( 2\sqrt{a^2 - 3b} \cos(\theta/3) - a \right)}{3K_T + 2\sqrt{(a^2 - 3b)\cos(\theta/3)} - a} \quad \text{(Equation 75)} \]
\[ [BmhMks] = \frac{|Bmh|_0 \left( 2\sqrt{a^2 - 3b} \cos(\theta/3) - a \right)}{3K_T + 2\sqrt{(a^2 - 3b)\cos(\theta/3)} - a} \quad \text{(Equation 76)} \]
\[ [Bmh] = |Bmh|_0 - |BmhMks] \quad \text{(Equation 77)} \]
\[ [Rtg2^{act}] = (|Rtg2|_0 - |Rtg2Mks|) \frac{1}{1 + K_T} \quad \text{(Equation 78)} \]
\[ [Rtg2^{na}] = (|Rtg2|_0 - |Rtg2Mks|) \frac{K_T}{1 + K_T} \quad \text{(Equation 79)} \]

where \( \theta = \cos^{-1} \left( \frac{2a^2 + 2ab - 2 \sqrt{a^2 - 3b}}{2\sqrt{(a^2 - 3b)^2}} \right) \).

### Analysis ultrasensitivity with relative amplification approach

The relative amplification approach is applied to quantify the local and global sensitivity by estimating the equivalent Hill coefficient (Legewie et al., 2005). When the response curve starts with a high basal level, this approach provides a more accurate approximation compared to the method using EC90:EC10 ratio (Legewie et al., 2005; Ferrell and Ha, 2014). The response coefficient \( R_{\text{output}}^{\text{input}} \) (Equation 80) is used to quantify the relative change in response to the stimulus.

\[ R_{\text{output}}^{\text{input}} = \text{input} \frac{d\text{output}}{d\text{input}} \quad \text{(Equation 80)} \]

Furthermore, to circumvent the deviation from a high basal level, the activated fraction \( f \) (Equation 81) is defined as the activated ratio of output between the maximum and basal response (Legewie et al., 2005).

\[ f(\text{output}) = \frac{\text{output} - \text{output}_{\text{basal}}}{\text{output}_{\text{max}} - \text{output}_{\text{basal}}} \quad \text{(Equation 81)} \]

where \( f \in [0, 1] \), output is the function of input. Besides, output_{max} and output_{basal} represents the maximum and minimum of outputs respectively, which makes activated fraction \( f \) independent from the basal activation.

The advantage of applying the response coefficient \( R \) and activated fraction \( f \) is that the classical Hill equation can be converted to a linear equation (Equation 82) (Legewie et al., 2005).

\[ R = nH (1 - f) \quad \text{(Equation 82)} \]

\[ n_R = \left| \frac{\int nH R_{\text{output}}^{\text{input}} df}{\int \text{output}_{\text{max}} df} \right| \quad \text{(Equation 83)} \]
where \( f_H \) and \( f_L \) are the maximum and minimum of activation fractions in a given region. \( n_H \) is Hill coefficient, and \( n_R \) is the relative amplification coefficient. Michaelis-Menten equation was used as the reference response.

**Frequency response of RTG signaling**

To measure the RTG response to the dynamical input, the original ODE model is converted into non-autonomous differential equations with mitochondrial damage signal \((s)\) as input (Equation 84).

\[
\frac{d[\text{Output}]}{dt} = Af(s(t); u^2, p)
\]

(Equation 84)

where \( A \) is the output map.

Furthermore, the sinusoidal function with frequency between \(10^{-3}\) Hz and 1 Hz was used to conduct the frequency analysis to reveal the break point in Bode diagram. In this study, we focus on the concentrations of Bmh/Mksp heterodimer and Rtg1/3p nucleus accumulation in response to the sinusoidal input.

The non-autonomous model is solved by TR-BDF2 ODE solver (Hosea and Shampine, 1996; Rackauckas and Nie, 2017; Kim et al., 2021). By the aid of automatic switching stiff/non-stiff method of DifferentialEquations.jl (Rackauckas and Nie, 2017), the simulation process can be significantly sped up.

**Stochastic simulation of Rtg1/3p translocation in response to mitochondrial damage**

The stochastic model is modified from the ODEs with the theory of chemical Langevin equation (CLE) (Gillespie, 2000). The derivative of nucleus Rtg1/3p is composed of the drifting term from the deterministic model, and the diffusion term caused by the Brownian motion. The process is described with the stochastic differential equation in Equation 85.

\[
d[\text{Rtg13n}]_t = \sum_{r=1}^{R} S_r f_r ([\text{Rtg13n}]_t) dt + \sum_{r=1}^{R} S^2_r f_r ([\text{Rtg13n}]_t) dW
\]

(Equation 85)

where \( f_r \) is the propensity of \( r \)th reaction, \( S_r \) the net change of the reaction \( r \), and \( dW \) represents the Wiener process (Schnoerr et al., 2014).

The CLE was solved using the Euler-Maruyama method with adaptive time stepping, and simulated for 100 s. Further, the time series simulation was used to derive the likelihood of the output state with kernel density estimation as illustrated in Figure 5.