Systems Biological Analysis of mTOR-Dependent Molecular Mechanisms of Autophagy

PhD thesis

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Introduction

Autophagy is an evolutionarily conserved cellular digestive process crucial for maintaining cellular homeostasis. While there are multiple forms of autophagy including chaperone-mediated autophagy, microautophagy, and macroautophagy, this study focuses exclusively on macroautophagy. Autophagy is active at a basal level at all times, performing essential functions like degrading damaged proteins and aged organelles to ensure cellular quality control. Beyond this constant surveillance, autophagy has a major role as a key cellular response to external and internal stimuli, including nutrient deprivation, hypoxia, infection, or oxidative stress.

Given its vital roles in the maintenance of cellular homeostasis, dysfunction in autophagy is correlated with multiple diseases, often exhibiting complex, context-dependent effects. Research has indicated a strong correlation between irregularities in autophagy processes and the pathogenesis of major neurodegenerative diseases, metabolic disorders through dysfunction in key metabolic tissues like the liver and pancreas, and aging-related conditions. In cancers, autophagy has a dual role, serving as a tumor suppressor mechanism during early oncogenesis while simultaneously being exploited by established malignancies to enhance their survival and therapeutic resistance.

The complexity of autophagy regulation and its multifaceted roles in disease necessitate quantitative approaches like systems biology. However, a central challenge is that the rate constants and parameters governing these biochemical networks are often not well known, requiring data-driven techniques such as parameter estimation or model calibration (Figure 1). Achieving reliable model calibration faces significant challenges including parameter identifiability, optimization complexity, and data limitations that become more pronounced as the size and complexity of the model increase.

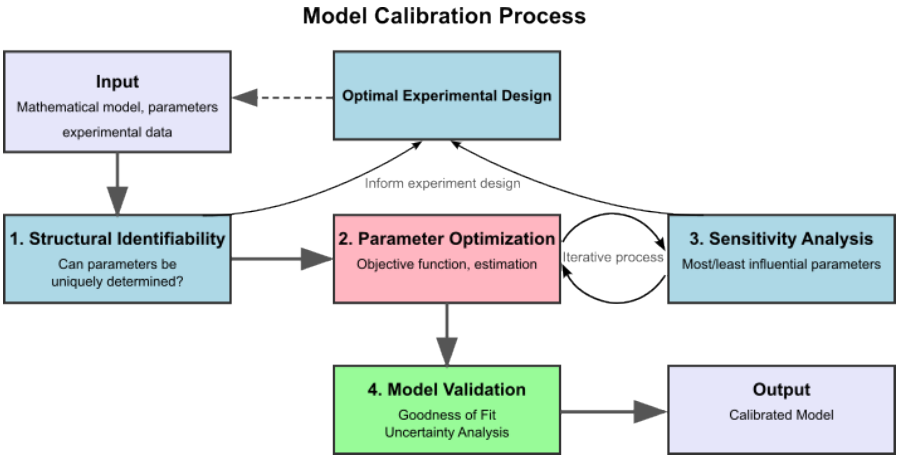

Figure 1: Workflow for ODE model calibration and validation. Starting with model structure, parameter estimates, and experimental data, the process involves: (1) structural identifiability analysis, (2) parameter optimization through iterative objective function minimization, (3) sensitivity analysis of parameter influence, and (4) model validation via goodness-of-fit and uncertainty assessment. Optimal experimental design guides data collection to maximize parameter identifiability. Output is a validated model ready for prediction.

This thesis presents a systematic framework for calibrating and validating ODE models against experimental data (Figure 1). This integrated approach combines structural identifiability analysis, parameter optimization, sensitivity analysis, and model validation to quantitatively characterize ULK1-mediated autophagy regulation.

Objectives

This PhD research aims to develop comprehensive autophagy induction models with future applications in translational medicine. The specific objectives are:

1. **Investigate the effect of the phosphatase PP2A on ULK1-mediated autophagy induction**

   Build and experimentally validate a small-scale chemical reaction network model incorporating PP2A-ULK1-mTORC1 interactions, focusing on feedback mechanisms and their contribution to robust autophagy induction.

2. **Determine the minimal dynamical requirements for ULK1-induced autophagy oscillation**

   Identify the minimal dynamical requirements needed to reproduce experimentally observed oscillatory behavior of ULK1-mediated autophagy induction.

3. **Develop a more comprehensive, top-down modeling approach for autophagy regulation**

   Construct a large-scale, computationally and biologically validated quantitative framework to decipher the dynamic interplay between autophagy and apoptosis regulation.

Methods

Experimental methods

Cell culture and cell treatments

Human embryonic kidney (HEK293T) cells were maintained in DMEM medium supplemented with 10% fetal bovine serum at 37°C. Cells were treated with rapamycin (100 nM, mTORC1 inhibitor), okadaic acid ($100-175$ nM, PP2A inhibitor), and Bafilomycin A1 (100 nM, autophagy flux inhibitor) for specified time periods. RNA interference was performed using ULK1 and PP2ACα siRNAs at 20 pmol/ml concentration.

SDS-PAGE and Western Blot Analysis

SDS-PAGE and Western blot analysis tracked the phosphorylation dynamics of key network components using specific antibodies: antiULK1-Ser757-P, antip70S6K-P (mTORC1 activity), antiPP2A-Tyr307-P, antiAMPK-P, and autophagy markers (antiLC3B, antip62). Densitometry analysis was performed using ImageJ software with appropriate normalization and statistical analysis.

Densitometry analysis was performed using ImageJ software with appropriate normalization controls and statistical analysis to quantify protein expression changes.

Computational methods

All molecular interactions were modeled using systems of ordinary differential equations (ODEs) with mass action kinetics. Three distinct model frameworks were developed:

The *minimal PP2A-mTORC1-ULK1 model* incorporated conservation laws to reduce system complexity, yielding three ODEs with 12 kinetic parameters. The *oscillation model* extended this framework by introducing an intermediary regulatory component ('REG') to capture delayed AMPK signaling effects, resulting in a four-variable system with 25 parameters.

The *comprehensive autophagy-apoptosis model* represented a substantial expansion, comprising 113 biochemical reactions and 84 molecular species. This framework built upon and extensively revised the published Liu et al. model, correcting identified biological inconsistencies and mechanistic gaps.

Parameter estimation

Parameter estimation was formulated as optimization problems to minimize Mean Squared Error (MSE) between model predictions and experimental data. The PP2A model utilized the L-BFGS-B algorithm for efficient parameter space exploration.

The comprehensive model employed the Optima++ framework with the FOCTOPUS algorithm, representing the first application of combustion kinetics optimization tools to biological systems modeling.

Dynamical analysis

Standard dynamical systems analysis was performed using XPP-AUT software. Phase plane analysis investigated system steady states and bistability through nullcline plotting. Bifurcation analysis mapped the dependence of steady states and periodic solutions on key parameters using numerical continuation algorithms.

Sensitivity analysis

Parameter influence on model behavior was assessed through complementary approaches. Global Sensitivity Analysis employed Sobol indices to quantify

parameter uncertainty impact on model outputs. Local sensitivity analysis identified the most influential parameters using the SUE impact measure, which integrates parameter sensitivity, uncertainty ranges, and experimental error considerations.

Model validation

Model performance was systematically validated using multiple initial condition sets. For the comprehensive model, 20 distinct parameter sets were generated through random sampling within physiologically plausible ranges.

Deviations from target basal states were quantified using RMSD error functions. Parameter optimization achieved substantial improvement, reducing overall model error from 1.3×10^9 to 2.22. This optimization involved 101 influential rate coefficients, each constrained within biologically realistic uncertainty ranges of ± 4 orders of magnitude.

Results

Parameterization of a Core Autophagy Regulatory Model using Western Blot Time-Series Data

A minimal biochemical reaction network model focusing on the mTORC1, ULK1, and PP2A regulatory triangle was constructed to investigate the phosphatase role in autophagy induction through ULK1. Following the systematic workflow outlined in Figure 1, experiments were designed according to the interaction network topology to ensure structural identifiability of the 12 kinetic parameters in the three-ODE system.

Time-series Western blot experiments tracked the phosphorylation dynamics of key network components following targeted perturbations with rapamycin (mTORC1 inhibitor) and okadaic acid (PP2A inhibitor). These experimental conditions provided complementary data: rapamycin treatment resulted in rapid mTORC1 inactivation followed by subsequent activation of both ULK1 and PP2A, while okadaic acid treatment led to sustained PP2A inhibition, resulting in mTORC1 hyper-activation and sustained ULK1 inhibition.

Parameter optimization using the L-BFGS-B algorithm successfully fitted the model to experimental data as demonstrated in Figure 2, demonstrating that noisy Western blot time-series can provide sufficient constraints for robust parameter estimation when experiments are designed according to network structure requirements. The parameterized model was subsequently validated against independent experimental conditions not used in the optimization process, including combined treatments with rapamycin plus okadaic acid or PP2A siRNA knockdown, where the model accurately predicted differential ULK1 responses depending on the method of PP2A inhibition.

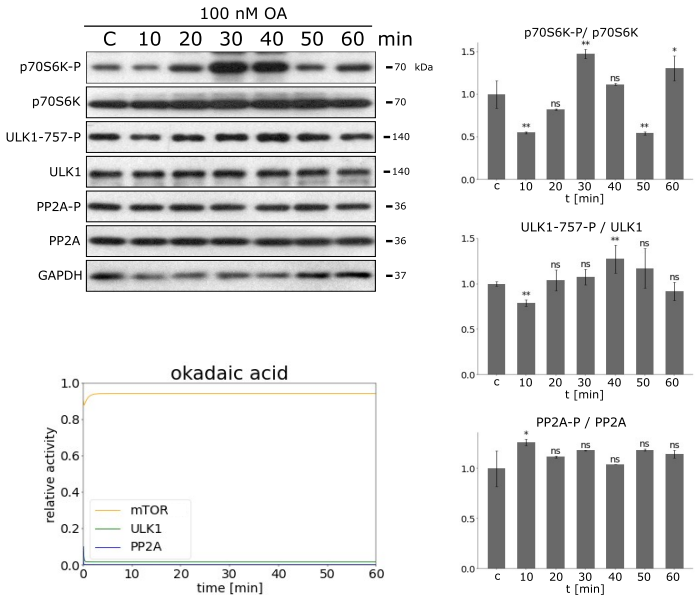

Figure 2: PP2A inhibition effects on mTORC1-ULK1-PP2A network. HEK293T cells treated with 100 nM okadaic acid. Top left: Immunoblots show ULK1-757-P, PP2A-P, and p70S6K-P with total proteins and GAPDH control. Bottom left: Model simulation shows protein concentration changes over time. Right: Densitometry quantification from three experiments; error bars show SD, asterisks indicate significance vs. control ns—nonsignificant; *—$p < 0.05$; **—$p < 0.01$

Time-delayed negative feedback enables autophagy oscillations

Like our previous minimal models, the PP2A-mTORC1-ULK1 regulatory triangle could not reproduce the experimentally observed oscillatory autophagy dynamics. To investigate this phenomenon, we reached back to our established AMPK-mTORC1-ULK1 model of similar size and network topology. Through phase plane and bifurcation analysis, we demonstrated that while this core network exhibits bistability enabling robust switching between autophagy and non-autophagy states, these direct feedback loops alone were insufficient to generate sustained oscillations across different treatment conditions.

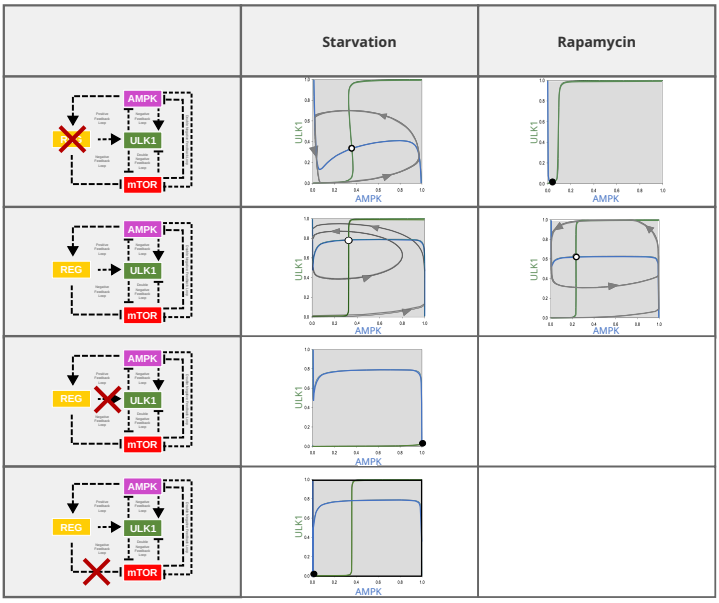

Figure 3: Phase plane analysis reveals bistability in AMPK-mTORC1-ULK1 network under different perturbations. Left column shows network topology with perturbations (red X indicates inhibition). Middle and right columns display phase portraits for starvation and rapamycin conditions, respectively. Trajectories demonstrate bistable switching between autophagy states, but lack sustained oscillations without time-delayed feedback mechanisms.

Global sensitivity analysis and systematic model refinement revealed that introducing an intermediary regulatory protein (REG) that propagates some of AMPK's effects provides the critical time delay necessary for oscillatory behavior. This REG component mediates delayed AMPK signaling to both ULK1 activation and mTORC1 inhibition, creating the temporal separation required for limit cycle dynamics.

Computational simulations demonstrated that both regulatory actions of REG are essential for reproducing experimental observations. Disrupting either the $REG \rightarrow ULK1$ positive effect or the $REG \dashv mTORC1$ inhibitory effect abolished the model's ability to oscillate and properly respond to multi-

ple treatment conditions including stress and rapamycin. Global sensitivity analysis confirmed the structural importance of this delay mechanism, with the $AMPK \rightarrow REG$ interaction ranking as the fourth most influential parameter governing system dynamics, highlighting the critical role of time-delayed negative feedback in autophagy regulation.

Top-down integration into a comprehensive autophagy-apoptosis model

Since constructing comprehensive models de novo presents significant computational and resource challenges, we adopted a top-down approach: integrating our validated small-scale models within an existing large-scale quantitative framework. We selected the autophagy-apoptosis decision model published by Liu et al. as our foundation, which necessitated complete reimplementation due to the absence of publicly available source code and essential simulation parameters.

The reconstructed model comprised 113 reactions and 84 species, requiring extensive modifications to achieve physiologically realistic behavior. Since the original model failed to maintain stable basal conditions and defaulted to apoptotic states regardless of initial conditions, we conducted an extensive literature review to establish plausible protein concentration ranges for all 84 species. Key biological inconsistencies were corrected, including PKA and PKC regulatory roles, mTOR-ULK1 interaction mechanisms, and stress pathway implementations.

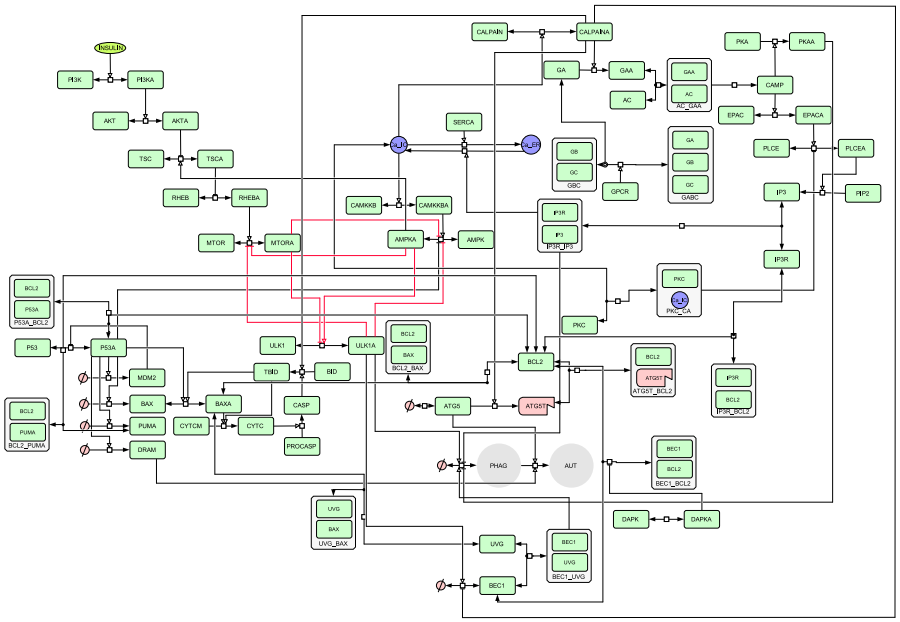

Figure 4: Wiring diagram of the enhanced autophagy-apoptosis signaling network model. This comprehensive network integrates key regulatory modifications including refined PKA/PKC/AKT signaling and streamlined stress response pathways, resulting in a model with 113 reactions and 84 molecular species. Critical regulatory connections within the AMPK-mTOR-ULK1 control hub are highlighted in red.

For the numerical framework, we adapted Optima++, a combustion kinetics computational platform, for cellular process simulation. This cross-disciplinary application provided significant advantages: standardized multi-source data handling through XML format, state-of-the-art algorithms for parameter optimization (FOCTOPUS), and integrated uncertainty analysis tools with sensitivity analysis capabilities. These features enabled seamless application of the Figure 1 workflow to the 101 influential parameters identified through local sensitivity analysis.

Parameter optimization successfully reduced the overall RMSD error from $1.3 \cdot 10^9$ to 2.22, with the calibrated model maintaining physiological

protein concentrations within target basal ranges over 24-hour simulations. This systematic calibration approach constrained 19 key reaction parameters to within one order of magnitude, establishing the first stable, reproducible comprehensive model of autophagy-apoptosis crosstalk.

Conclusions

This thesis investigated ULK1-mediated autophagy regulatory mechanisms using systems biology approaches, successfully addressing all outlined objectives through integrated experimental-computational methodologies.

Objective 1 - PP2A regulatory model: A small-scale chemical reaction network model incorporating PP2A-ULK1-mTORC1 interactions was successfully built and experimentally validated. By designing experiments according to structural identifiability analysis, model parameters were uniquely determined from Western blot time-series data. The approach demonstrated that noisy experimental data can provide sufficient constraints for robust parameter estimation when experimental design follows network topology requirements. Importantly, the model accurately predicted cellular responses to combined treatments not used in parameterization, confirming its predictive capacity.

Objective 2 - Oscillation mechanisms: Time-delayed negative feedback was identified as the minimal dynamical requirement for ULK1-mediated autophagy oscillations. Through systematic analysis, an intermediary regulatory component (REG) was found to be essential for mediating delayed AMPK effects on both ULK1 and mTORC1. Disrupting either REG function abolished oscillatory behavior, while global sensitivity analysis confirmed the structural importance of this delay mechanism in the control network.

Objective 3 - Comprehensive model development: An open-source, comprehensive autophagy-apoptosis model was developed by extensively reconstructing Liu et al.'s framework. Using Optima++ for biochemical networks for the first time, parameter optimization reduced RMSD error from $1.3 \cdot 10^9$ to 2.22, achieving stable homeostatic behavior while prioritizing reproducibility through open-source code availability and detailed documentation.

Broader impact: These mechanistic insights provide a foundation for understanding autophagy dysregulation in disease contexts and enable systematic investigation of therapeutic interventions. The methodological framework facilitates integration of diverse experimental data sources for complex biological systems modeling, advancing both fundamental understanding and translational applications in systems biology.

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