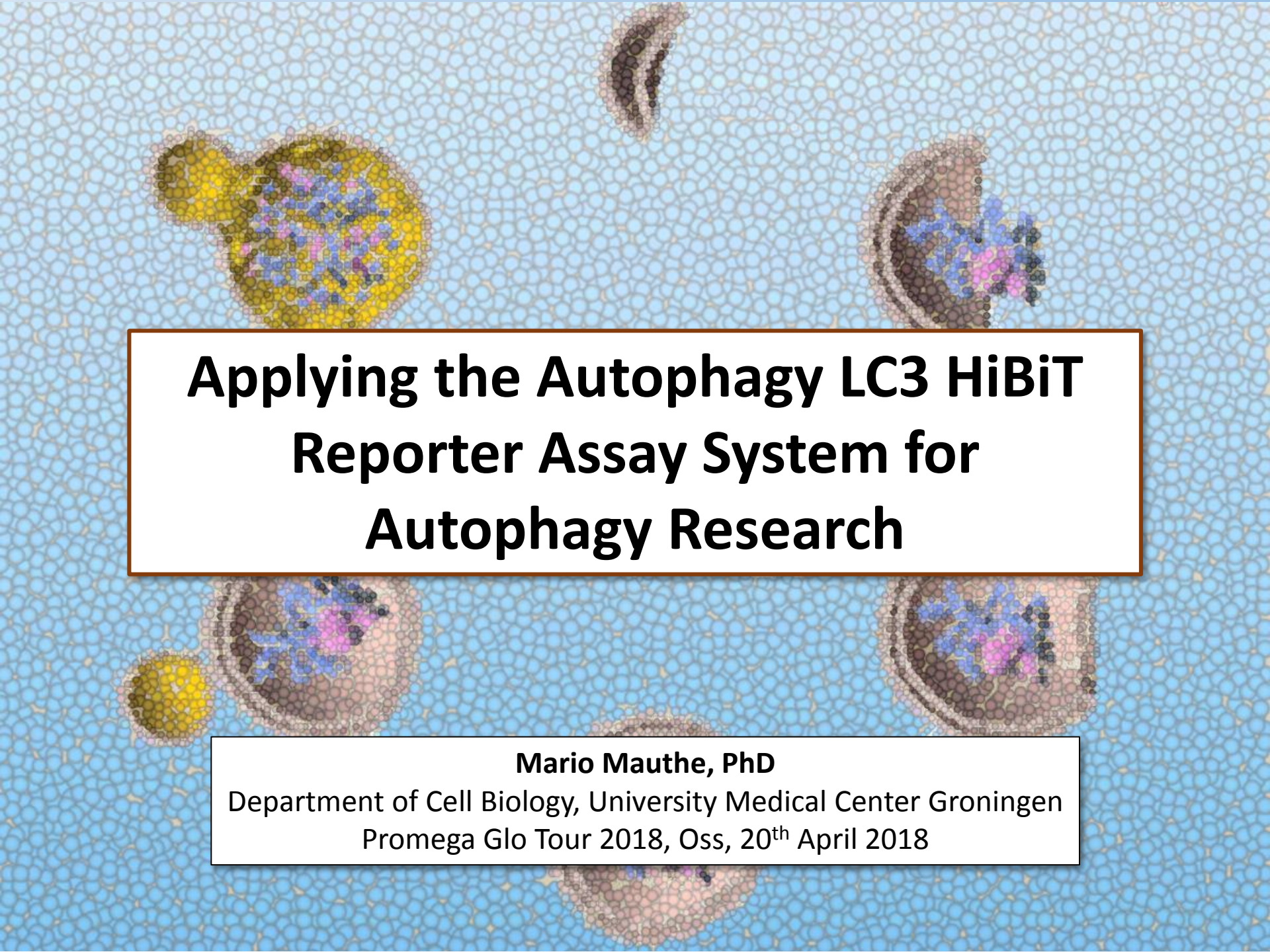


The background of the slide is a composite of several fluorescence microscopy images. These images show cells with various organelles and structures labeled with different fluorescent dyes, appearing in colors like yellow, green, blue, and magenta. The images are arranged in a grid-like pattern, with some showing individual cells and others showing clusters or specific organelles. The overall background has a light blue, textured appearance.

Applying the Autophagy LC3 HiBiT Reporter Assay System for Autophagy Research

Mario Mauthe, PhD

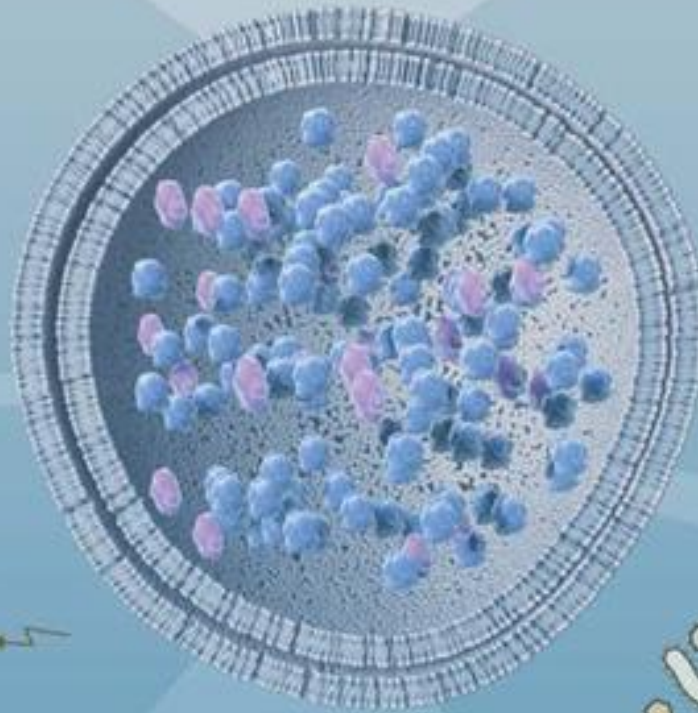
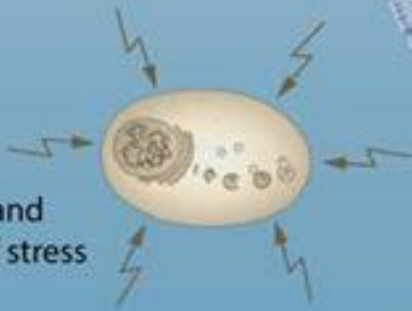
Department of Cell Biology, University Medical Center Groningen
Promega Glo Tour 2018, Oss, 20th April 2018

Autophagy in Health and Disease

Embryogenesis
and cell
differentiation



Starvation and
other types of stress



Neurodegenerative
diseases



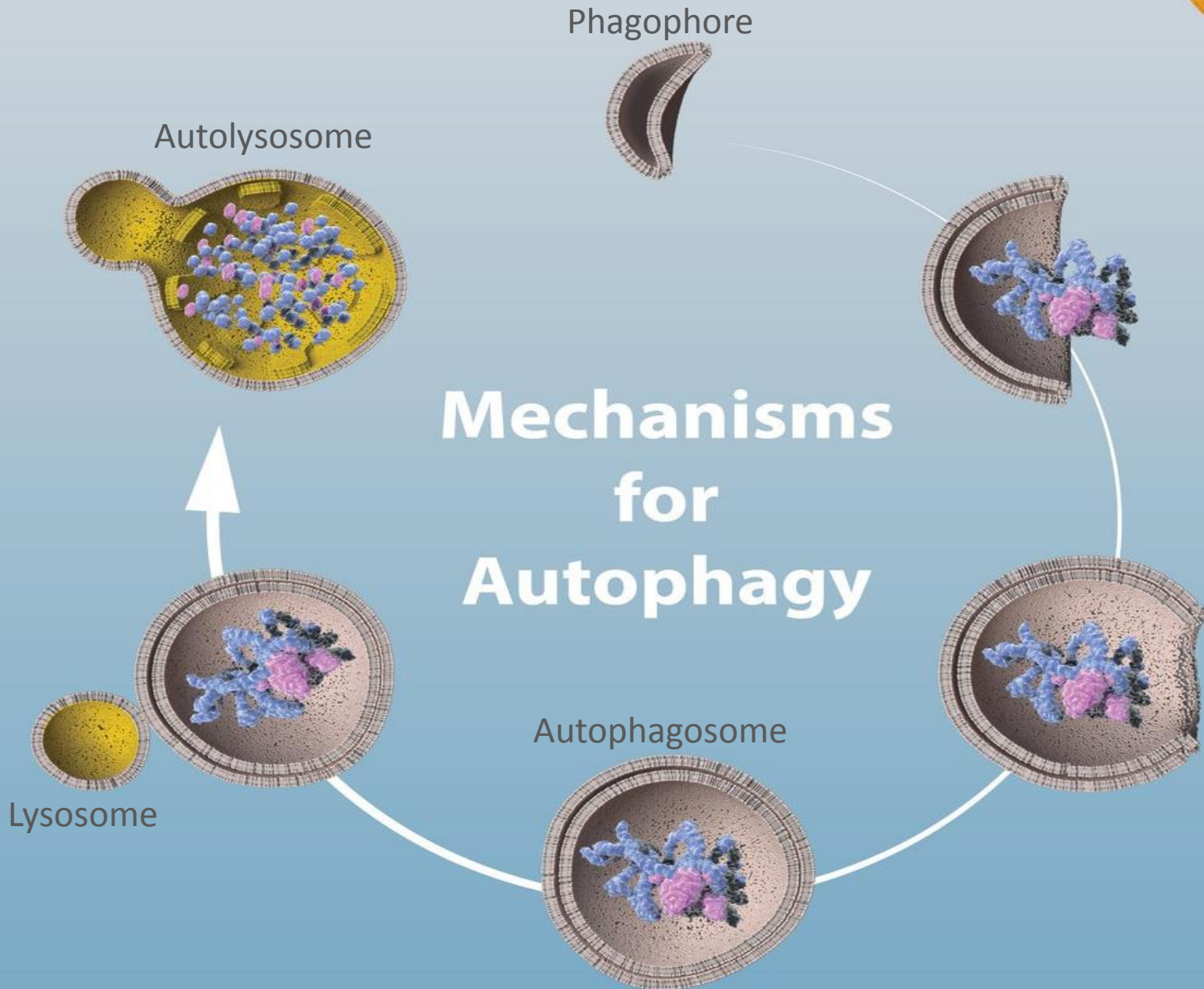
Cancer



Bacterial and
viral infections



The Nobel Prize in Physiology or Medicine 2016



Methods to Monitor Autophagy

I. Direct Measurement of Autophagic Activity

Turnover of long-lived proteins

LDH sequestration

Western blot-based assays (LC3 lipidation, SQSTM1/p62 turnover and post-translational modifications)

II. Indirect Measurement of Autophagic Activity

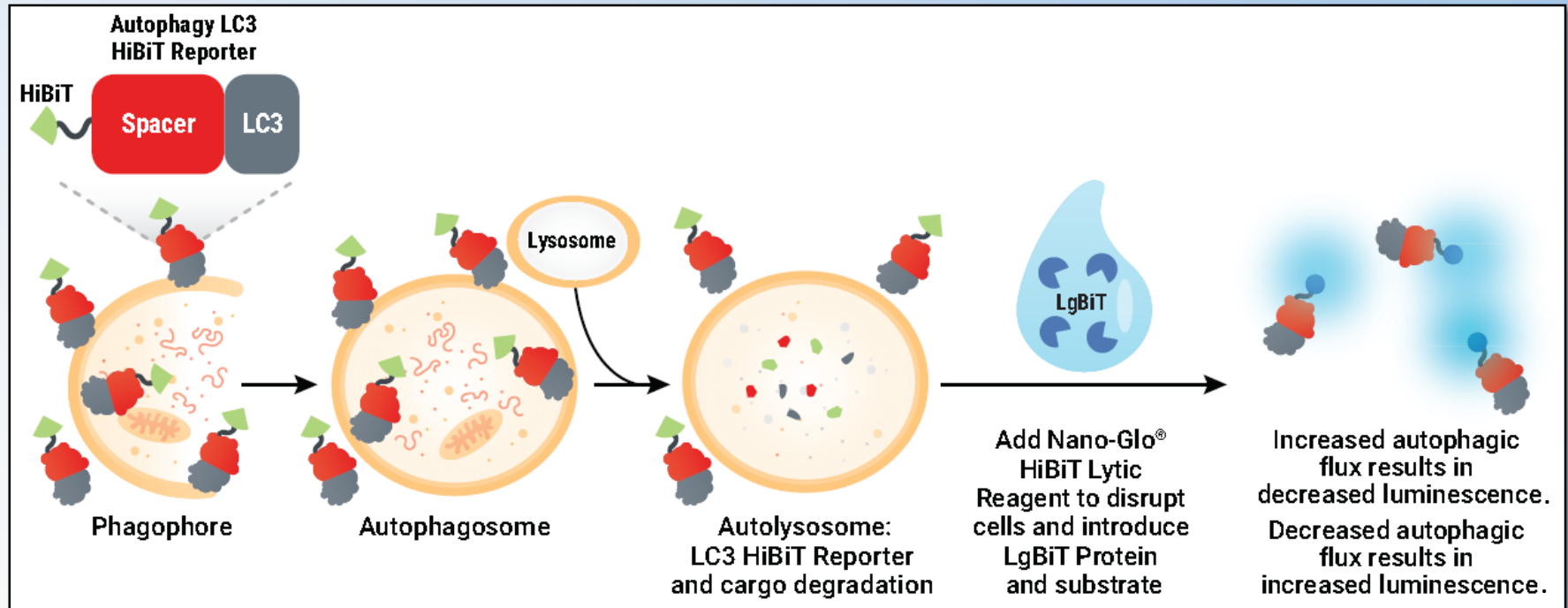
Fluorescence microscopy-based methods (distribution of autophagosomal protein markers and molecular tandem probes)

Electron microscopy

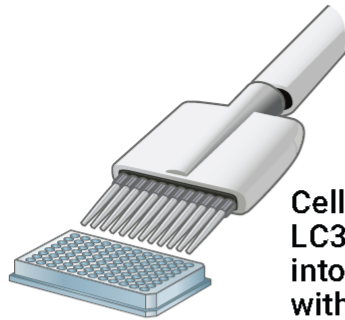
Flow cytometry and imaging flow cytometry

The Principle of the Autophagy LC3 HiBiT Reporter Assay

Theory behind the assay



Autophagy Assay Protocol and Interpretation of the Results



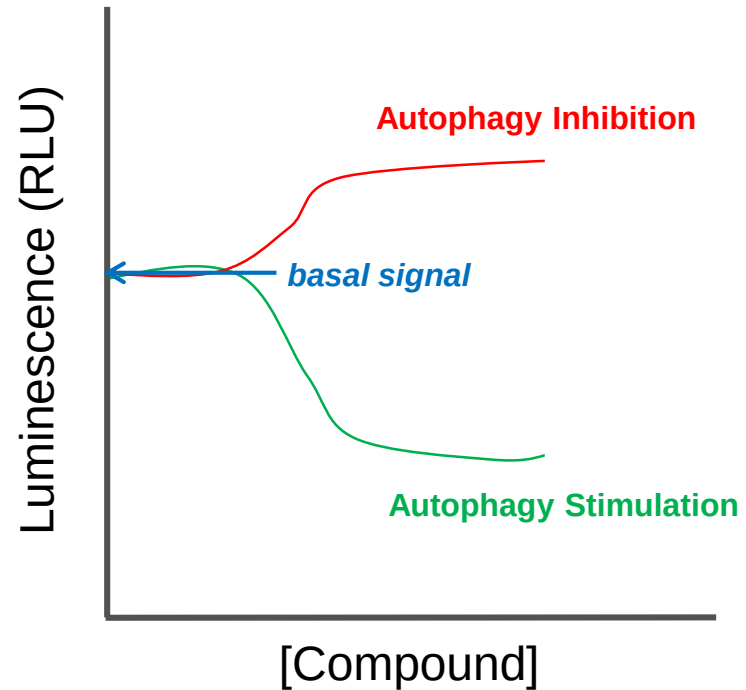
Cells expressing Autophagy LC3 HiBiT Reporter dispensed into assay plate and treated with test agents for desired time.



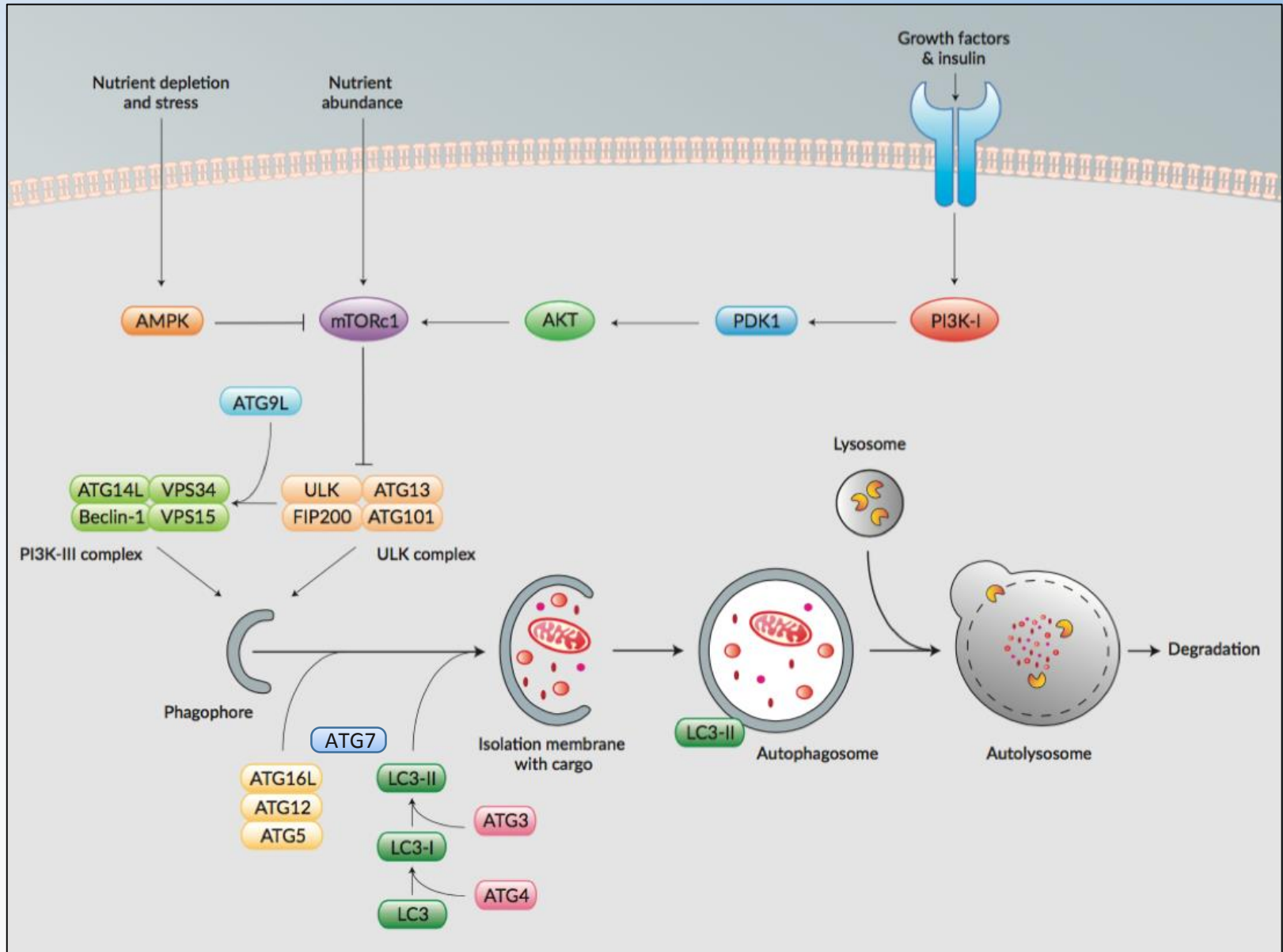
Add the Nano-Glo[®] HiBiT Lytic Reagent.



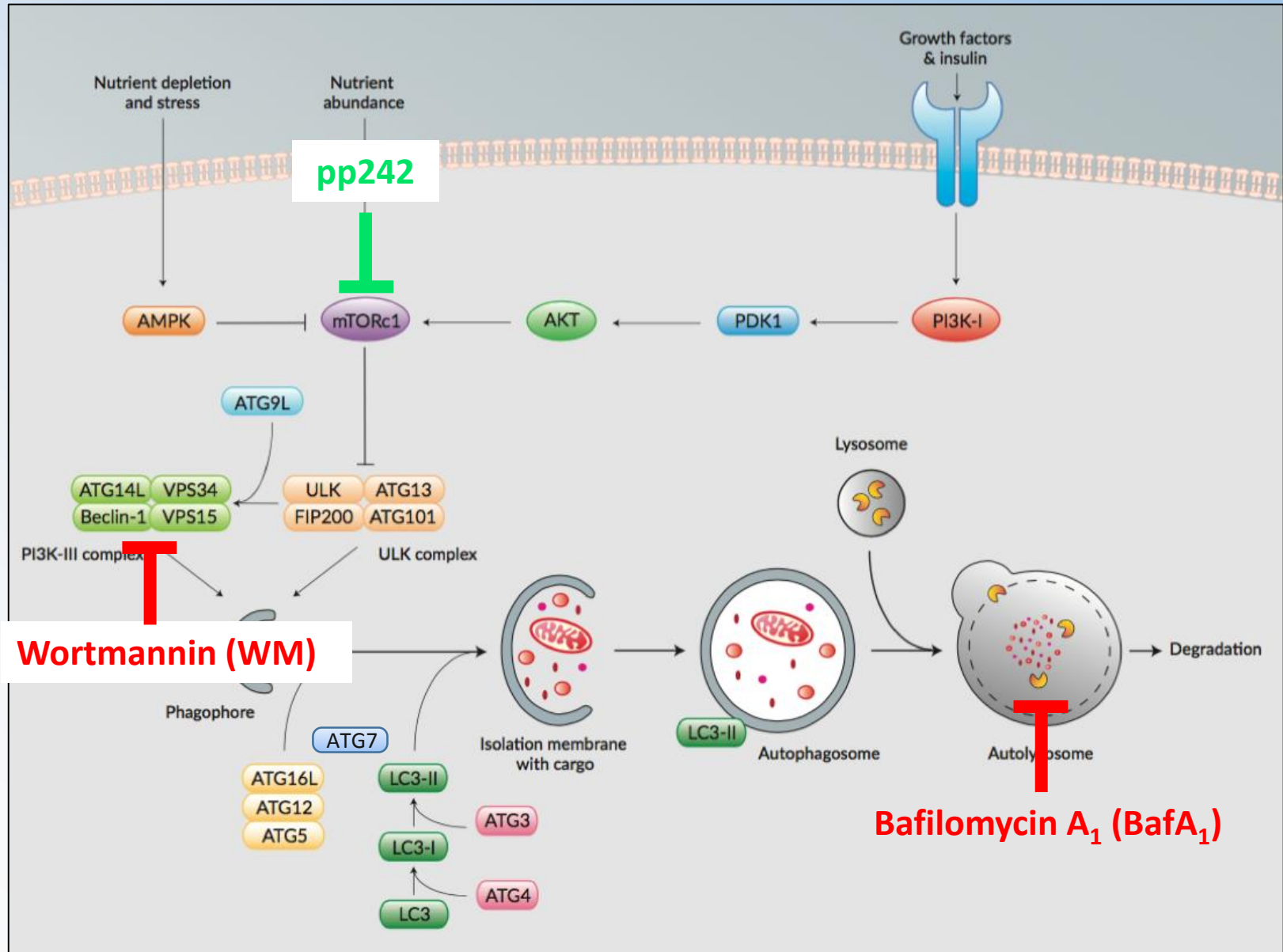
Record luminescence after 10 minutes or up to 3 hours.



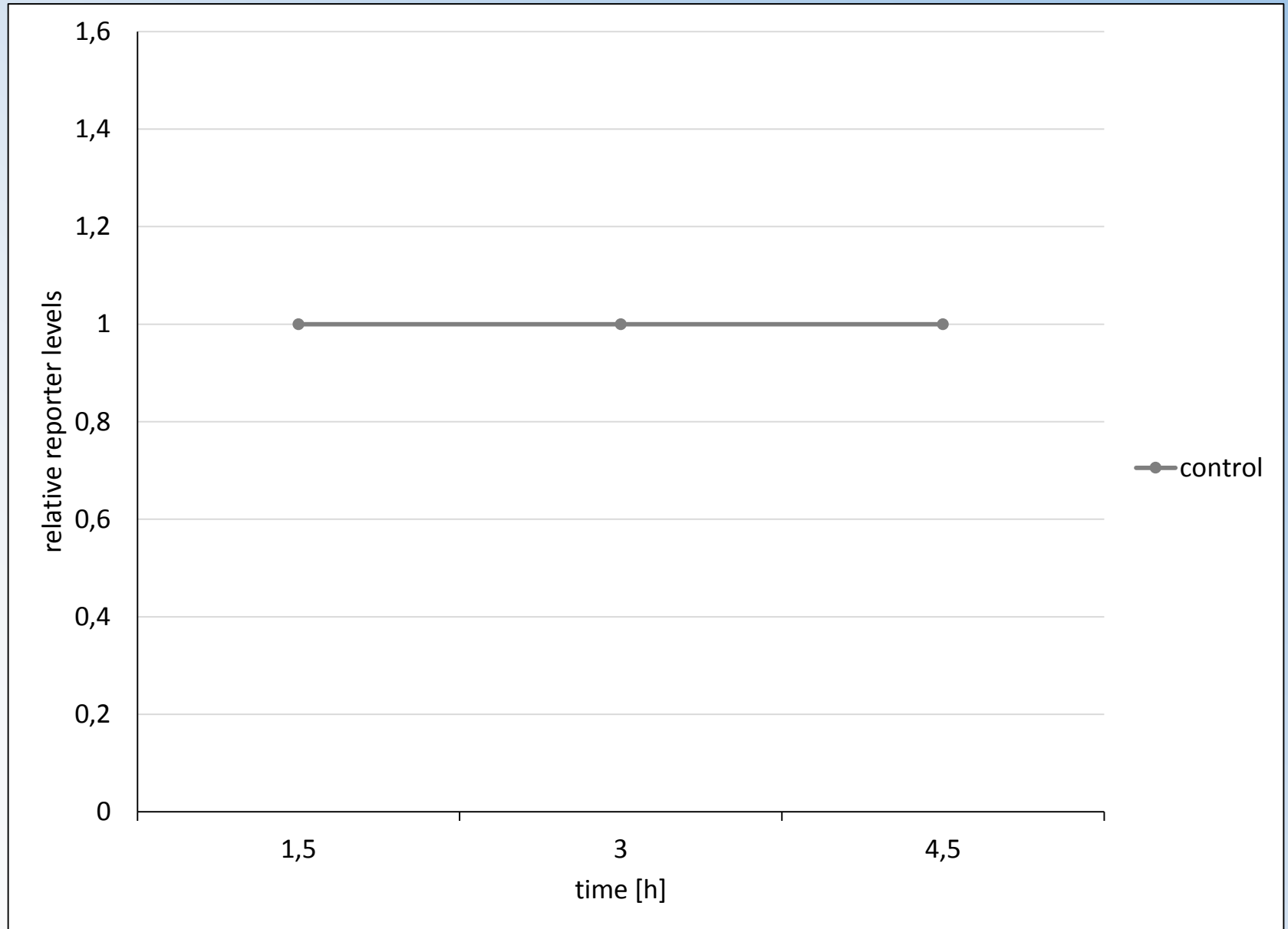
The Autophagy Pathway in More Detail



The Autophagy Pathway in More Detail

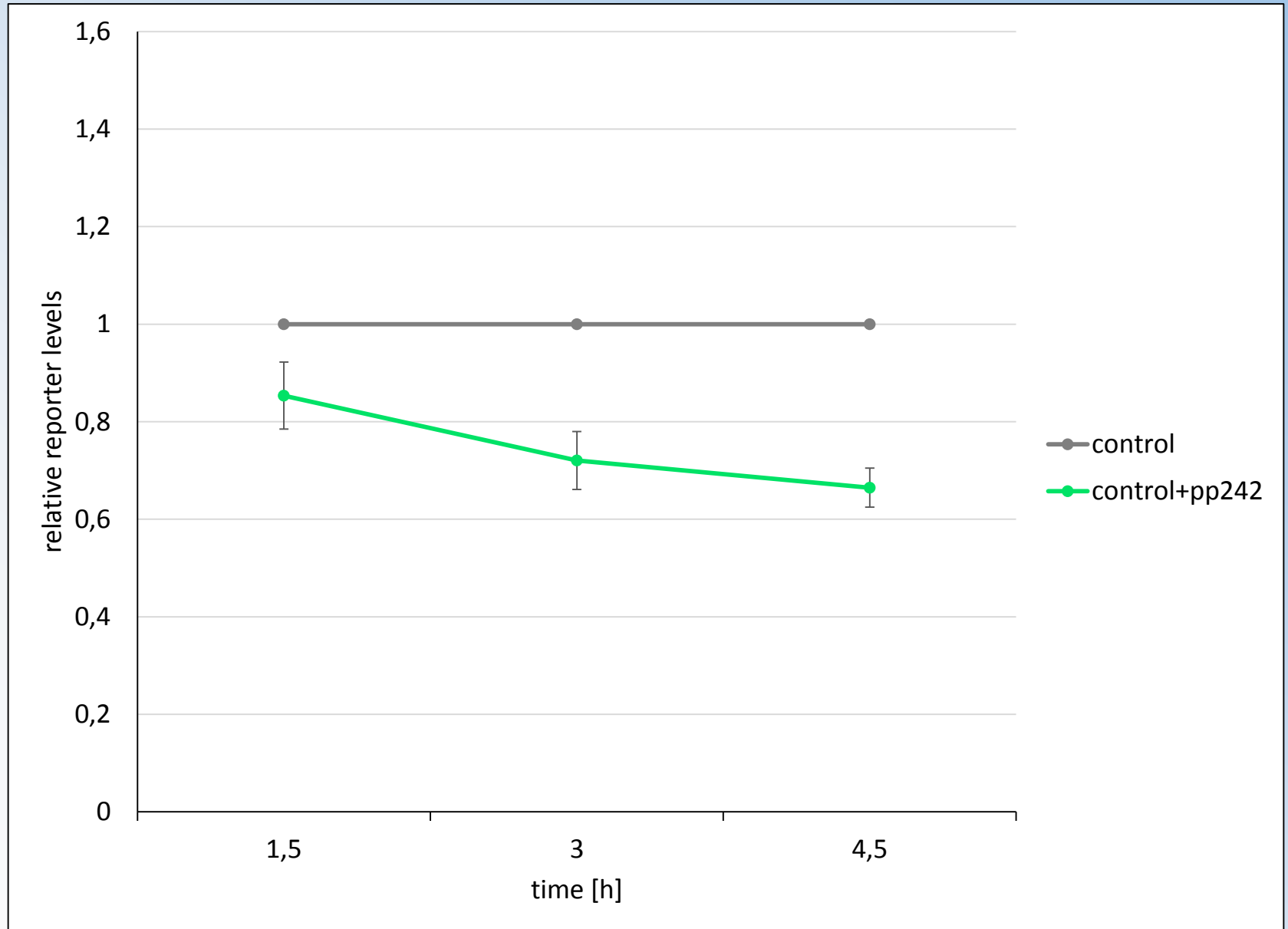


LC3 HiBiT Reporter Assay & Autophagy Modulators



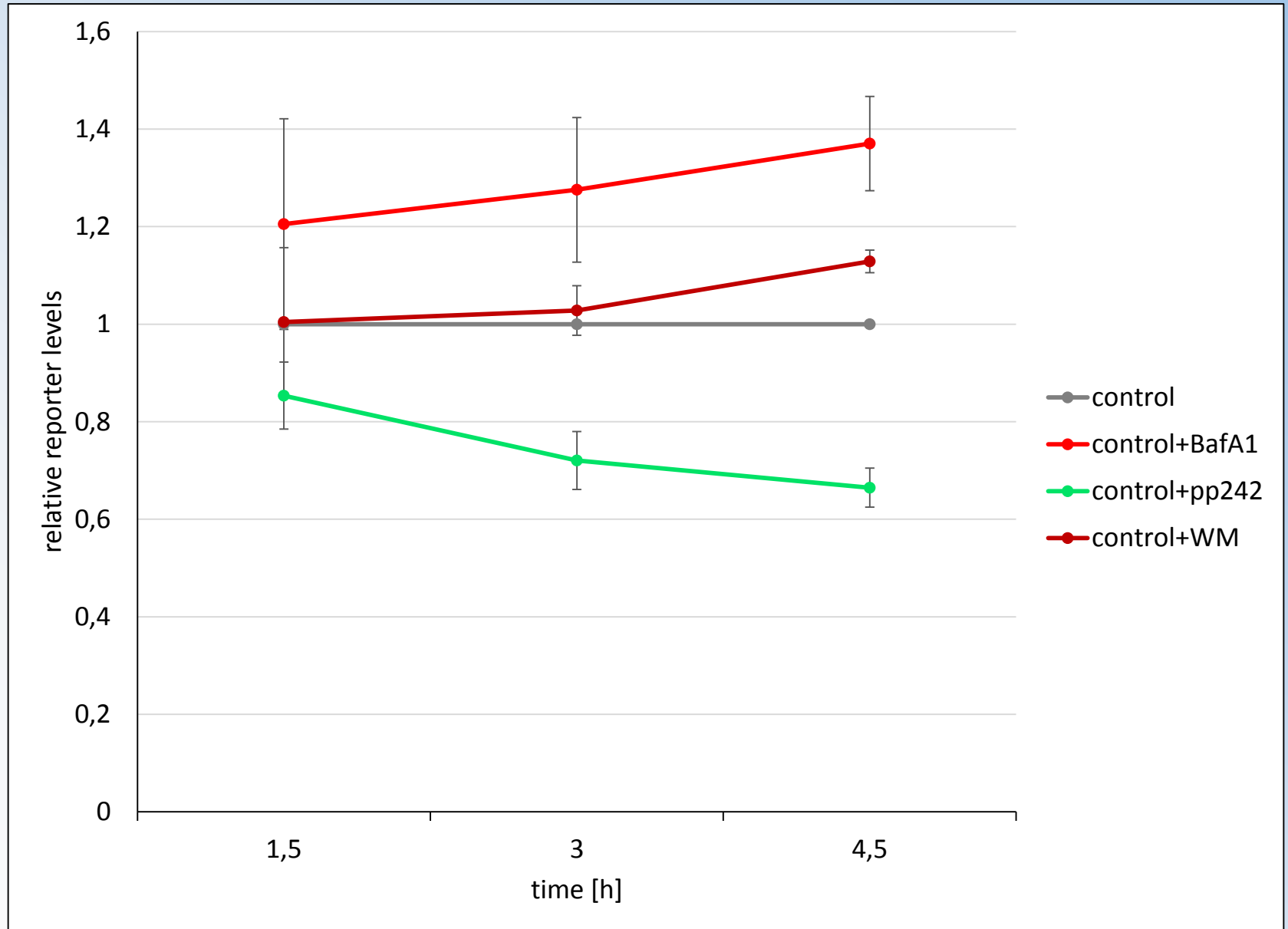
U2OS reporter cells

LC3 HiBiT Reporter Assay & Autophagy Modulators



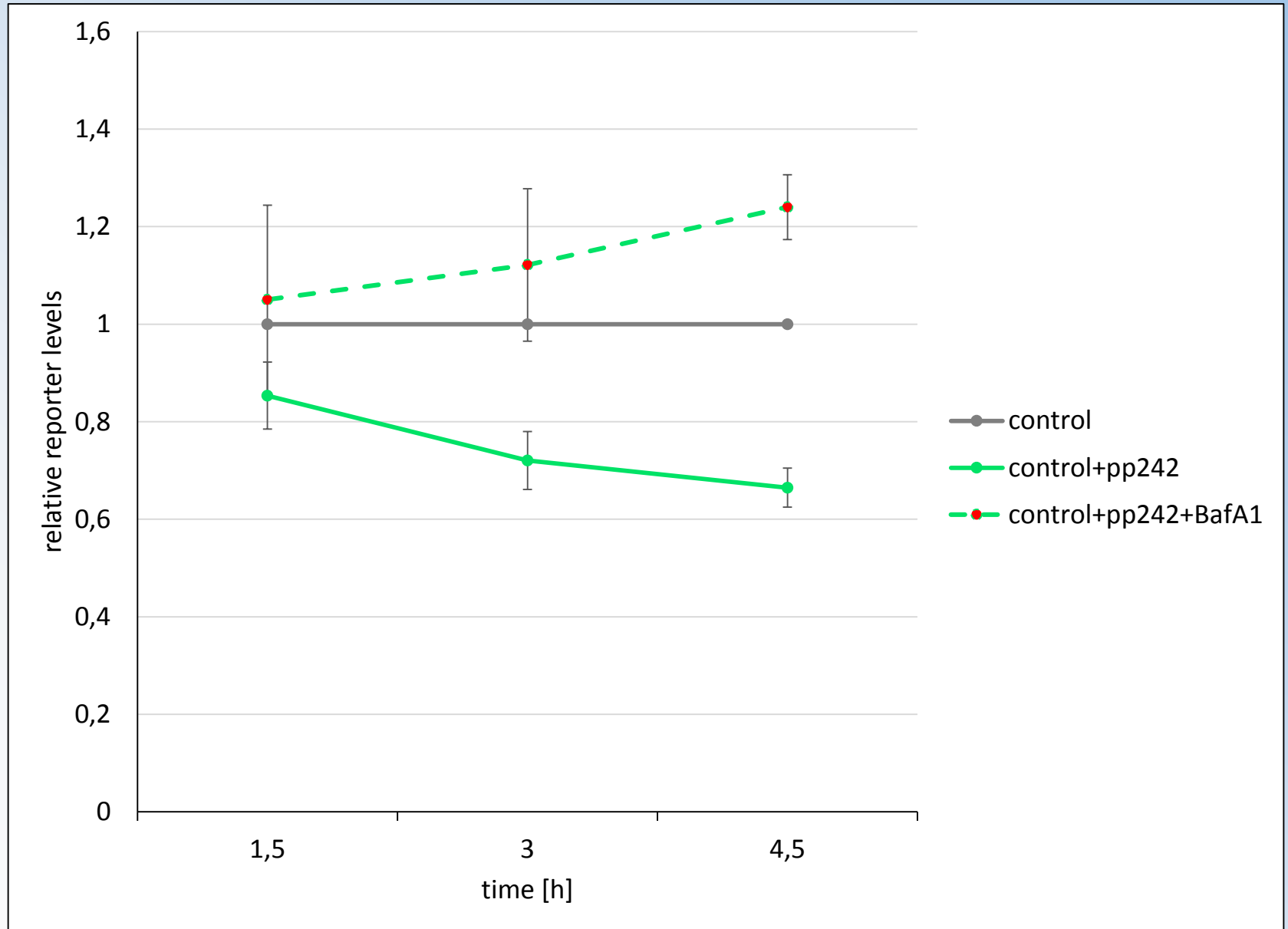
U2OS reporter cells

LC3 HiBiT Reporter Assay & Autophagy Modulators



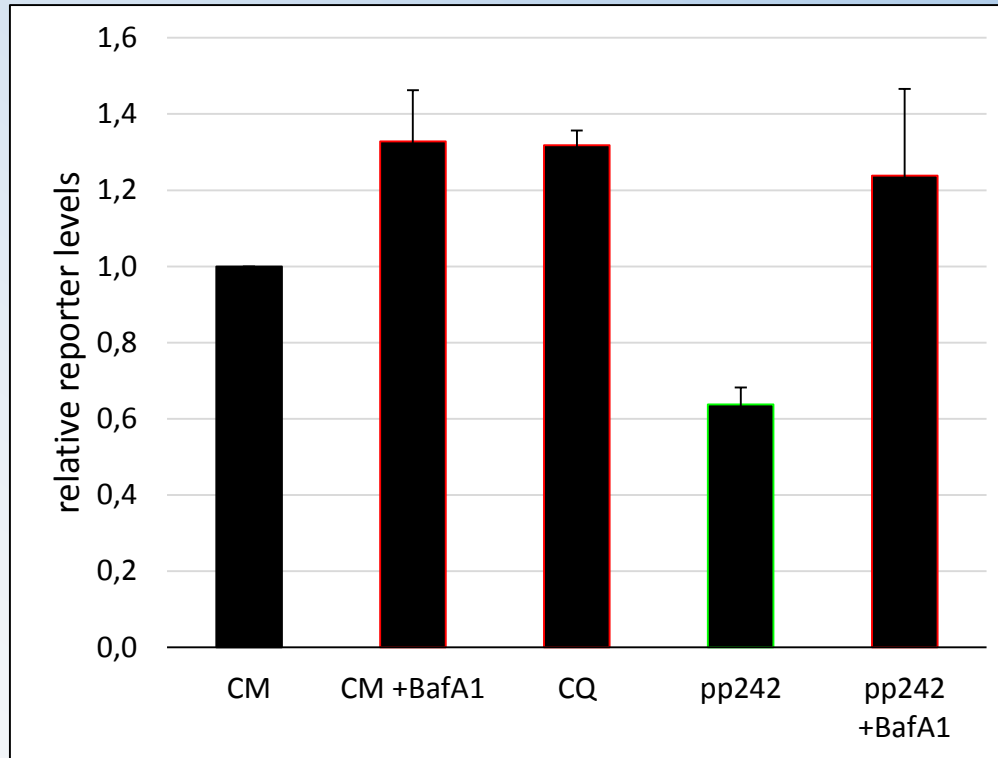
U2OS reporter cells

LC3 HiBiT Reporter Assay & Autophagy Modulators



U2OS reporter cells

LC3 HiBiT Reporter Assay & Autophagy Modulators



CM: control medium

BafA₁: 100 nM bafilomycin A₁

CQ: 100 μ M chloroquine

pp242: 2 μ M

LC3 HiBiT Reporter Assay & Autophagy Modulators

- Autophagy stimulation results in decreased reporter levels (decreased luminescence)
- Autophagy inhibition results in increased reporter levels (increased luminescence)
- Easily detects both early- and late-phase autophagy pathway inhibitors
- Autophagy reporter level decreases/increases over time and in a concentration-dependent manner in U2OS and HEK293 cells (data from Dan Lazar, Promega)

Challenge the assay!

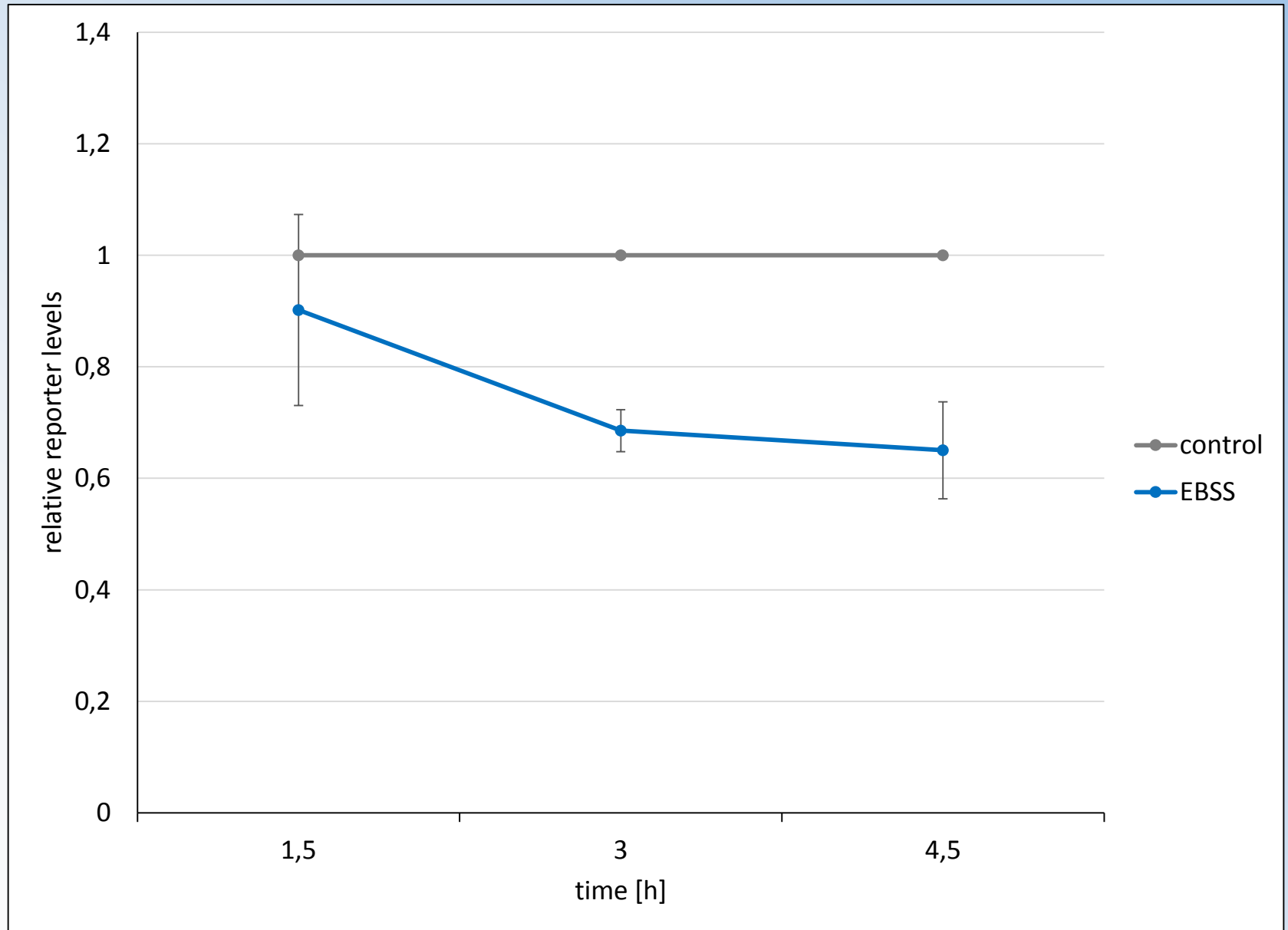
LC3 HiBiT Reporter Assay under Physiological Conditions

- Autophagy stimulation by starvation (EBSS medium)
- Autophagy inhibition by siRNA-mediated depletion of ATG proteins
- Autophagy inhibition by viruses

LC3 HiBiT Reporter Assay under Physiological Conditions

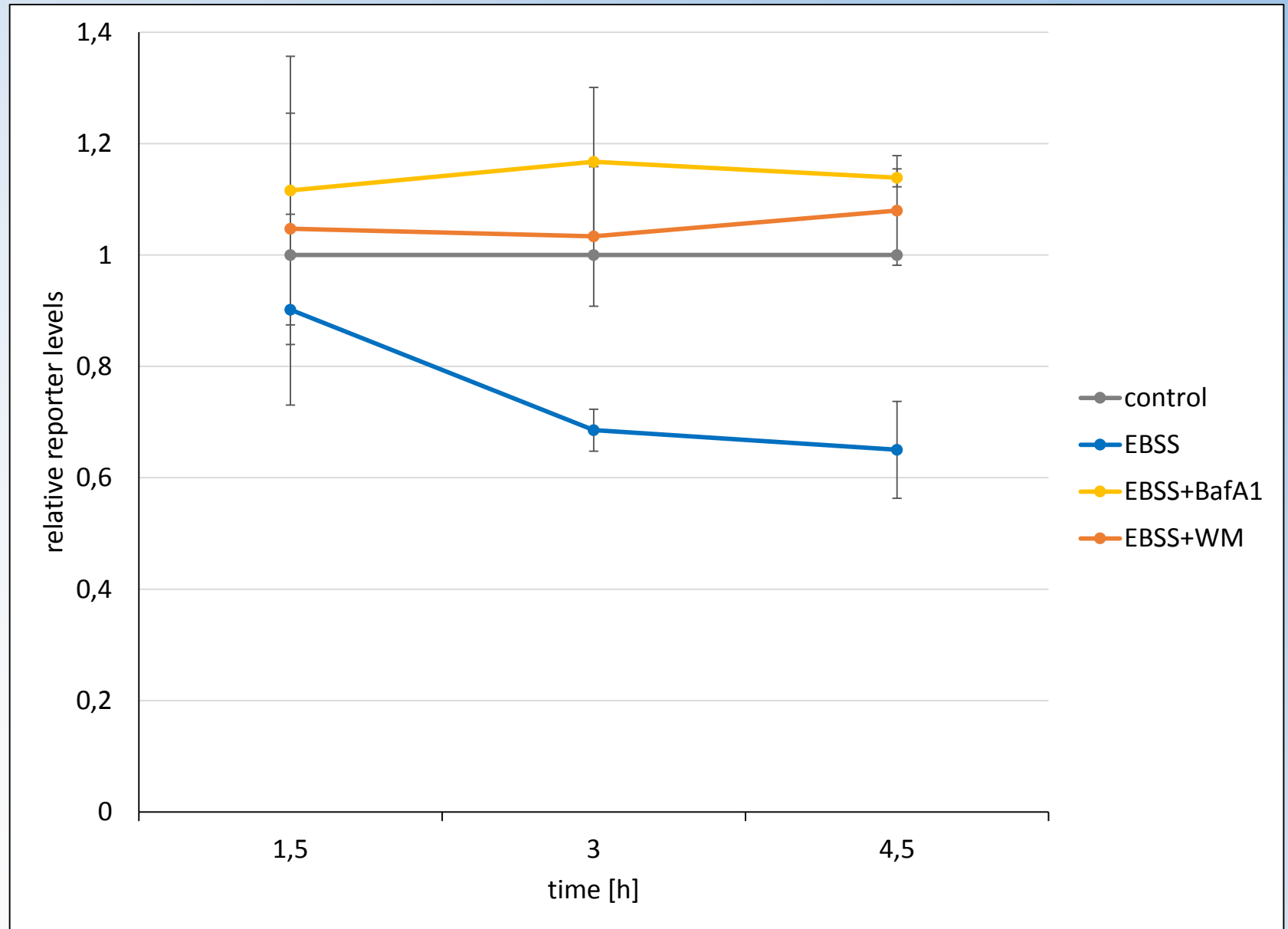
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- Autophagy inhibition by siRNA-mediated depletion of ATG proteins
- Autophagy inhibition by viruses

LC3 HiBiT Reporter Assay under physiological conditions



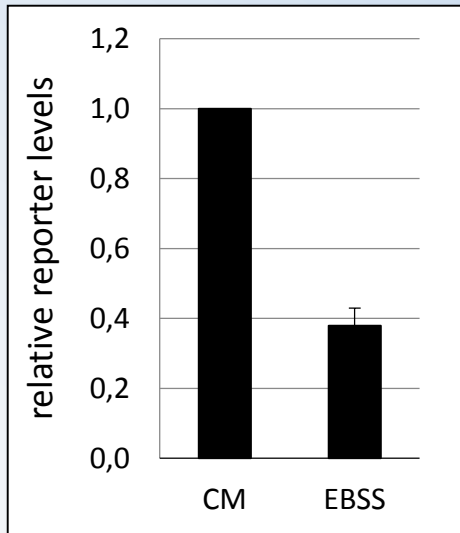
U2OS reporter cells

LC3 HiBiT Reporter Assay under Physiological Conditions

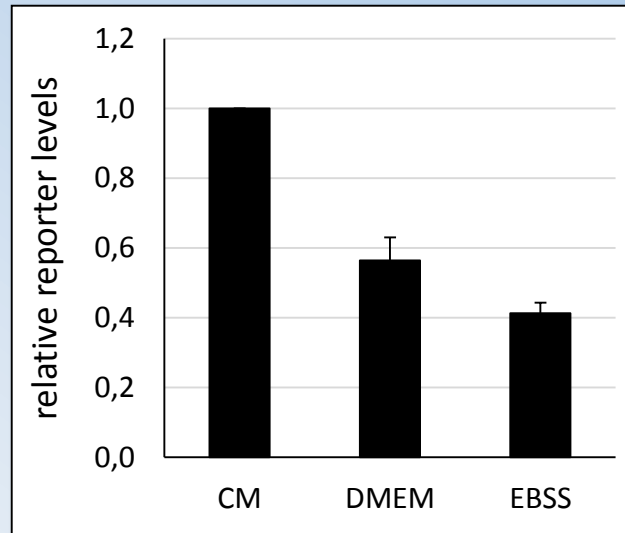


U2OS reporter cells

LC3 HiBiT Reporter Assay under Physiological Conditions



HEK293 reporter cells

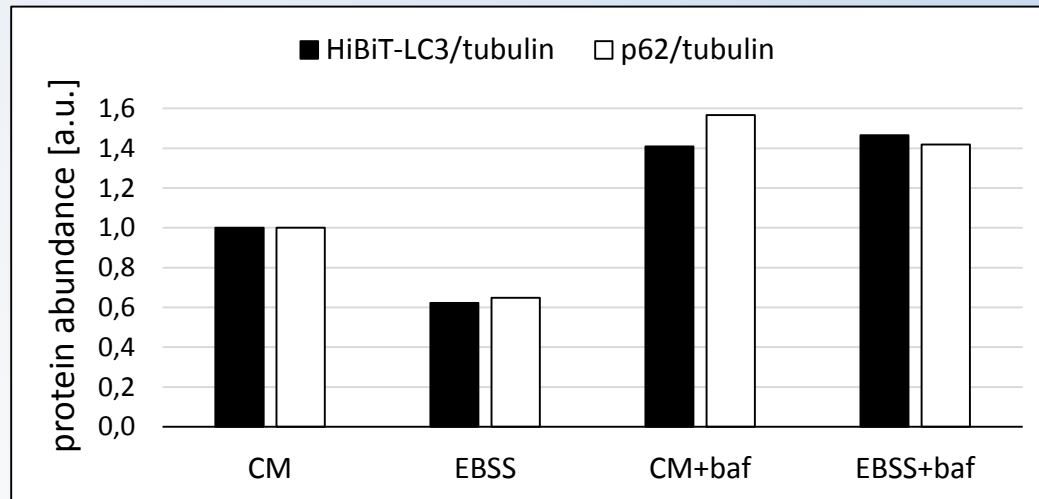
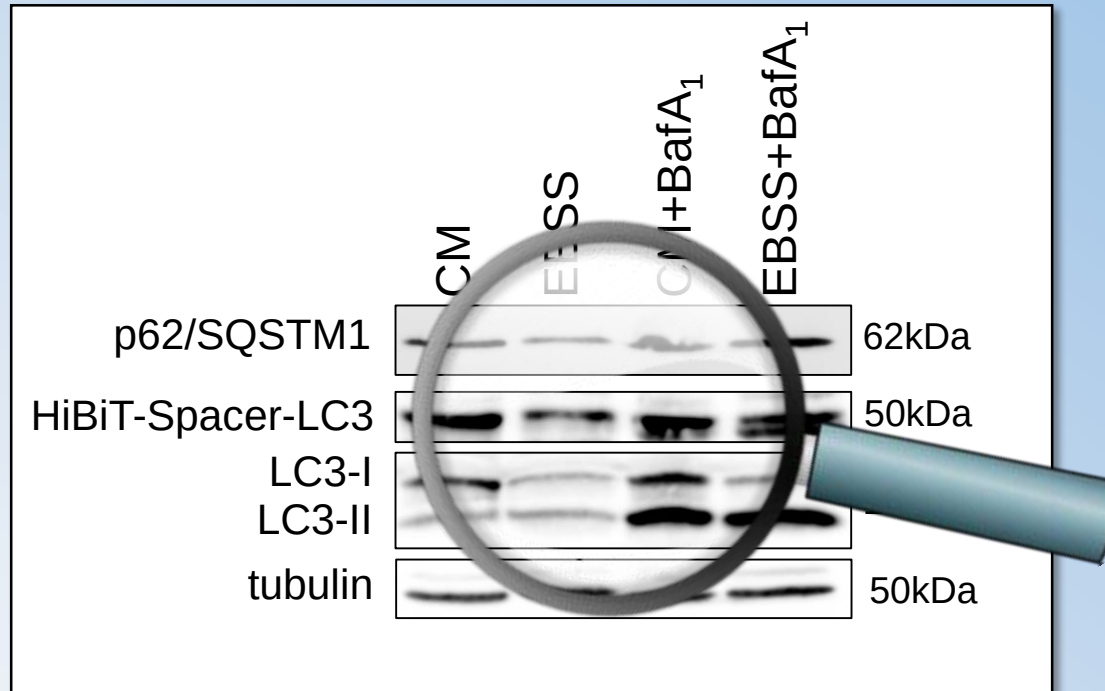


U2OS reporter cells

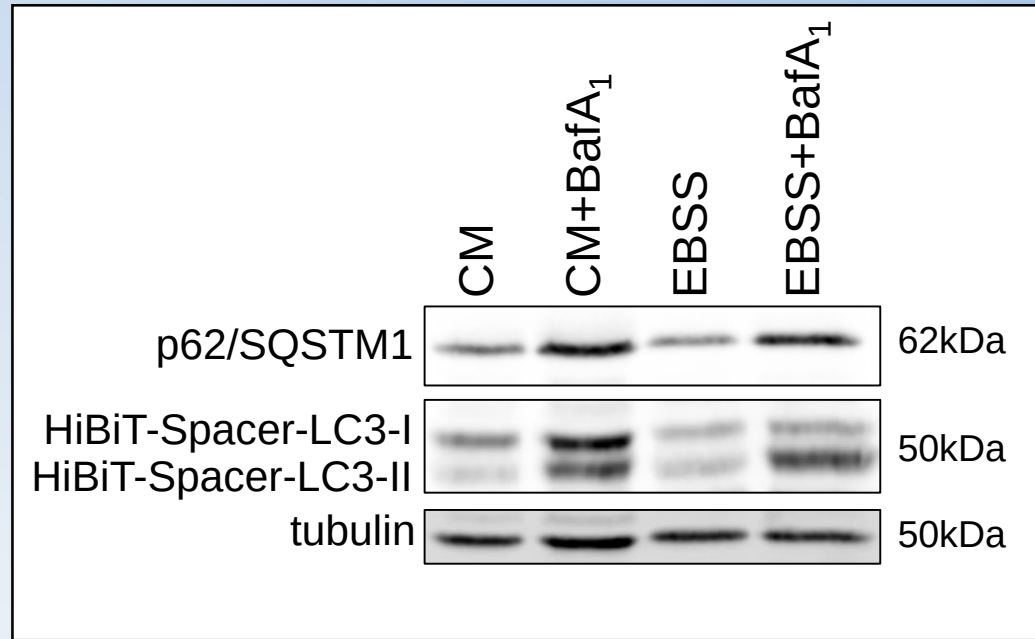
CM: control medium
DMEM: serum starvation
EBSS: amino acid + serum
starvation

➔ Autophagy induction by starvation reduces reporter levels

LC3 HiBiT Reporter Assay under Physiological Conditions

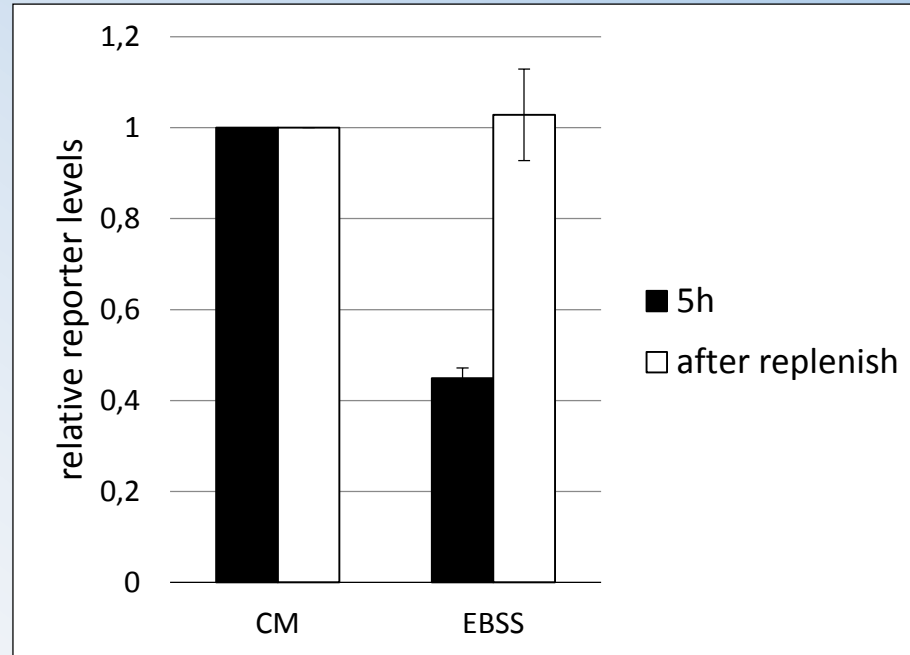


LC3 HiBiT Reporter Assay under Physiological Conditions



➔ **HiBiT-Spacer-LC3 protein participates in autophagosome formation**

LC3 HiBiT Reporter Assay under Physiological Conditions



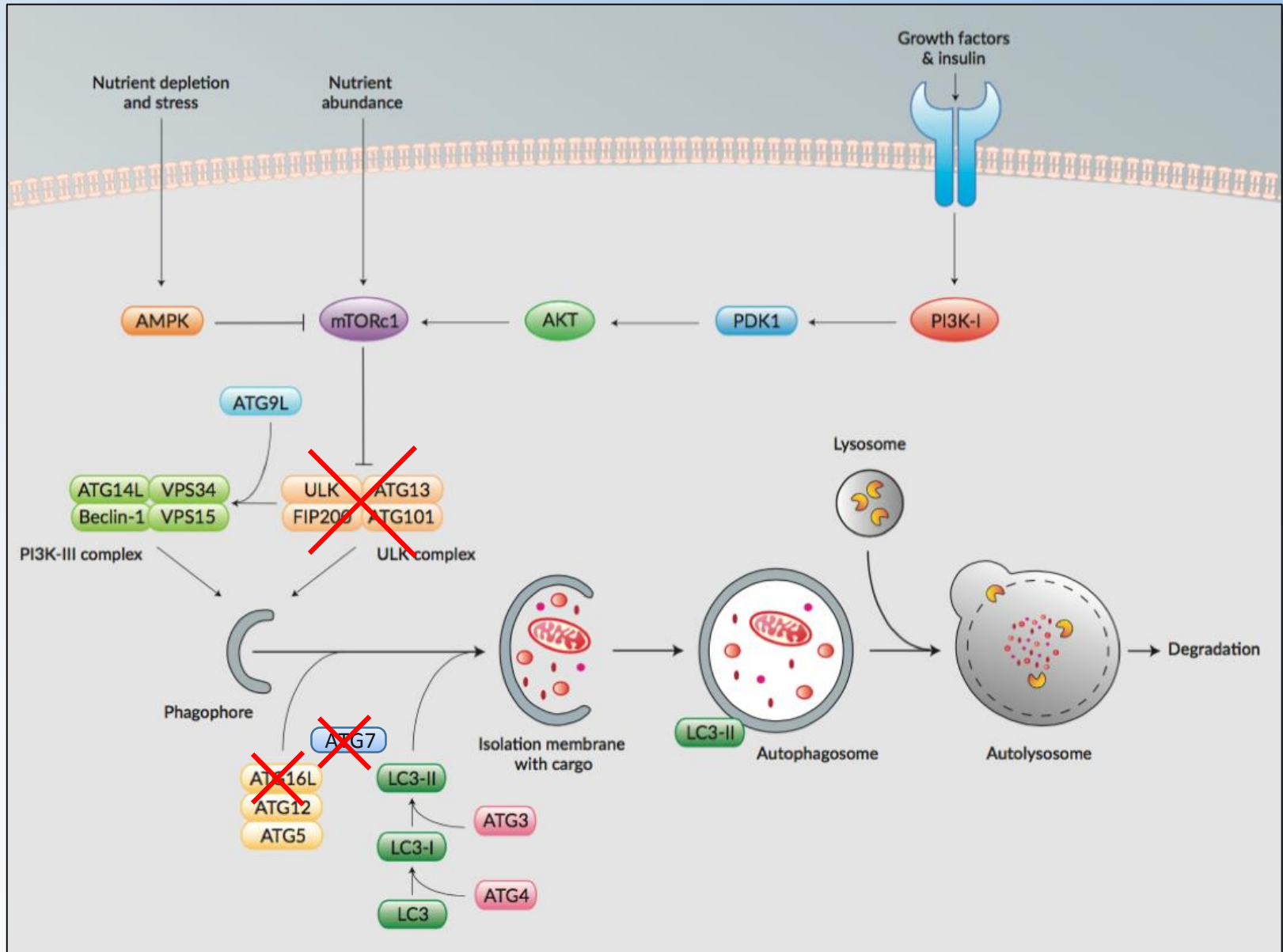
LC3 HiBiT Reporter Assay under Physiological Conditions

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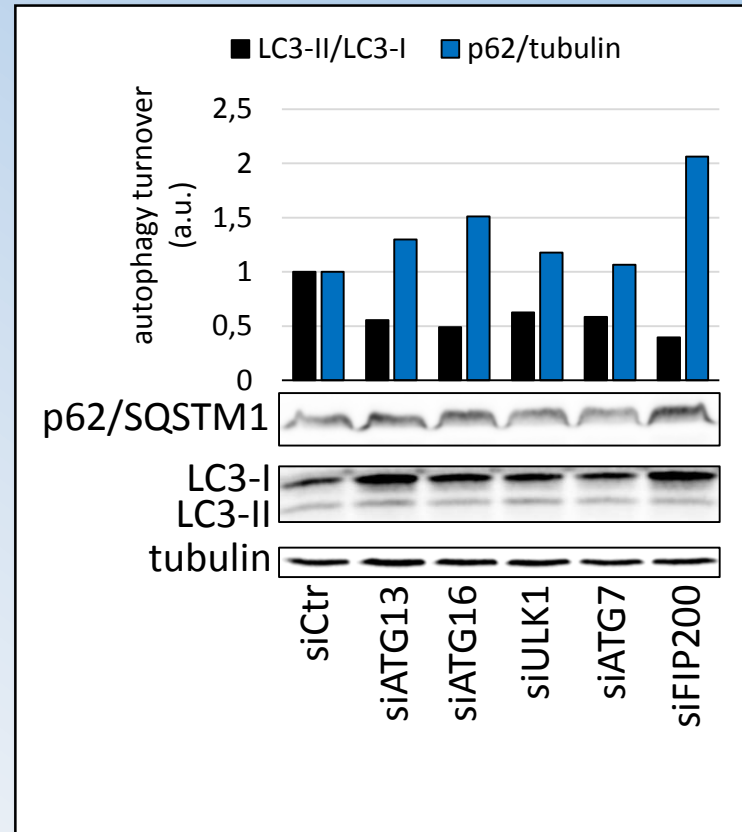
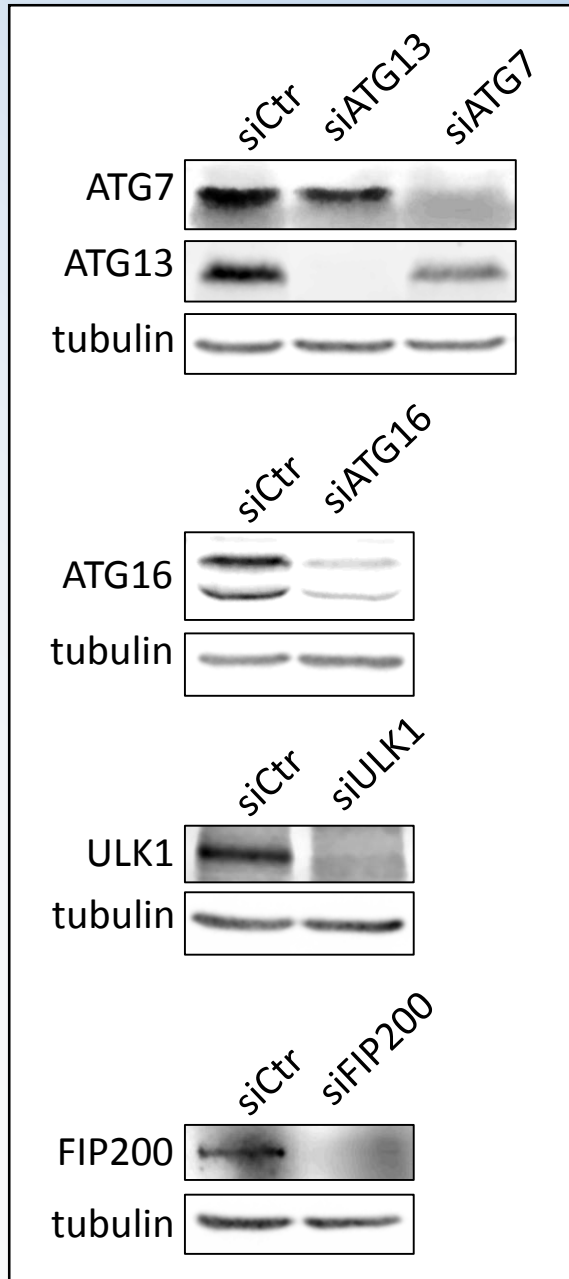
LC3 HiBiT Reporter Assay under Physiological Conditions

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The Autophagy Pathway in More Detail

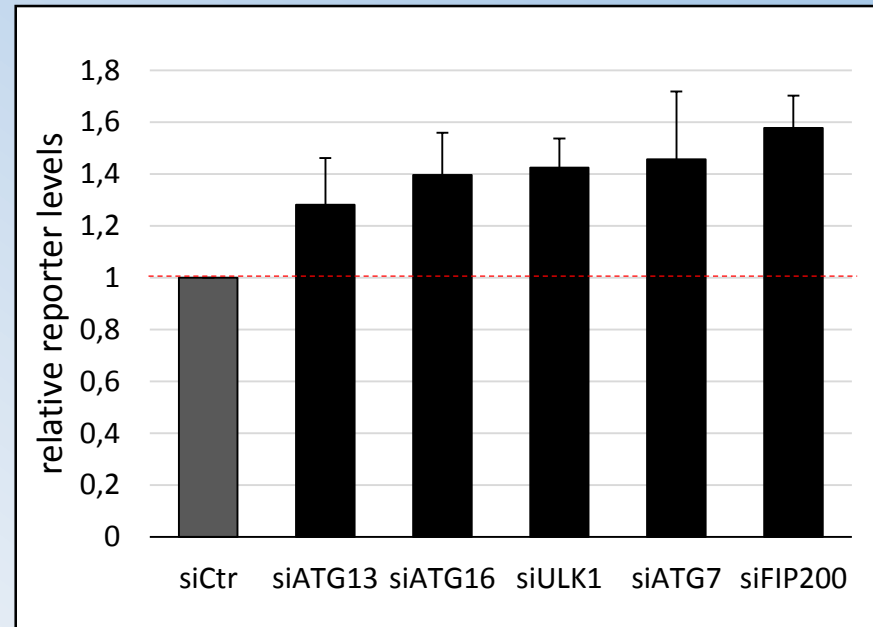
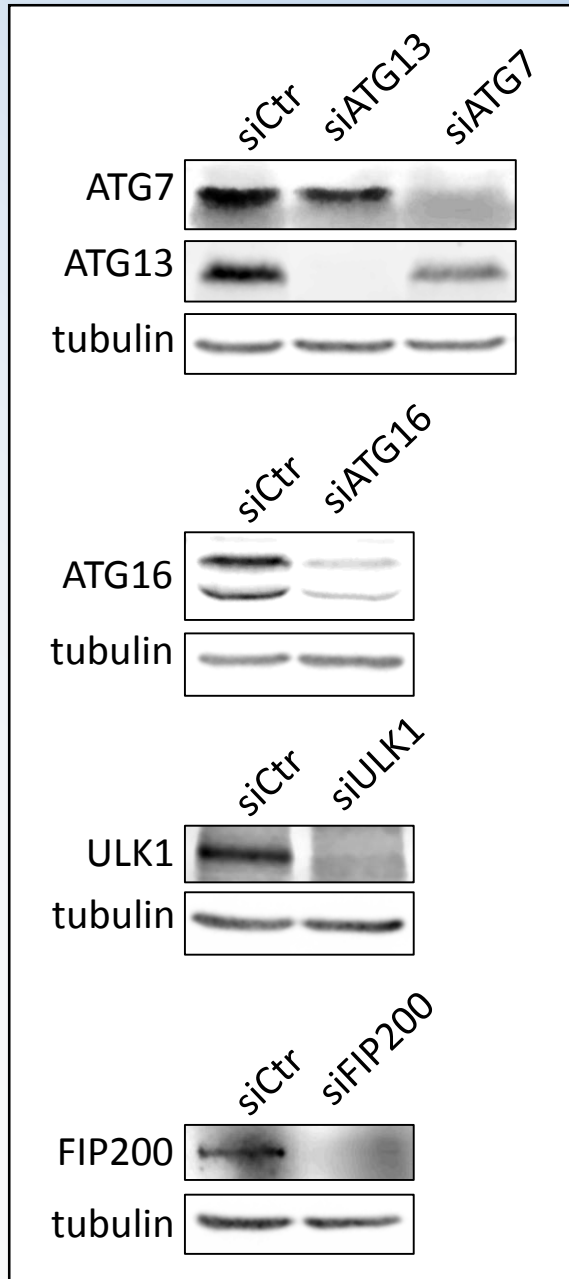


LC3 HiBiT Reporter Assay under Physiological Conditions



Endogenous protein levels

LC3 HiBiT Reporter Assay under Physiological Conditions



LC3 HiBiT analysis in EBSS

LC3 HiBiT Reporter Assay under Physiological Conditions

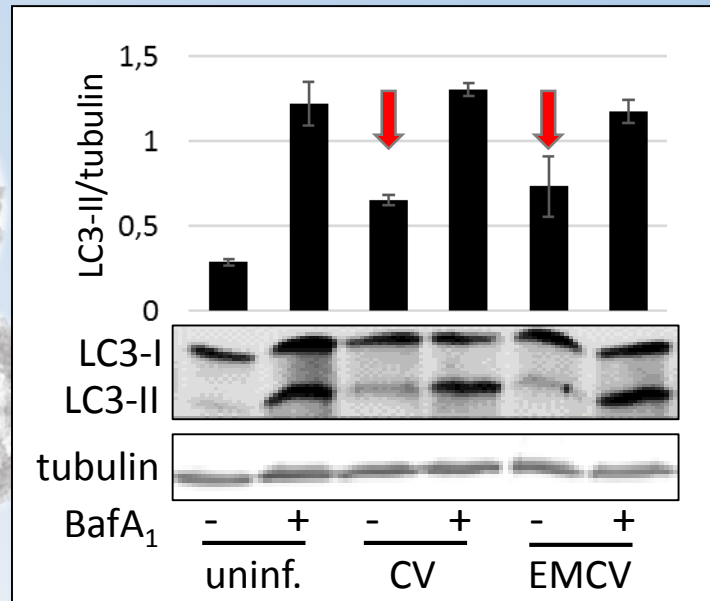
- Autophagy stimulation by starvation (EBSS medium)
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LC3 HiBiT Reporter Assay under Physiological Conditions

- Autophagy stimulation by starvation (EBSS medium)
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LC3 HiBiT Reporter Assay under Physiological Conditions

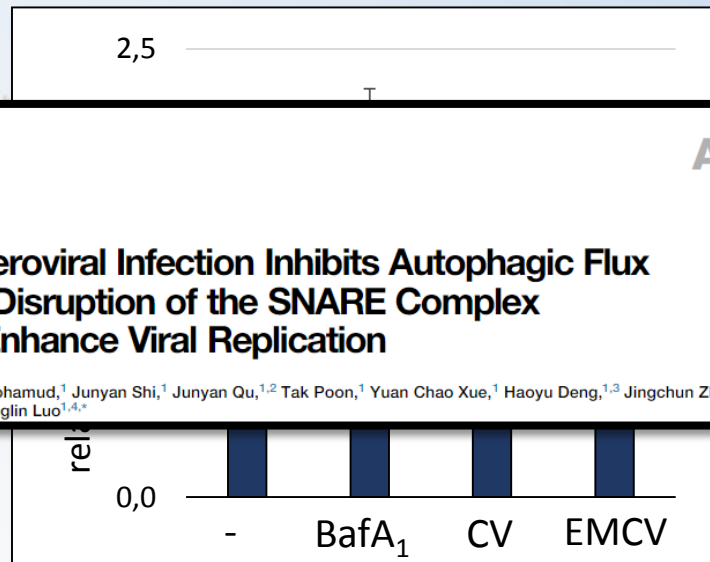
Endogenous LC3 analysis



CV: coxsackievirus B3

EMCV: encephalomyocarditis virus

LC3 HiBiT analysis



Cell Reports
Article

Enteroviral Infection Inhibits Autophagic Flux via Disruption of the SNARE Complex to Enhance Viral Replication

Yasir Mohamud,¹ Junyan Shi,¹ Junyan Qu,^{1,2} Tak Poon,¹ Yuan Chao Xue,¹ Haoyu Deng,^{1,3} Jingchun Zhang,¹ and Honglin Luo^{1,4,*}

U2OS reporter cells

LC3 HiBiT Reporter Assay: Conclusions

- Autophagy LC3 HiBiT Reporter expressed at low levels in stable cell lines
- Easy-to-handle autophagy reporter assay, “add-mix-read” protocol
- Monitors autophagosomal-lysosomal degradation
- Can be used to detect autophagic activity in HEK293 3D culture models (Dan Lazar)
- Mostly suitable for compound screening and automated high-throughput assays
- siRNA applications: normalization parameter(s) *under preparation*

Acknowledgments



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Fulvio Reggiori

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Ralph Hardenberg

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Idil Orhon

Qianliang Yuan

Alex Jones

Promega

Dan Lazar, Promega Corporation, Madison, WI

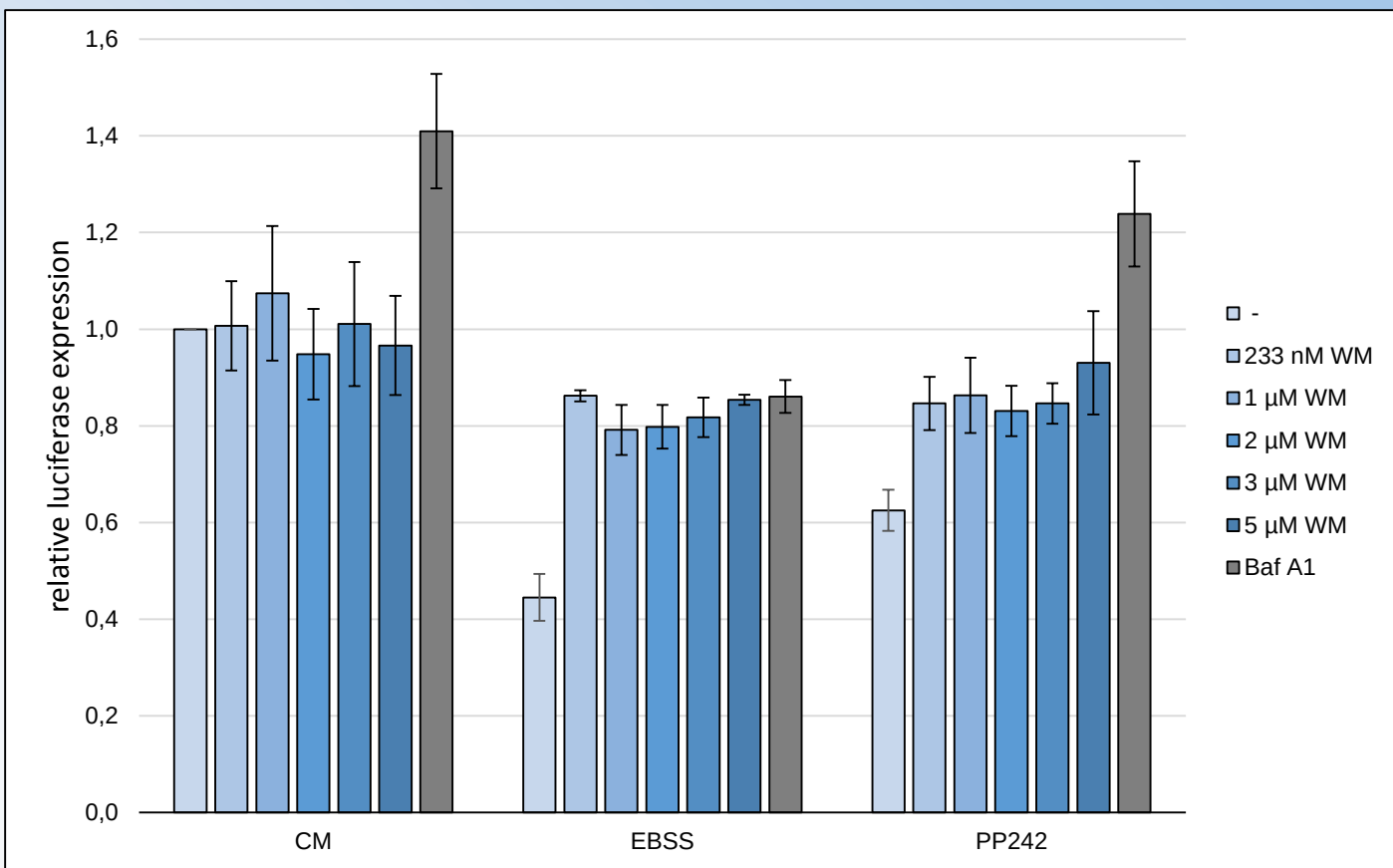


umcg

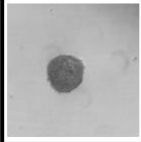
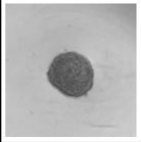
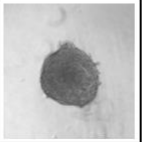
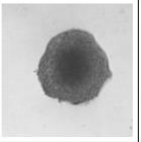
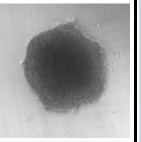


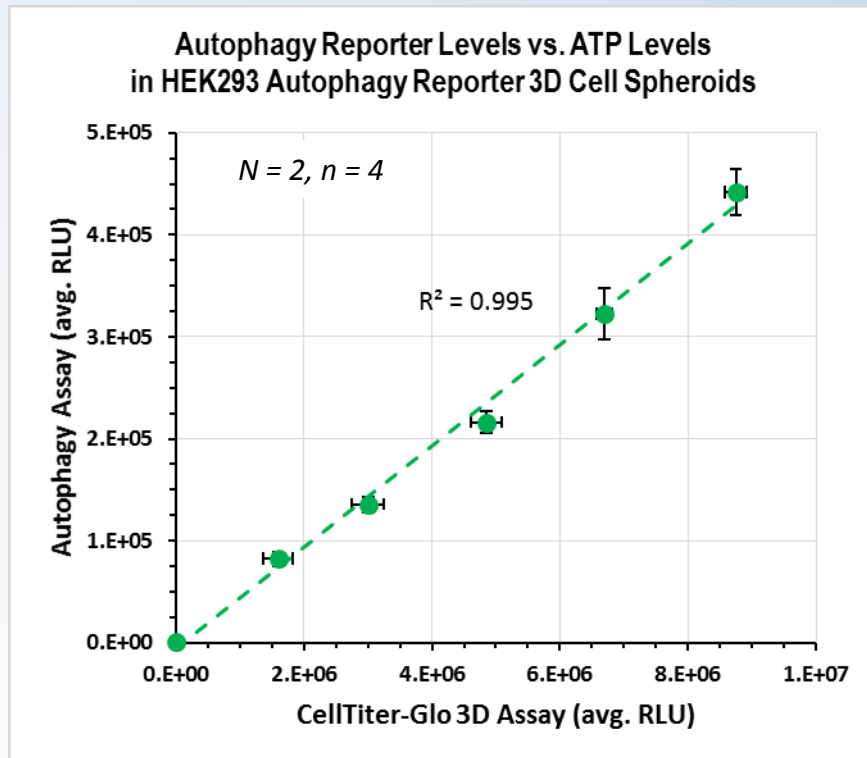
UMC Utrecht





Assay Application to HEK293 Autophagy LC3 HiBiT Reporter 3D Cell Spheroids

Diameter (µm) (day 4)	278 +/- 8	353 +/- 6	453 +/- 15	543 +/- 6	647 +/- 20
Brightfield image					



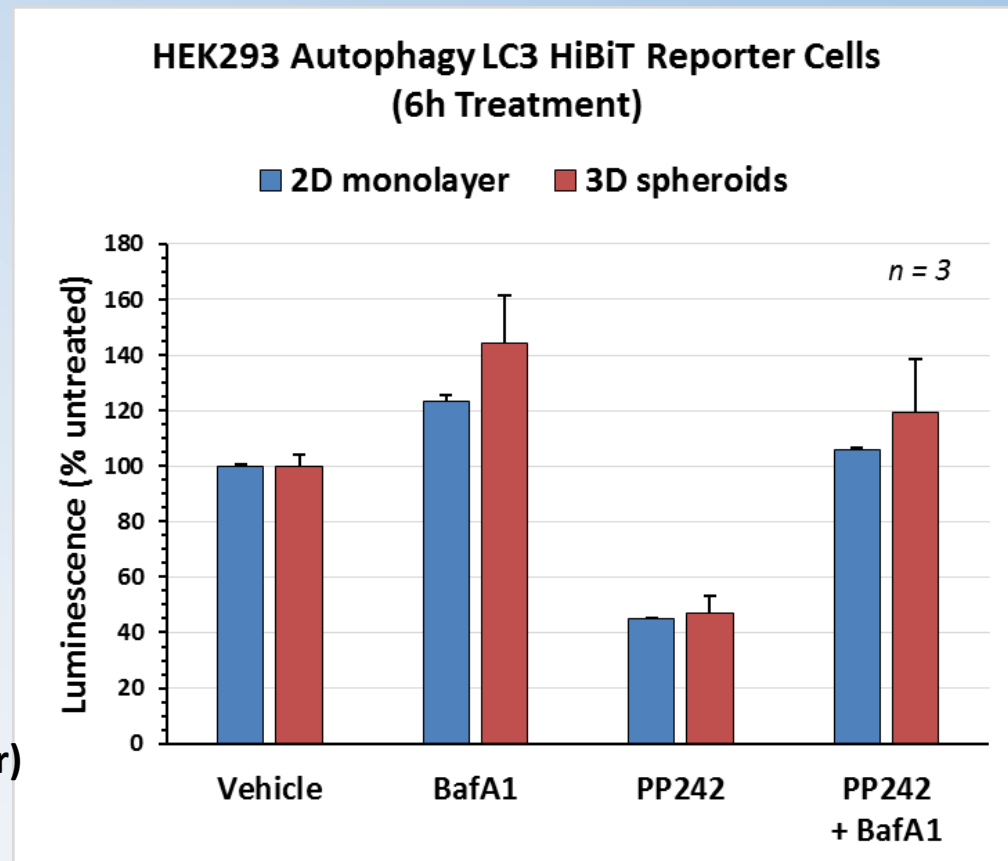
- Generated HEK293 Autophagy LC3 HiBiT Reporter day-4, cell spheroids in Corning #7007 ULA 96-well plates (round bottom)
- Compared autophagy reporter recovery and detection to ATP levels measured with CellTiter-Glo 3D Cell Viability Assay in parallel samples (multiplex not possible)
- Increased shake time** to 30 min after addition of Nano-Glo HiBiT Lytic Reagent (*shorter shake times may be sufficient for complete reporter recovery and detection*)
- Linear relationship between autophagy assay and cell viability assay signals indicates modified autophagy assay protocol effectively recovers Autophagy LC3 HiBiT Reporter from 3D cell spheroids

Autophagy Assay Performance Enables Comparison of Compound Effects in Both 2D and 3D Models

- HEK293 Autophagy LC3 HiBiT Reporter Cells
- Day-4, 3D spheroids (~540µm diameter) vs. normal 2D monolayer cultures

Assay Protocol

1. Prepare day-4 cell spheroids and normal 2D monolayer cultures in 96-well plates
2. Treat with reference compounds for 6 h in 37°C, CO₂ incubator
 - Vehicle (0.2% DMSO)
 - 50nM Baf A1
 - 2µM PP242
 - 2µM PP242 + 50nM Baf A1
3. Equilibrate plates to room temperature
4. Add equal volume of Nano-Glo HiBiT Lytic Reagent (100uL)
5. **Plate shake (extended shake for 3D)**
6. **Transfer 3D reaction volume to 96-well, white assay plate (because ULA plate's clear)**
7. Read luminescence on a GloMax Discover



Functional complexes of the ATG proteins during autophagy

Autophagy initiation and Phagophore biogenesis

ULK
complex

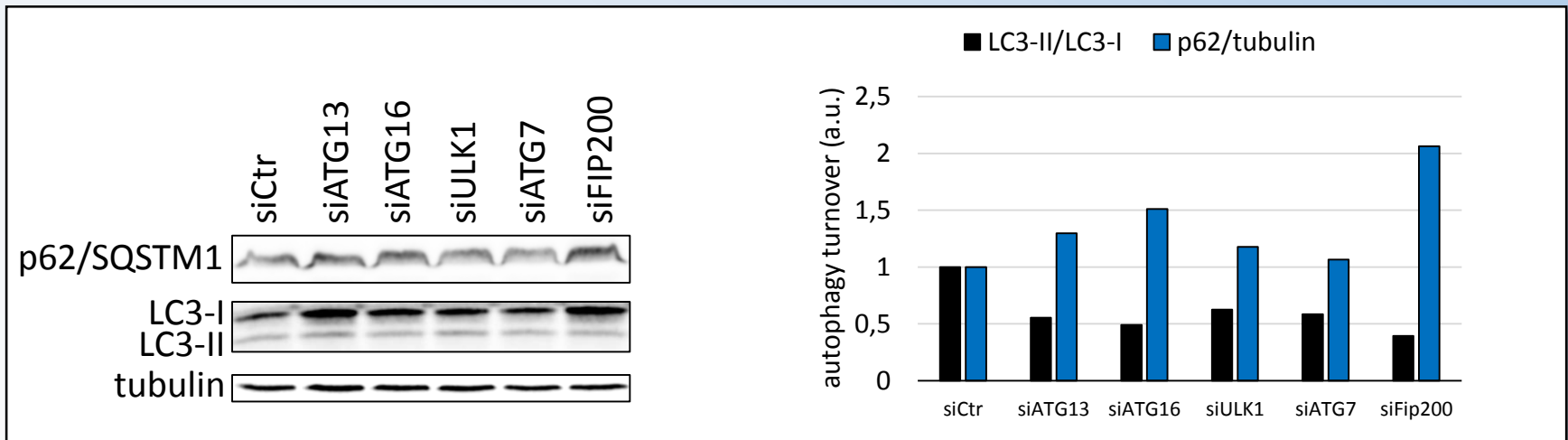
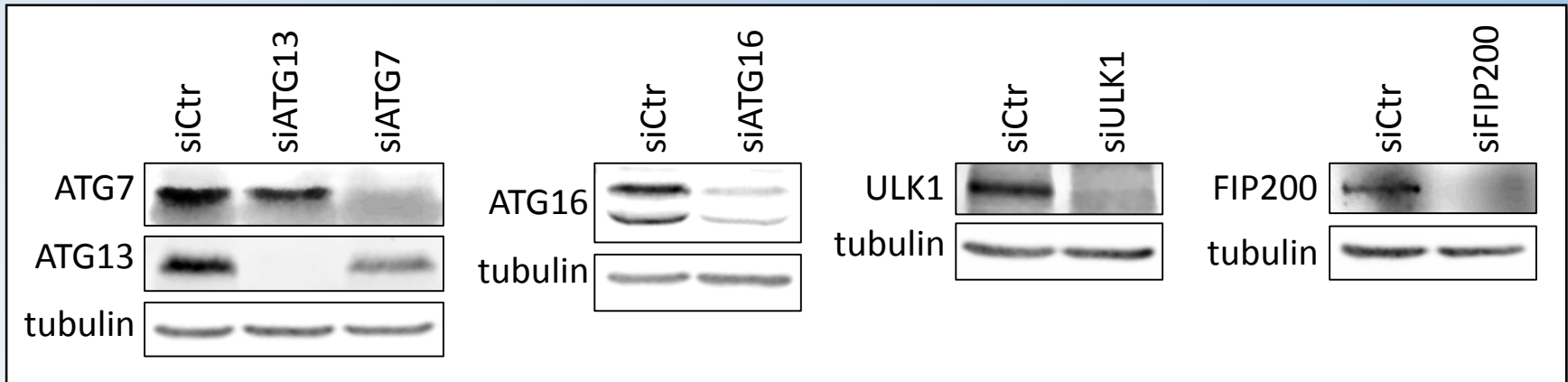
PI-3 kinase
complex

ATG9 cycling
complex

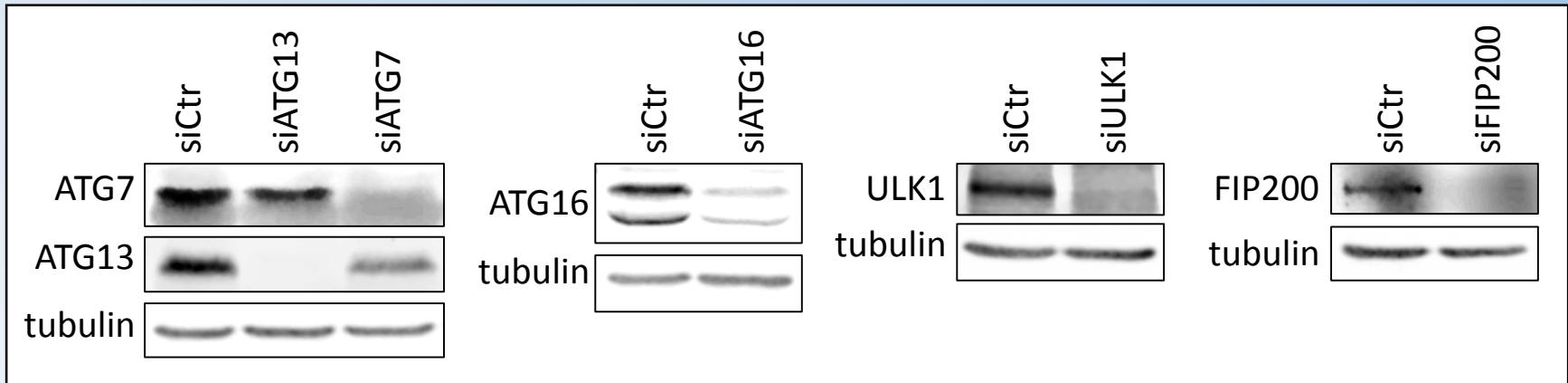
Phagophore elongation

Ubiquitin-like
conjugation systems

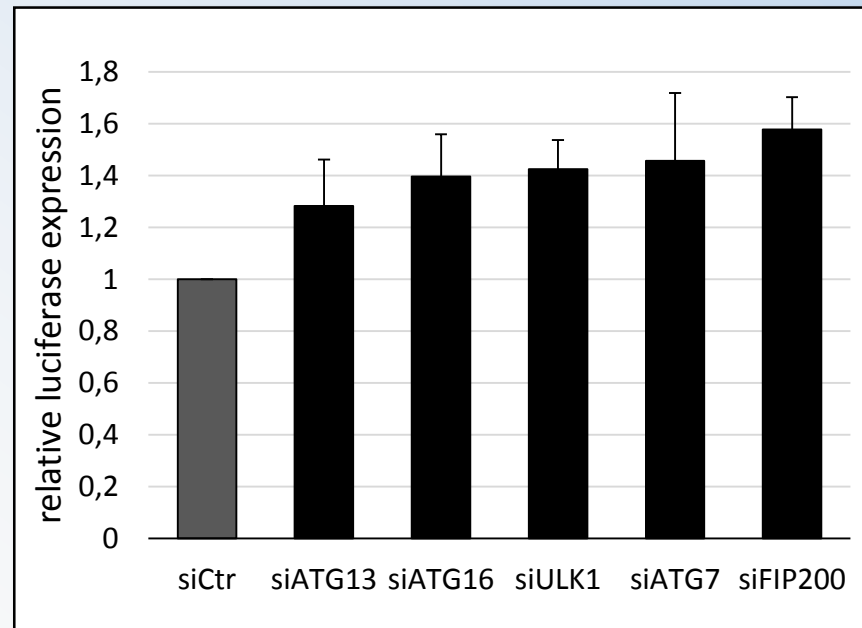
LC3 HiBiT Reporter Assay under physiological conditions



LC3 HiBiT Reporter Assay under physiological conditions



LC3 HiBiT analysis
in EBSS



U2OS reporter cells