Sweet times to study sour organelles

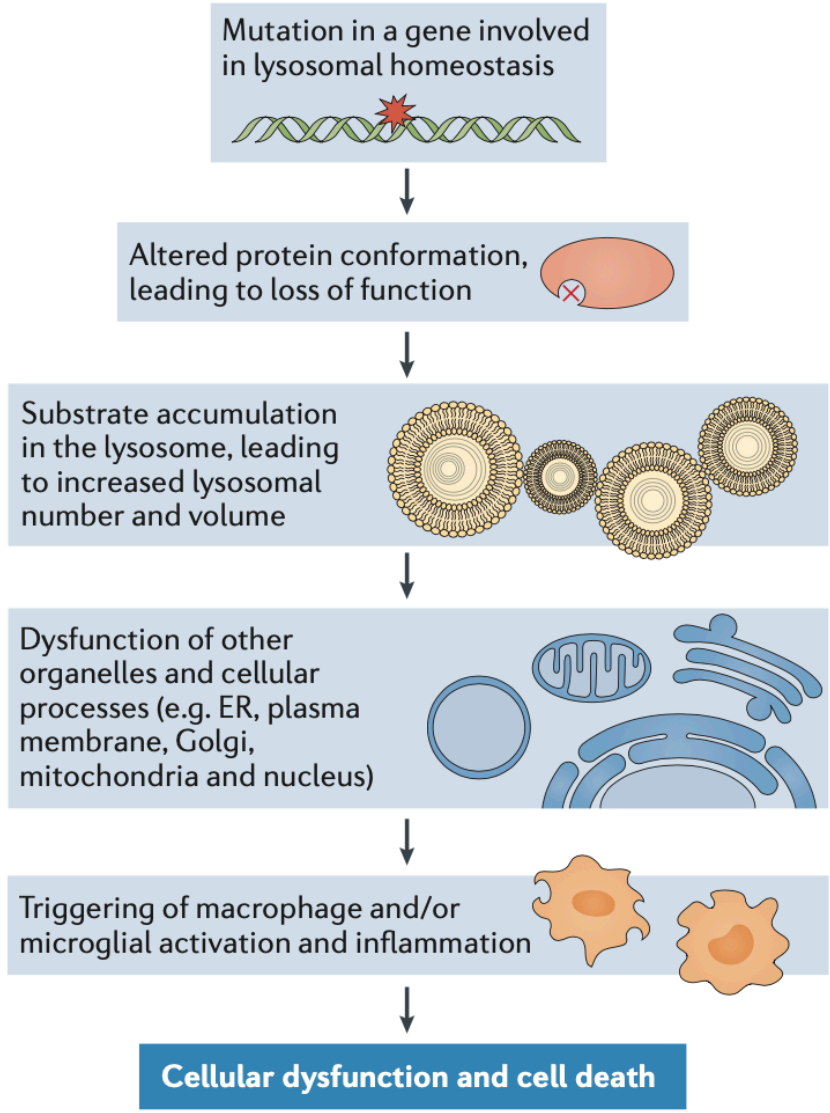
New tools to explore lysosomal dysfunction in neurodegeneration

Lysosomes in health

- Christian de Duve 1955: "Exploring Cells with a Centrifuge”
- membrane-enclosed organelles
- Acidic lumen (pH ~ 4.5), contain > 50 hydrolytic enzymes
- Cellular “waste bins”
  - Sites of macromolecular degradation, nutrient recycling
- Important signaling hubs, nutrient sensing, phagocytosis and antigen presentation
- Protein quality control (**autophagy**-lysosome pathway)
  - Together with ubiquitin-proteasome system

Lysosomes in disease

- > 50 lysosomal storage disorders (GD, FD)
- Substrate accumulation
  - (peripheral and central) **nervous system**, eye, bone, muscle, reticulo-endothelial system
- Lysosomal dysfunction also associated with
  - Aging
  - Rheumatological disorders (SLE, RA)
  - **Neurodegenerative disorders** (AD, PD, FTD/ALS, HD)



Boustany, R.-M. N. *Nat. Rev. Neurol.* 2013

Lysosomal dysfunction in neurodegenerative disorders

- Function/survival of neurons critically dependent on an efficient cellular waste clearance system
  - Post-mitotic state
  - High metabolic demands
  - Often large polarised morphology
- α-syn and huntingtin are autophagy substrates
- Lysosomes also involved in inflammation

Existing probes to study lysosomal function

- **pH**: Small molecule pH-sensitive dyes, organic fluorophores, synthetic probes
  - LysoSensor, LysoTracker (fluorophore linked to a weak base only partially protonated at neutral pH, can freely permeate cell membranes and label live cells)
- **Hydrolytic activity**: Artificial substrates of lysosomal enzymes
  - 4-MU-b-Glc,…
- **Limitations**:
  - Poor specificity for subcellular targeting
  - Cytotoxicity
  - Autofluorescence/imaging artifacts
  - Modification of cellular metabolic activity
  - Leakage from cells
  - Measure total enzymatic content (often in cell lysates)

New tools to study lysosomal (dys)function

- Genetically encoded biosensors for probing lysosomal pH
- Activity-based probes suitable for live-cell imaging

ACS SENSORS

pubs.acs.org/acssensors

Article

Genetically Encoded, pH-Sensitive mTFP1 Biosensor for Probing Lysosomal pH

Marcus Y. Chin,[⊥] Anand R. Patwardhan,[⊥] Kean-Hooi Ang, Austin L. Wang, Carolina Alquezar, Mackenzie Welch, Phi T. Nguyen, Michael Grabe, Anna V. Molofsky, Michelle R. Arkin,* and Aimee W. Kao*

Cite This: *ACS Sens.* 2021, 6, 2168–2180

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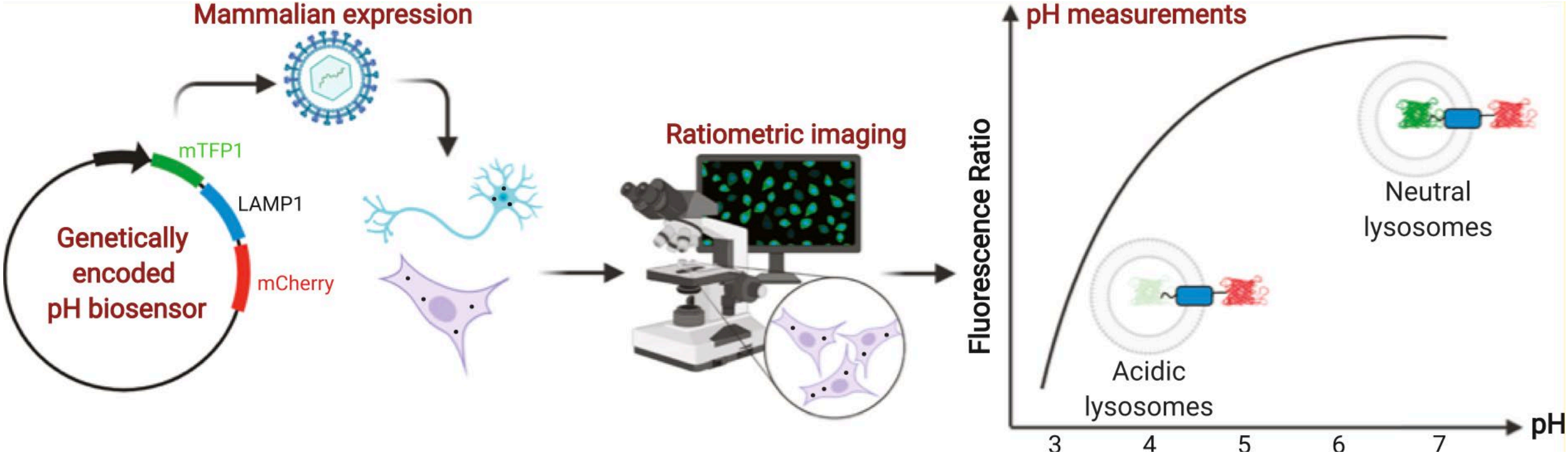
Genetically encoded biosensors for probing lysosomal pH



Overall Aim

Design a lysosomal pH biosensor with broad applicability for investigating pH dynamics in lysosome-related diseases and drug discovery

Lysosomal acidity

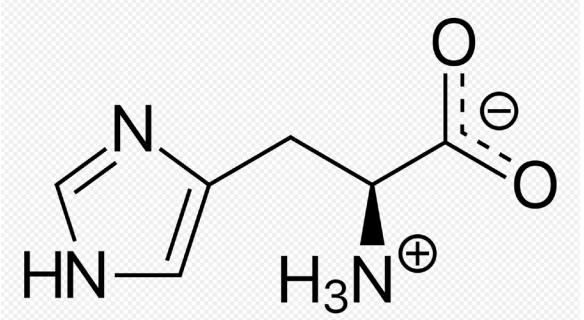
- Maintained by vacuolar-type H^+-ATPase (**V-ATPase**) proton pump
  - (F-type in mitochondria and P-type in prokaryotes, plants)
- Electrogenic proton pump, evolutionarily conserved
- Generates proton gradient across membranes, depends on ATP hydrolysis
- Loss of function: embryonically lethal
  - Hyperacidity associated with aggressiveness in cancer
  - Loss of lysosomal acidity observed in aging

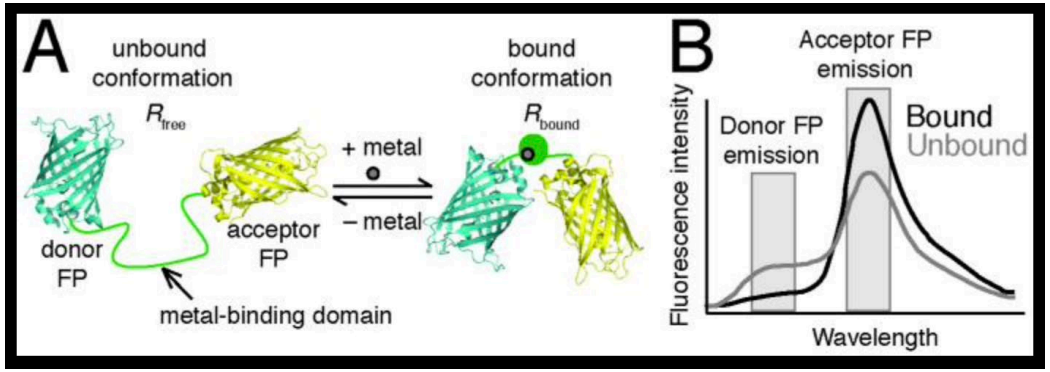
Advantages of genetically encoded biosensors based on fluorescent proteins

- Controlled expression in different cell types
- Enhanced intracellular specificity
- Bypassing of dye-incubation steps
- Enable long-term, live imaging studies in cells

Genetically encoded (pH) biosensors

- pHluorins
  - Directed mutations of specific residues of GFP to pH-sensitive histidine residues
  - Correlation between pH and fluorescent readout
- Genetically encoded biosensors exist to quantify Ca^{2+}, Zn^{2+}, Cl^{-}, Mg^{2+}, K^{+}





Park JG. Methods Mol Biol. 2014

Ratiometric biosensors targeted to lysosomes

- **mCherry-pHluorin-mouseLAMP1**
  - Both FPs within lysosomal lumen
- **sfGFP-ratLAMP1-mCherry**
  - sfGFP inside lysosome, mCherry faces cytosol
- Both use **LAMP1** (lysosomal-associated membrane protein 1) to target the lysosome
- **pK_a of 6.5/5.9** (lysosomal pH: 4.5)
  a biosensor with a lower pK_a would be desirable

$pK_a = -\log[K_a]$

Acid dissociation constant

$$K_a = \frac{[A^-][H^+]}{[HA]}$$

K_a = acid dissociation constant

$[A^-]$ = concentration of the conjugate base of the acid

$[H+]$ = concentration of hydrogen ions

$[HA]$ = concentration of chemical species HA

Design principles for a ratiometric lysosomal pH sensor

- **Ratiometric system**: relative brightness of two reporters to quantify pH measurement (dual reporter system)
- One fluorophore changes its signal in response to the **proton concentration**
- Second fluorophore: **stable reference point** for identifying lysosomes and normalizing fluorescent signal (expression control)
- Advantage: no bias between samples/experiments

Requirements of a ratiometric pH biosensor

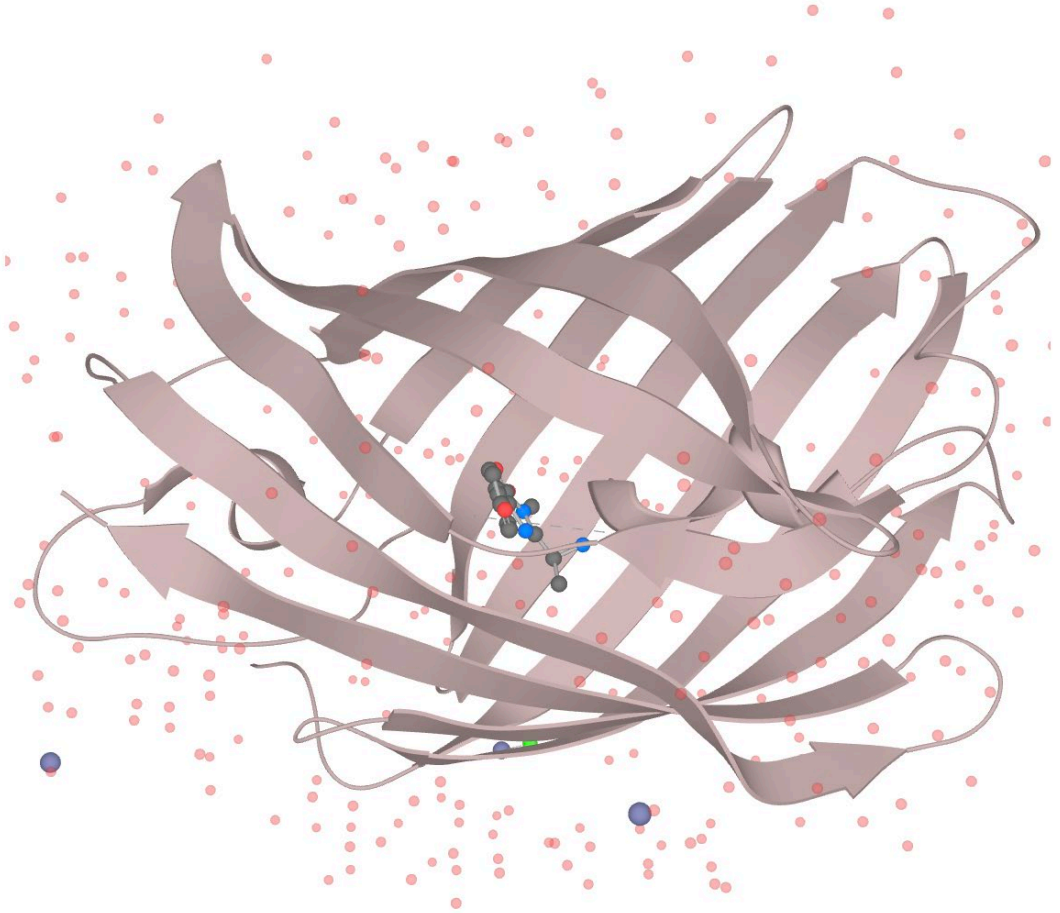
1. Domain for lysosomal targeting
2. Cytosolically facing fluorescent protein
   stable brightness at physiological intracellular pH (6.8 – 7.2)
3. Lysosomal lumen-facing fluorescent protein providing dynamic lysosomal pH sensing at highly acidic pH

Requirements of a ratiometric pH biosensor

1. **Domain for lysosomal targeting**
   LAMP1: membrane protein harbouring a tyrosine-based lysosomal sorting motif in its cytoplasmic tail (“GYQT1”)
2. **Cytosolically facing fluorescent protein**: stable brightness at physiological intracellular pH (6.8 – 7.2)
   mCherry: brightness, fluorescent stability at physiol. intracell pH
3. **Lysosomal lumen-facing fluorescent protein providing dynamic lysosomal pH sensing at highly acidic pH**
   mTFP1 (variant of cyan fluorescent protein): low pK_a, high brightness, photostability, preserved folding/integrity within acidic lysosomal environment

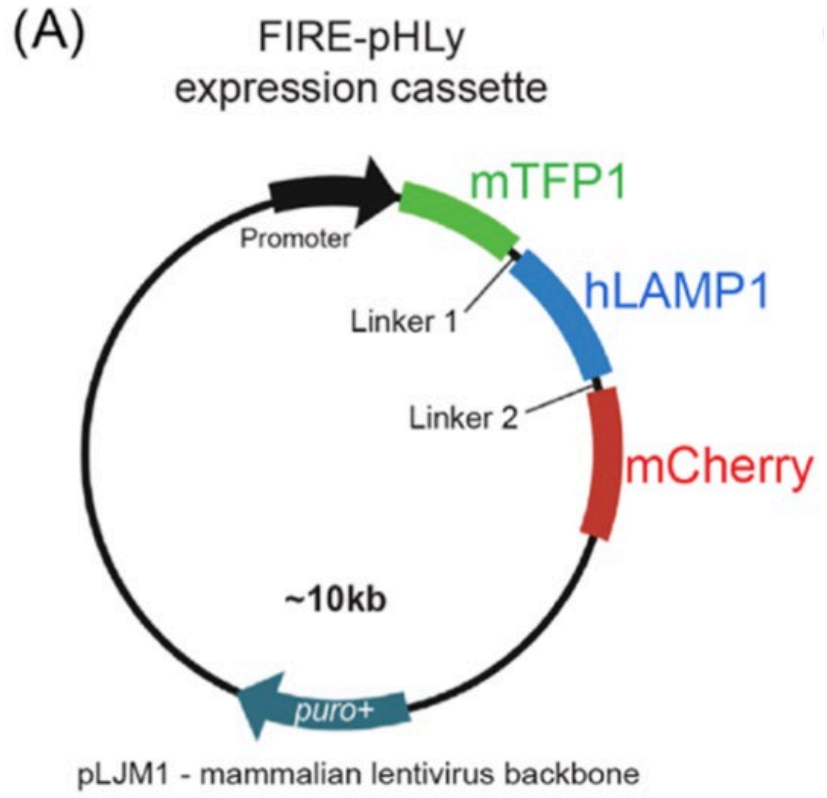
Monomeric teal fluorescent protein 1 (mTFP1)

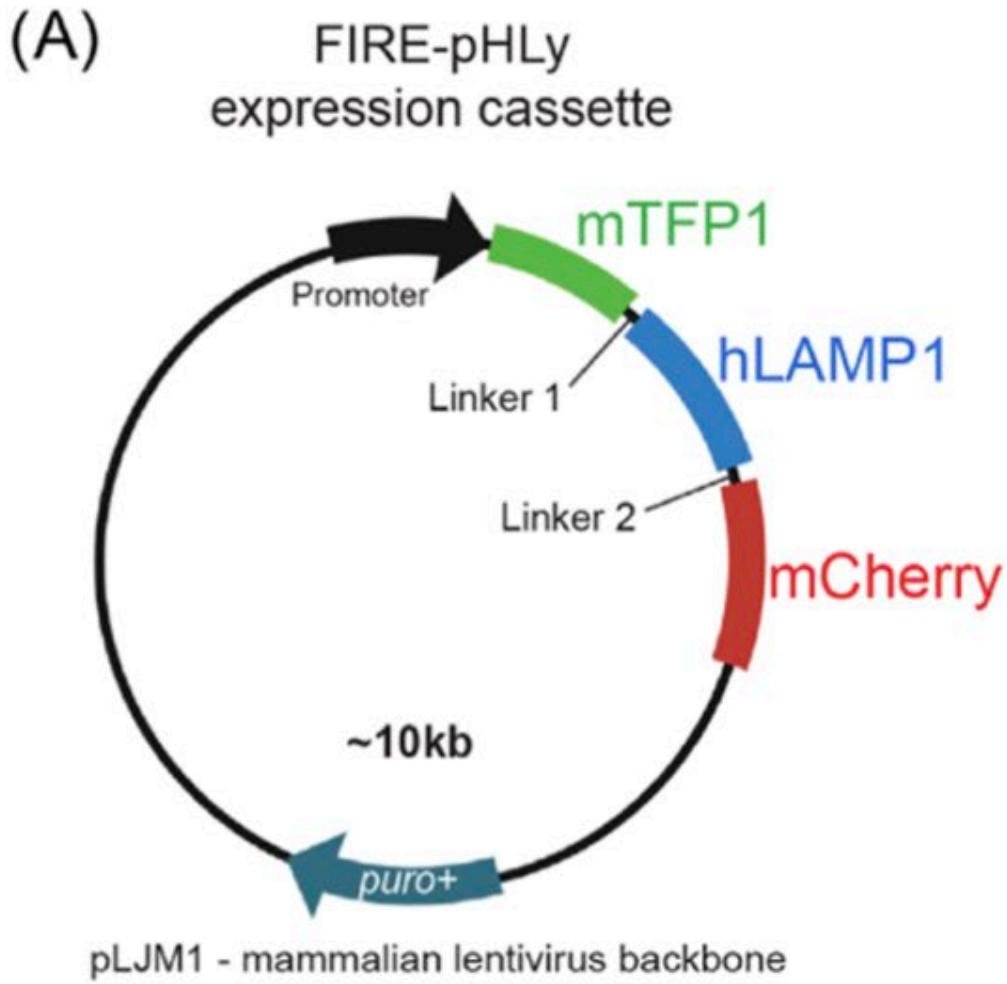
- Derived from corals (Clavularia)
- pK_a of 4.3
- Sigmoidal pH response from 3.5 – 6
- no photobleaching or aggregation

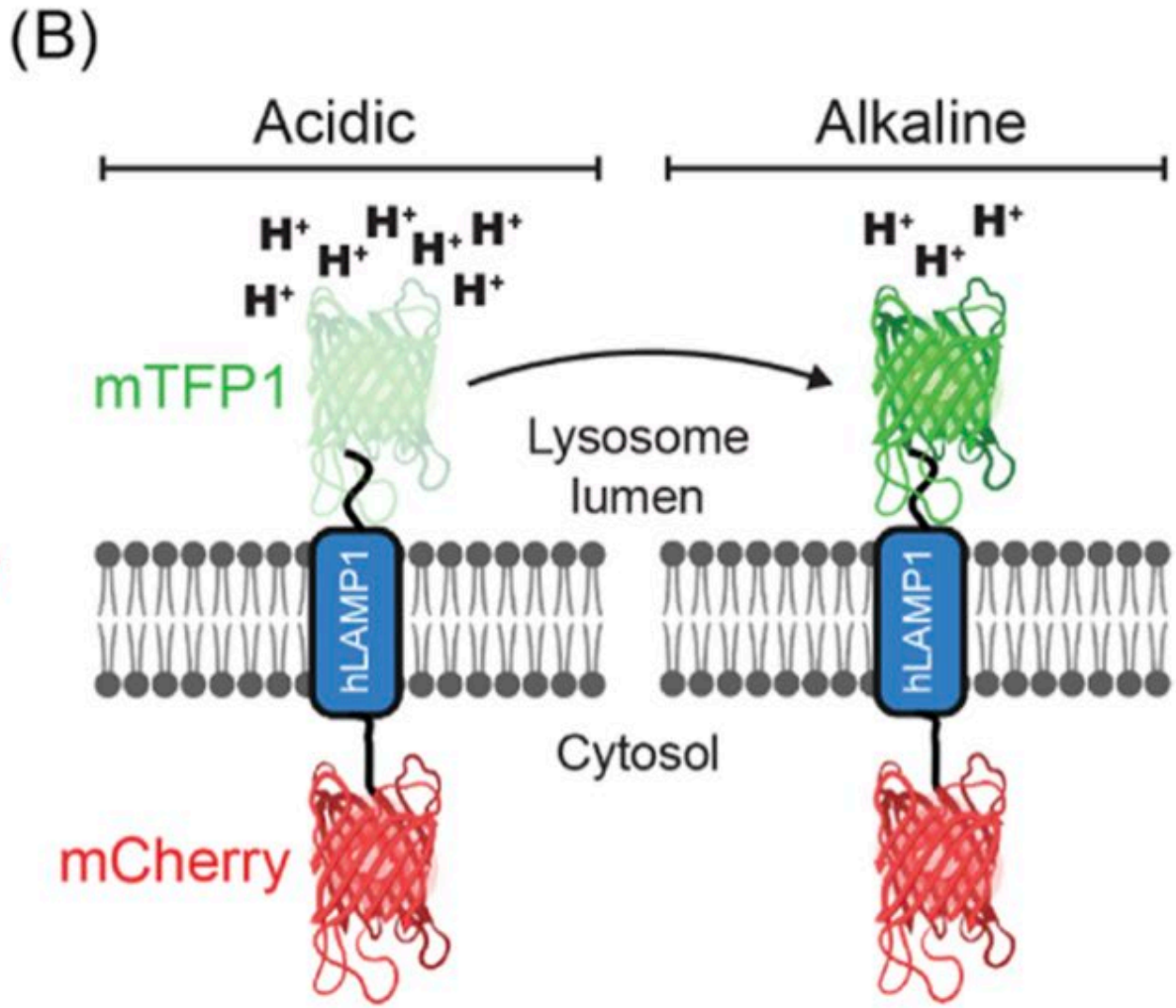


Fluorescenece Indicator REporting pH in Lysosomes (**FIRE-pHLy**)

- N-terminal, lysosomal lumen-facing, pH-sensitive **mTFP1** fused to transmembrane portion of **LAMP1**
- C-terminal, pH-insensitive **mCherry** outside the lysosome
- **Flexible linker** (small and polar amino acids) between mTFP1 and LAMP1 for correct folding and retention of fluorescent properties
- **Rigid linker** between LAMP1 and mCherry for correct sorting and minimization of risk of aggregation
- Driven by **CMV** or **ubiquitin C** (UbC) promotor within lentiviral backbone





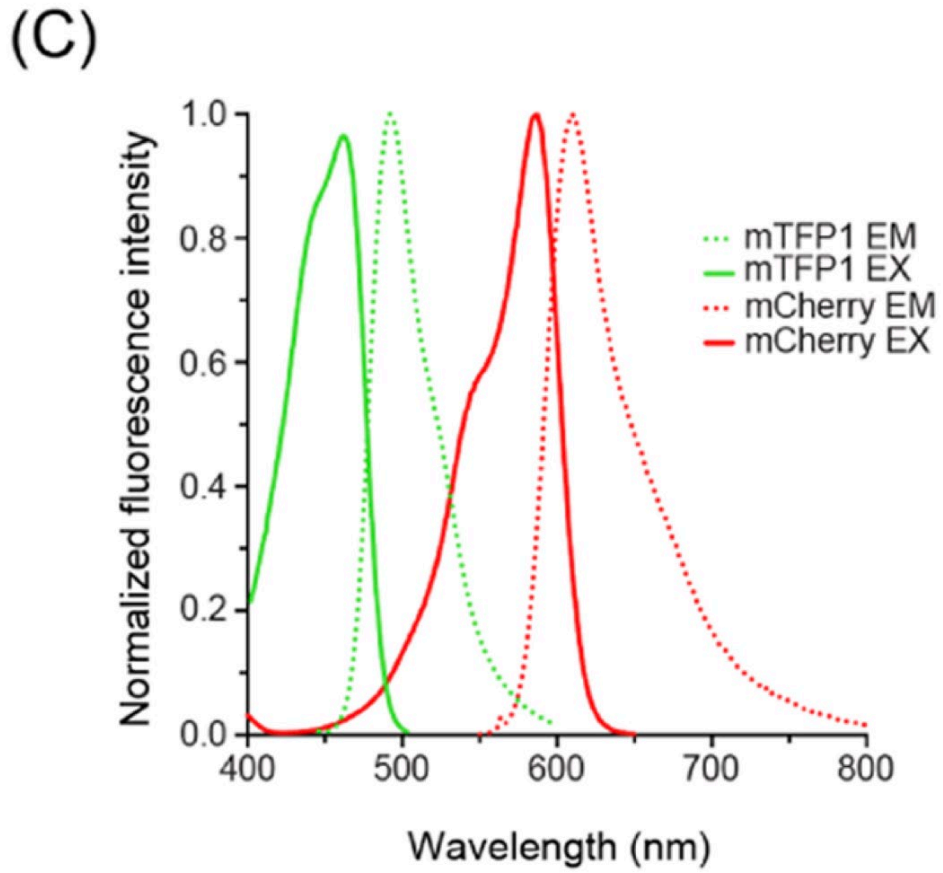


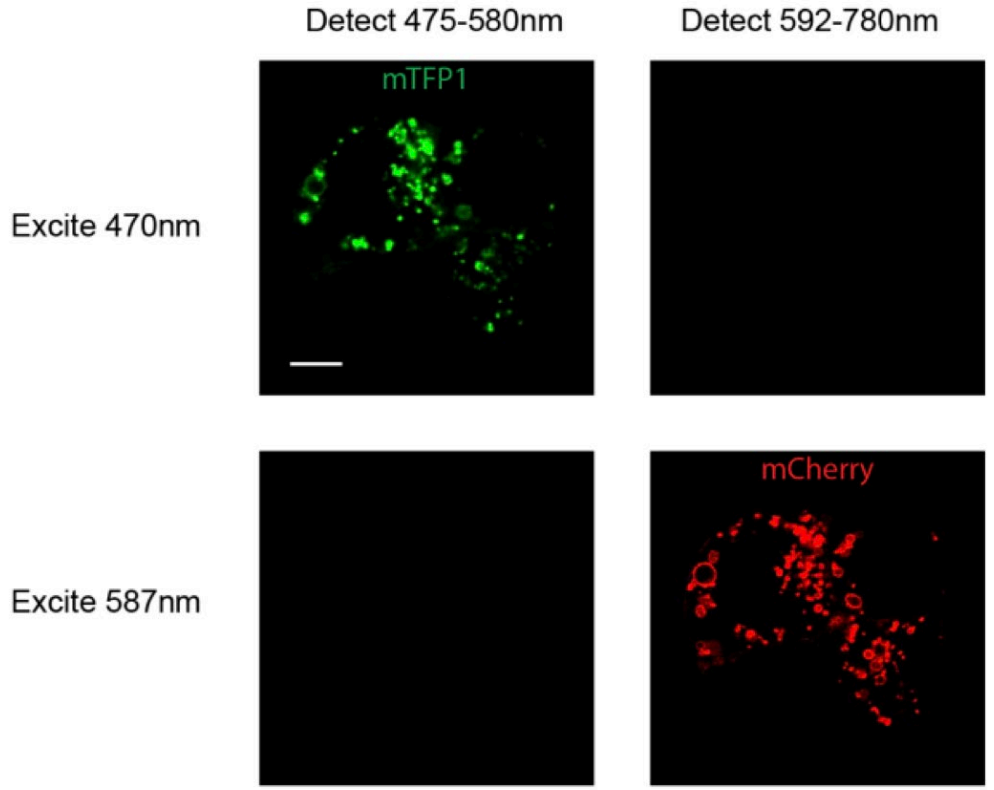
pH-sensitive **mTFP1**

Important properties to assess

- Spectral compatibility
- Expression of the construct in different cell lines
- Specificity of localization
- Quantification of pH-dependent mTFP1 fluorescence in live cells

Spectral overlap



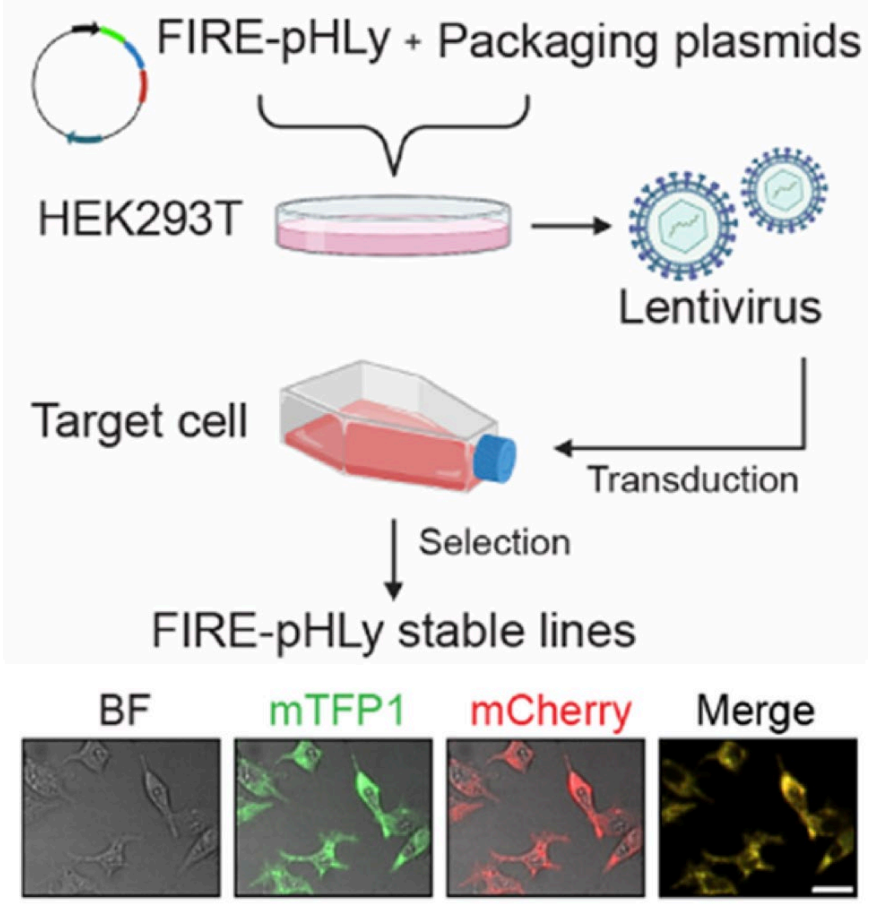


Minimal cross-talk/bleed-through

Suitable spectral compatibility

Expression of the construct in different cell lines

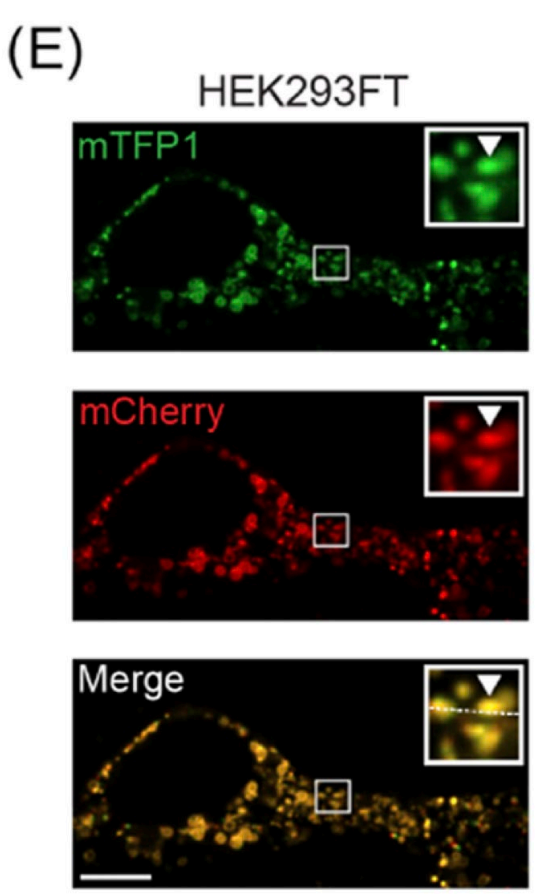
- Lentiviral transduction into HEK293FT and SH-SY5Y neuroblastoma cells
- generation of stable lines

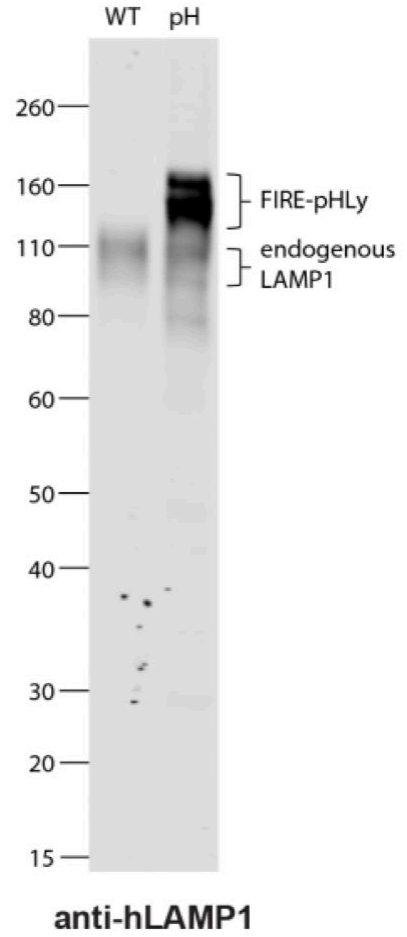


Expression of the construct

- **Co-localization** of mCherry and mTFP1
- Sensor is **glycosylated**
  glycosylated LAMP1: ~90-120 kD
  FIRE-pHLy ~130-160 kD
  (mCherry and mTFP1 27 kD each)

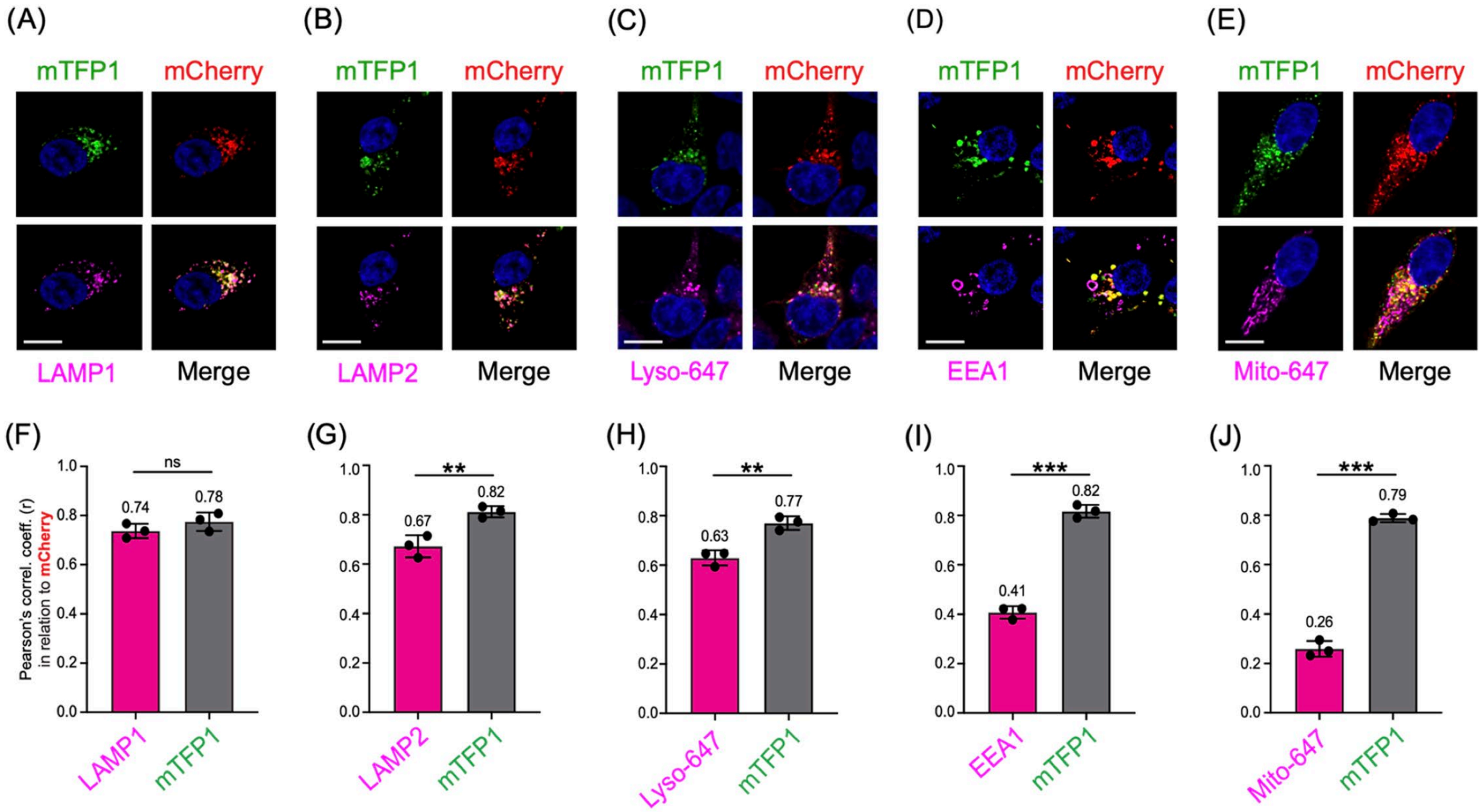
➡ successful expression





Specificity of localization

-



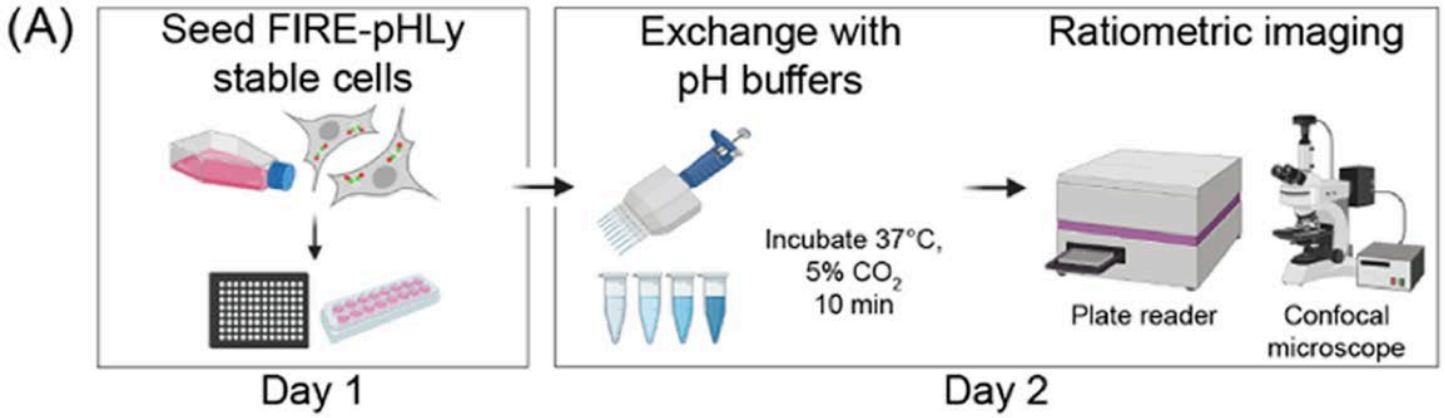
<1: quenching of mTFp1 at physiological pH in lysosomes?

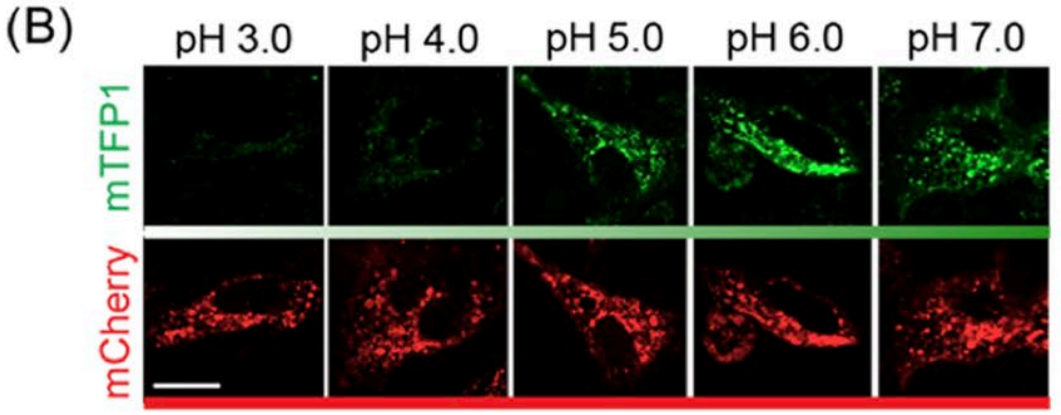
Acidic compartments (late endosomes, lysosomes)

Maturation through endolysosomal system

Mitochondrial-lysosome crosstalk

Quantification of pH-dependent fluorescence in live cells





Equilibrate external pH with that of lysosomes using ionophores nigericin (K^+/H^+) and monensin (Na^+/H^+)

In MES buffer (5 mM NaCl, 115 mM KCL, 1.3 mMMgSO4, 25 mM MES), with the pH adjusted to within the range 3.0–7.0 for 10 min

Suitability for HTS application

High-content confocal ratiometric imaging

IN Cell Analyzer 6500 HS

Workflow

Auto-focus optimization

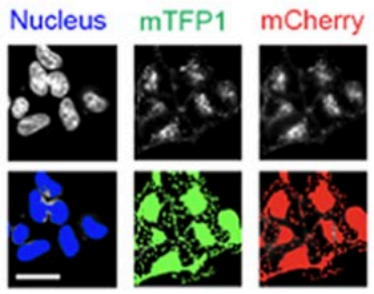
↓

FITC,dsRED,DAPI

↓

Image acquisition

High-content analysis



Segmented regions for quantification

Segmentation & feature extraction

↓

Object recognition

↓

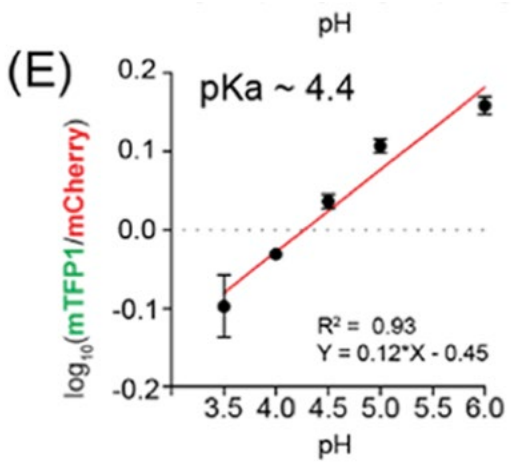
Overlay mask

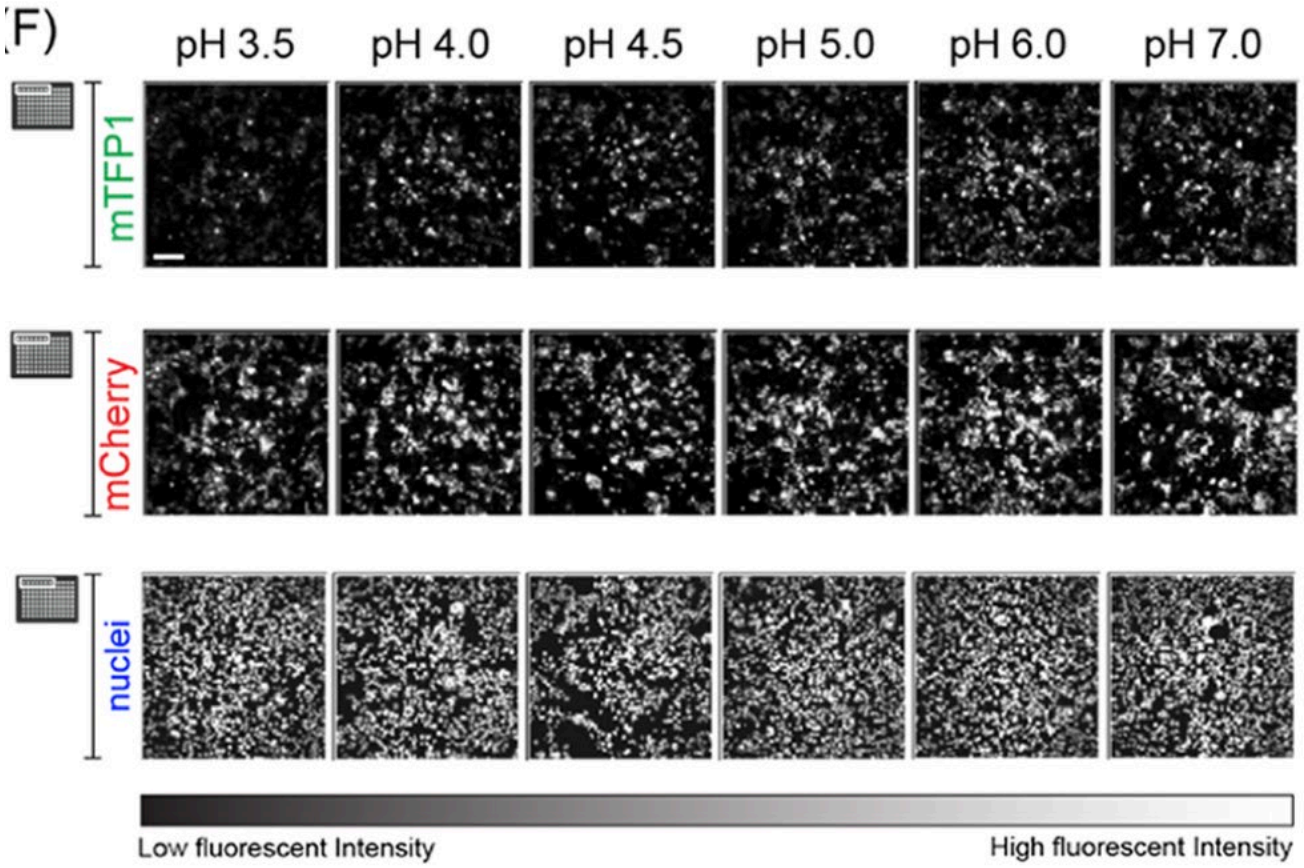
↓

Feature Extraction

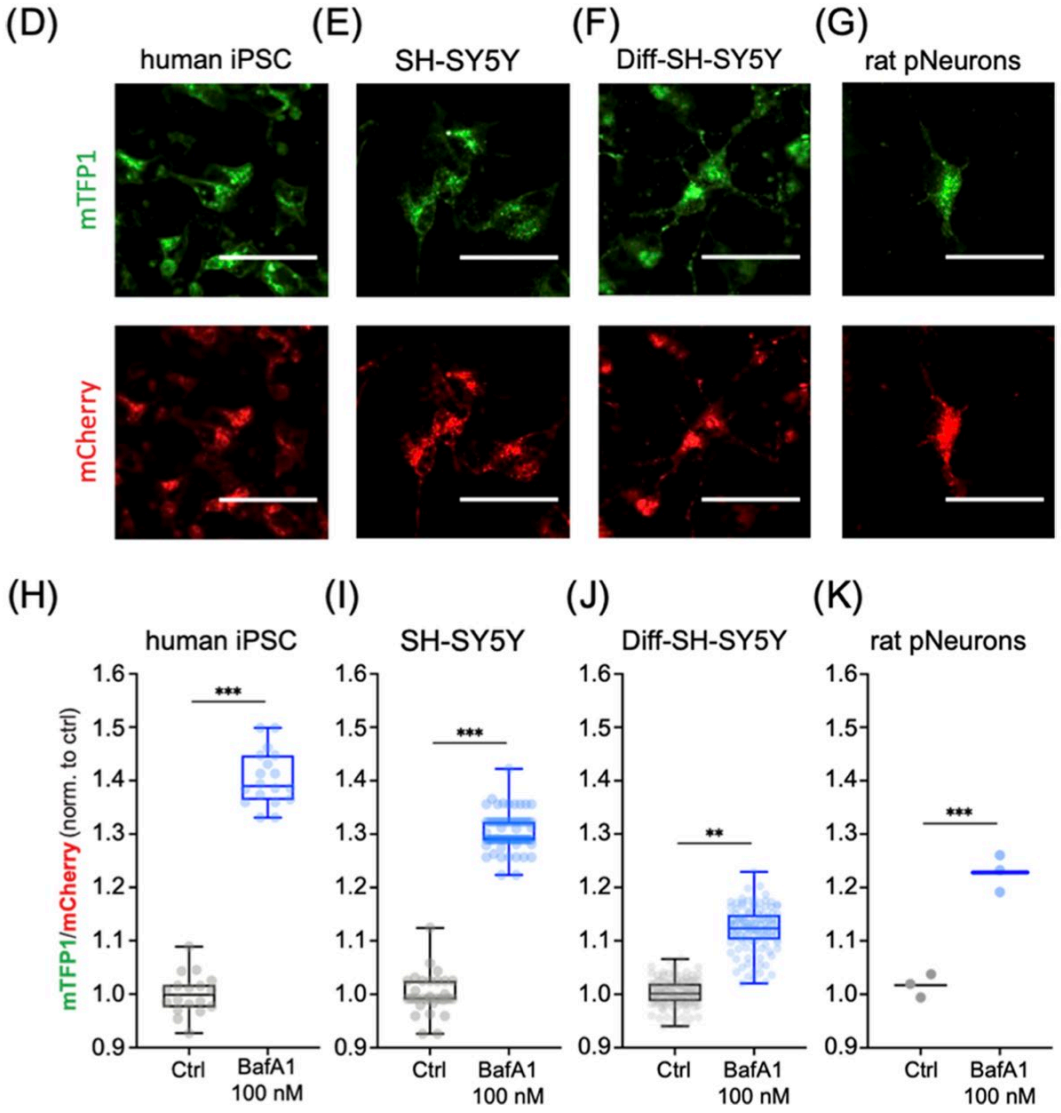
↓

Raw intensity values





Functional validation in different cell lines



- UbC promotor, as CMV promotor is silenced by DNA methylation during differentiation
- ± BafA1 treatment (6h)
- analysis of 5,000 cells/condition
- Lysosomal alkalinization detected across all cell lines
- Cell line differences:
  - Differential BafA1 sensitivity
  - Different pH set point (V-ATPase expression level)

Conclusion: FIRE-pHLy

- Responds robustly to pH changes
- Amenable to stable integration to multiple cellular models, including primary cells
- Suitable for live- and **fixed-cell assays**
- Suitable for high-resolution confocal and **quantitative high-content imaging**

ACS Chemical Neuroscience

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Research Article

Phenotypic Screening Using High-Content Imaging to Identify Lysosomal pH Modulators in a Neuronal Cell Model

Marcus Y. Chin, Kean-Hooi Ang, Julia Davies, Carolina Alquezar, Virginia G. Garda, Brendan Rooney, Kun Leng, Martin Kampmann, Michelle R. Arkin,* and Aimee W. Kao*

Cite This: *ACS Chem. Neurosci.* 2022, 13, 1505−1516

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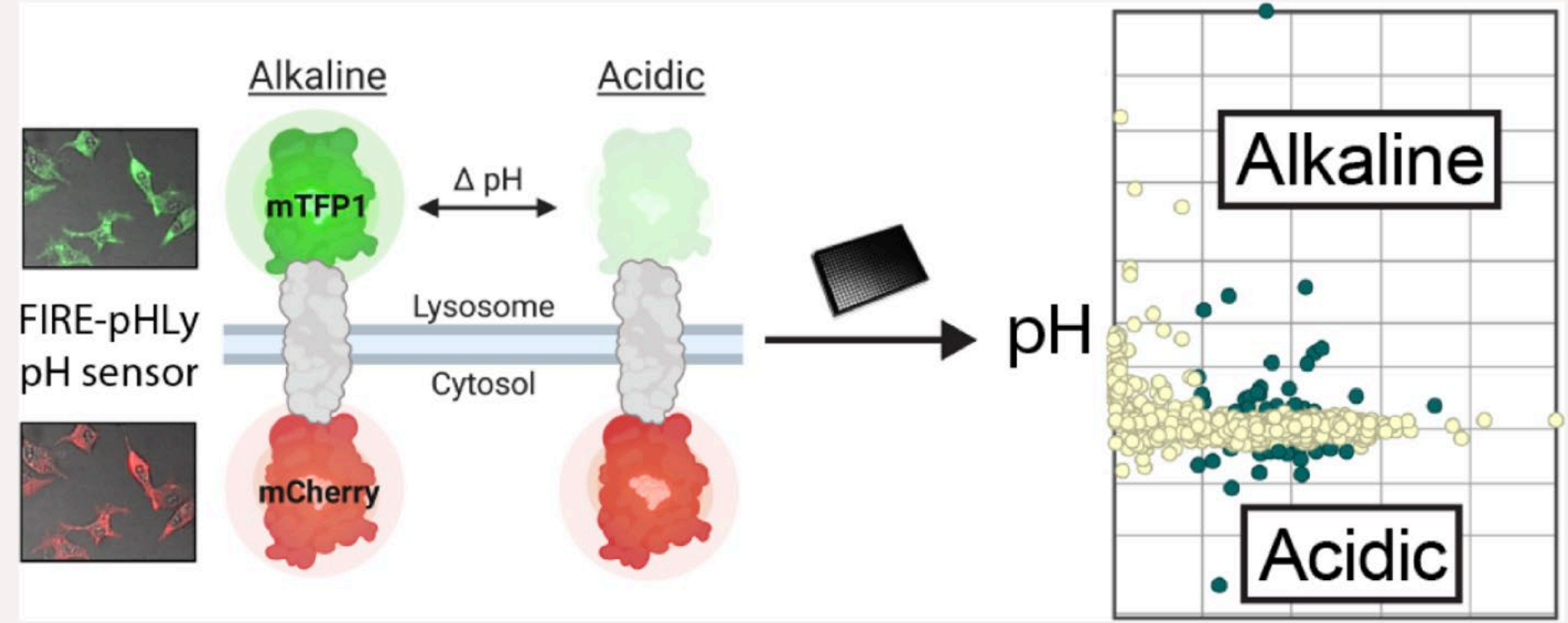
High-Content Imaging to Identify Lysosomal pH Modulators



Goals of the study

1. Screen for small molecules acidifying lysosomal pH
2. Identify molecular targets and pathways regulating lysosomal pH

Correcting lysosomal function and pH regulation might be a therapeutically tractable strategy for future drug development

Experimental approach

- Cell-based phenotypic screen on lysosomal pH
- **Differentiated SH-SY5Y** to model neurodegenerative disorders
  - Endogenous expression of aggregation-prone tau
  - Differentiated cells (retinoic acid treatment): elongated and branching neurites
  - Lysosomes: perinuclear and along the processes
- FIRE-pHLy
  - Stable expression in cells
  - Accurate targeting to lysosomes
  - Resistance to quenching during fixation

Experimental approach II

- Small molecule library comprising 1,835 compounds with annotated targets
- Exposure for 6 hours:
  - Capture fast-acting mechanisms
    (ion channels and transporters generating proton gradient)
  - Avoid longer-term global changes (proliferation, survival)
  - $10\ \mu M\ \mathrm{compound}, 0.2\%\ \mathrm{DMSO}\ (\mathrm{non-toxic})$
- Single-plain measurements
  - mTFP1, mCherry, nuclei target sets

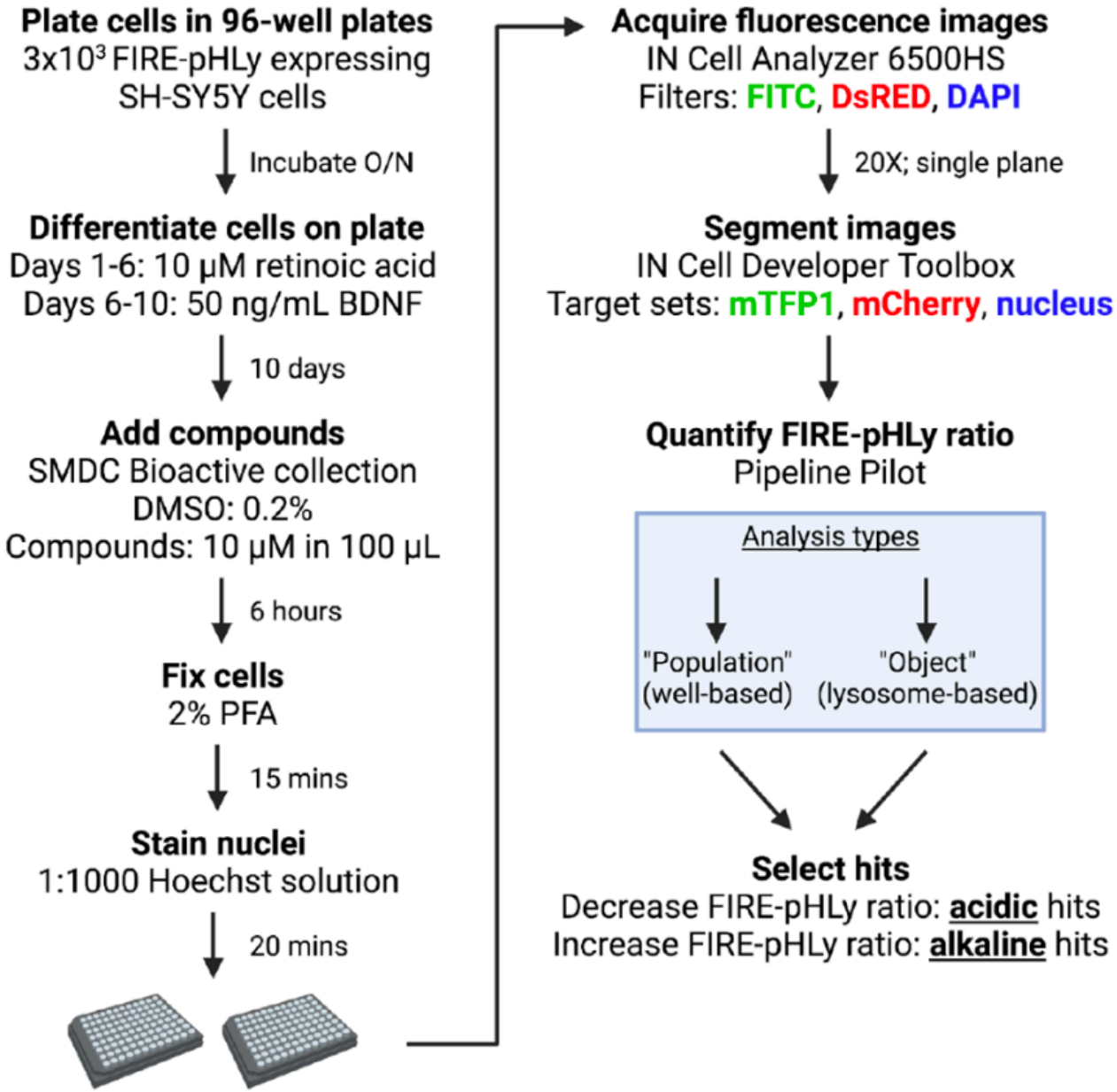


Figure 1. HTS flowchart for identifying lysosomal pH modulators.

Analysis-approaches for hit-selection

- **Population-based analysis** approach
  - FIRE-pHLy ratio averaged across the entire well
- **Object-based analysis** approach
  - focus on lysosomes, optimize sensitivity, account for **different populations of lysosomes** based on coordinate location (perinuclear or in neurites)

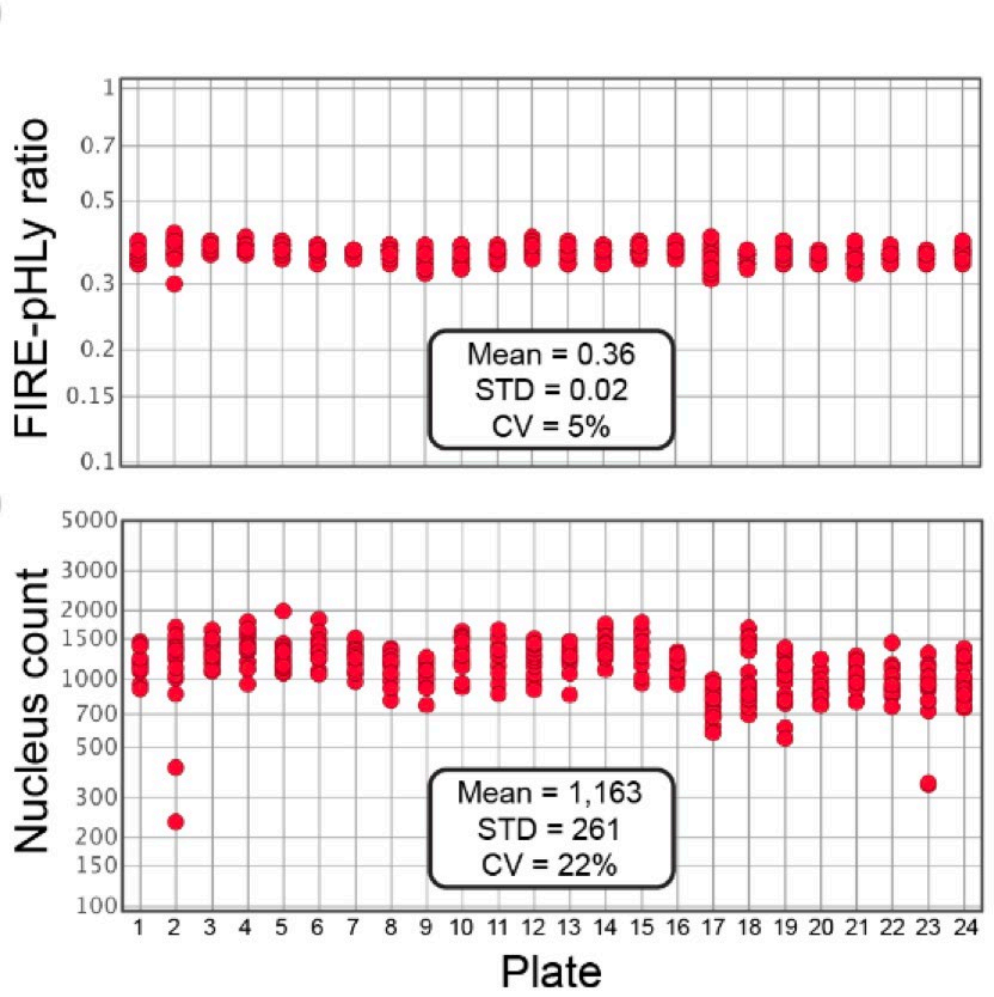
Population-based analysis approach

- establish variability by the percent **coefficient of variation** (CV) of the FIRE-pHLy ratio in DMSO controls

$$CV = \frac{\sigma}{\mu}$$

σ = population standard deviation

μ = population mean

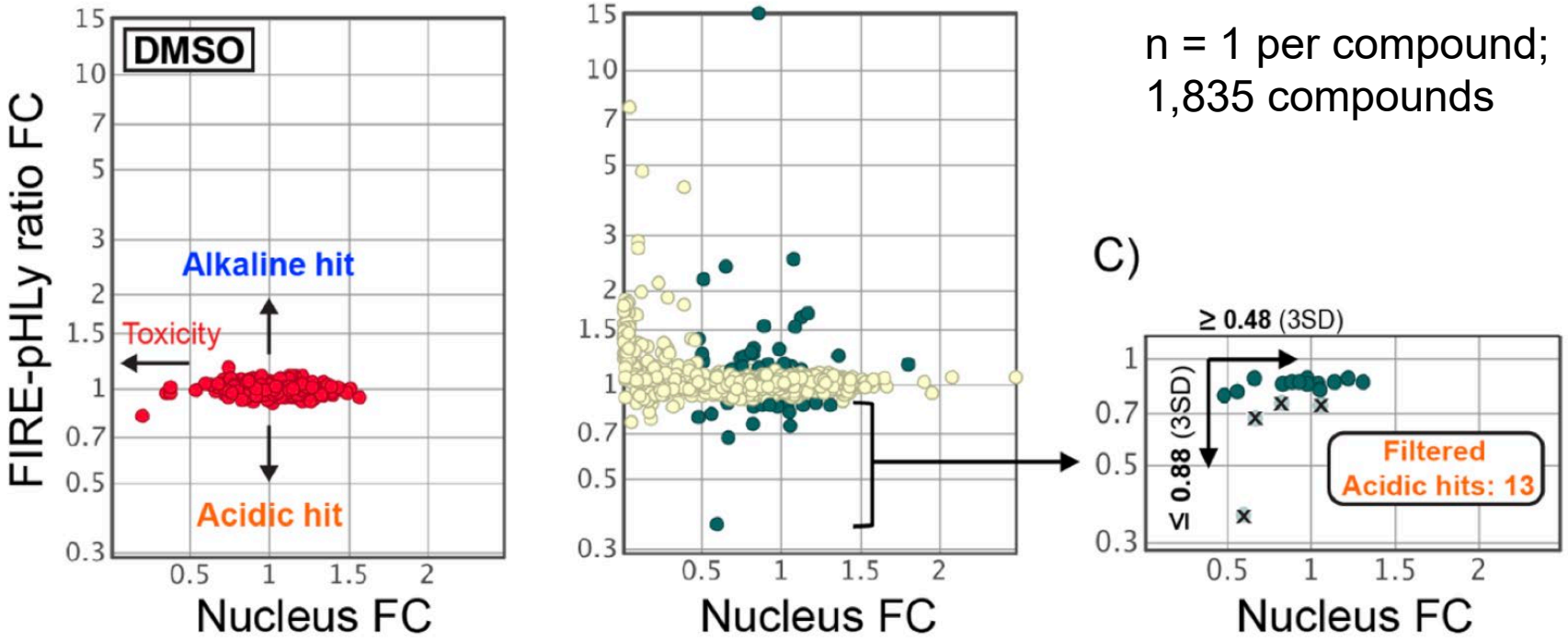


Each red circle represents 1 negative control well (n = 16 per plate).

Primary Hit Selection: Population-based analysis

- **Cytotoxicity**: nucleus FC <0.48 [−3 SD] excluded.
- **Inactive**: Compounds with FIRE-pHLy ratio FCs within ± 3SD of control
- **Primary alkaline hits**: FIRE-pHLy ratio FC of $\geq$1.12 and nucleus FC of $\geq$ 0.48
- **acidic hits**: FIRE- pHLy ratio FC $\leq$ 0.88 and nucleus FC of $\geq$ 0.48
- Alterations in mCherry fluorescence (e.g. compound autofluorescence; off-target pH changes in cytosol)
  –> Exclude compounds with mCherry fluorescence FC > 0.7 or FC < 1.5

Population-based analysis



n = 1 per compound;
1,835 compounds

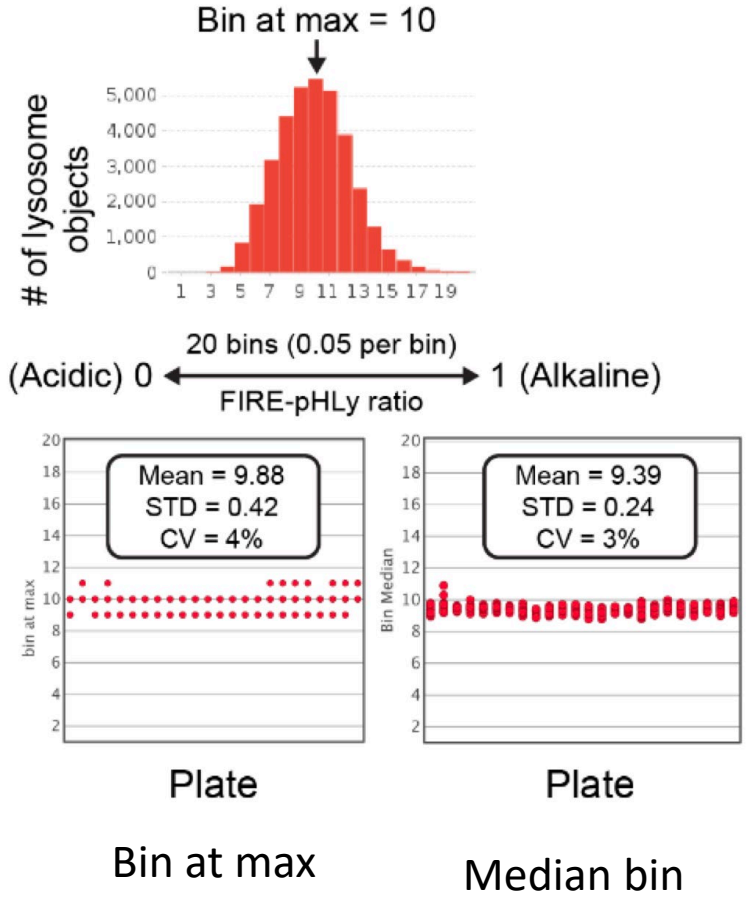
Compounds altering FIRE-pHLy ratio through mCherry fluorescence excluded (black cross marks)

Green dots: primary hit compounds
yellow dots: toxic or inactive compounds.

Acidic hits: nucleus FC ≥ 3SD and FIRE-pHLy ratio FC ≤ 3SD compared to controls (n =384)

Object-based analysis approach

- FIRE-pHLy ratios from individually segmented lysosomes in the DMSO controls
- Bins normalized from 0.0 to 1.0, increment of 0.05/bin, plotted as histogram
- **Bin at max, Median bin**
- Low CV: consistency of assay



Bin at max

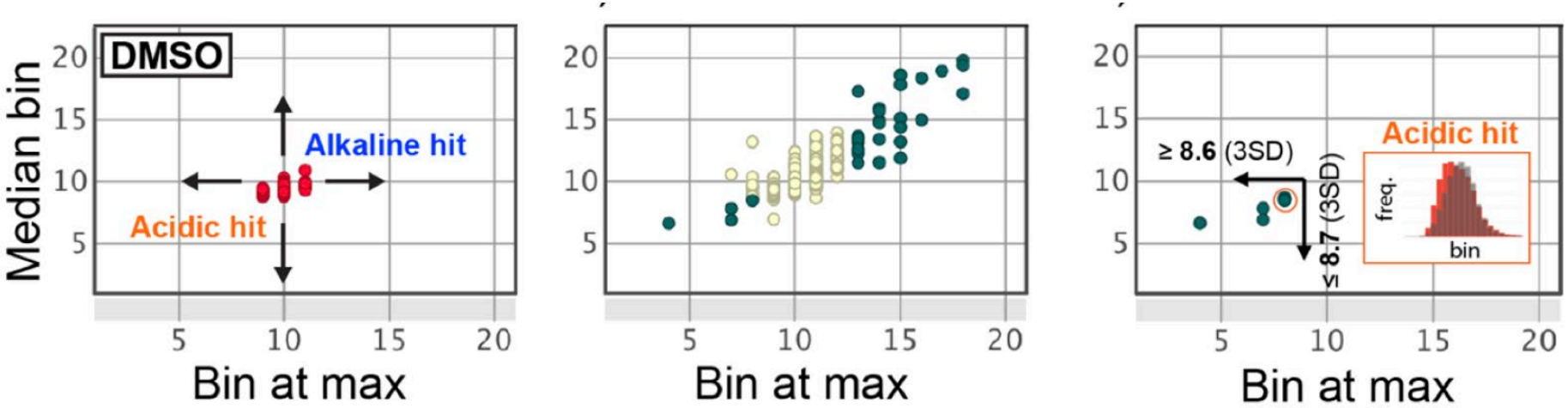
Median bin

Bin median across 24 assay plates
Each red circle represents 1 negative control well
(n = 16 per plate)

Primary hit selection: Object-based analysis approach

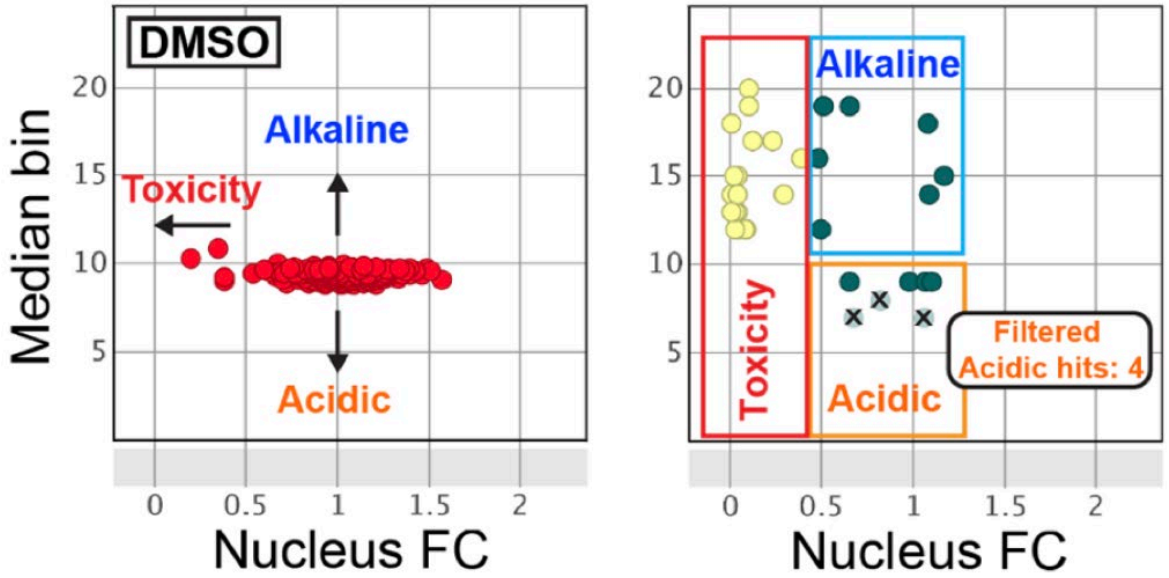
- Cut-offs: increase or decrease in median bin and bin at max by at least 3SD
- **Alkaline hits**: bin at max and median $\geq$ 13 and 11.5
- **Acidic hits**: bin at max and median $\leq$ 8.7 and 8.6
- Exclude compounds reducing nucleus FC compared to control
- Remove compounds altering mCherry fluorescence intensity

Green dots
primary hit compounds



median bin
and
bin at max
≤ 3SD of neg. ctrl

Filtering hits for cell toxicity



Compounds altering FIRE-pHLy ratio through mCherry fluorescence excluded (black cross marks)

Hit selection combining both approaches

Combine both pipelines

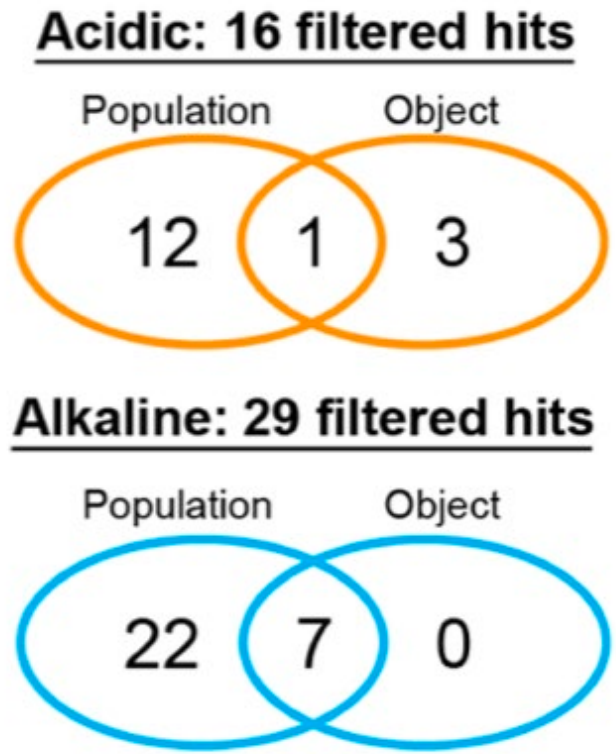


Table 1. Primary Filtered Acidifying Compounds Identified from Population-Based Analysis

Hit No.	SMDC ID	Compound	Ratio FC	Nuclei FC
1	972531	OSI-027[a]	0.88	0.98
2	972462	AZ 960	0.79	0.48
3	972875	3,4-Methylenedioxy-β-nitrostyrene	0.81	0.56
4	751824	Erlotinib	0.82	1.05
5	972846	LY2835219	0.85	0.98
6	131834	Nocodazole	0.85	1.04
7	972322	BMS-707035	0.85	0.83
8	972264	ABT-751 (E7010)	0.86	1.14
9	973076	Vinblastine	0.86	0.93
10	972537	Buparlisib	0.86	0.88
11	972406	Teniposide	0.86	1.31
12	972458	NVP-BHG712	0.88	1.22
13	130715	Nifedipine	0.88	0.66

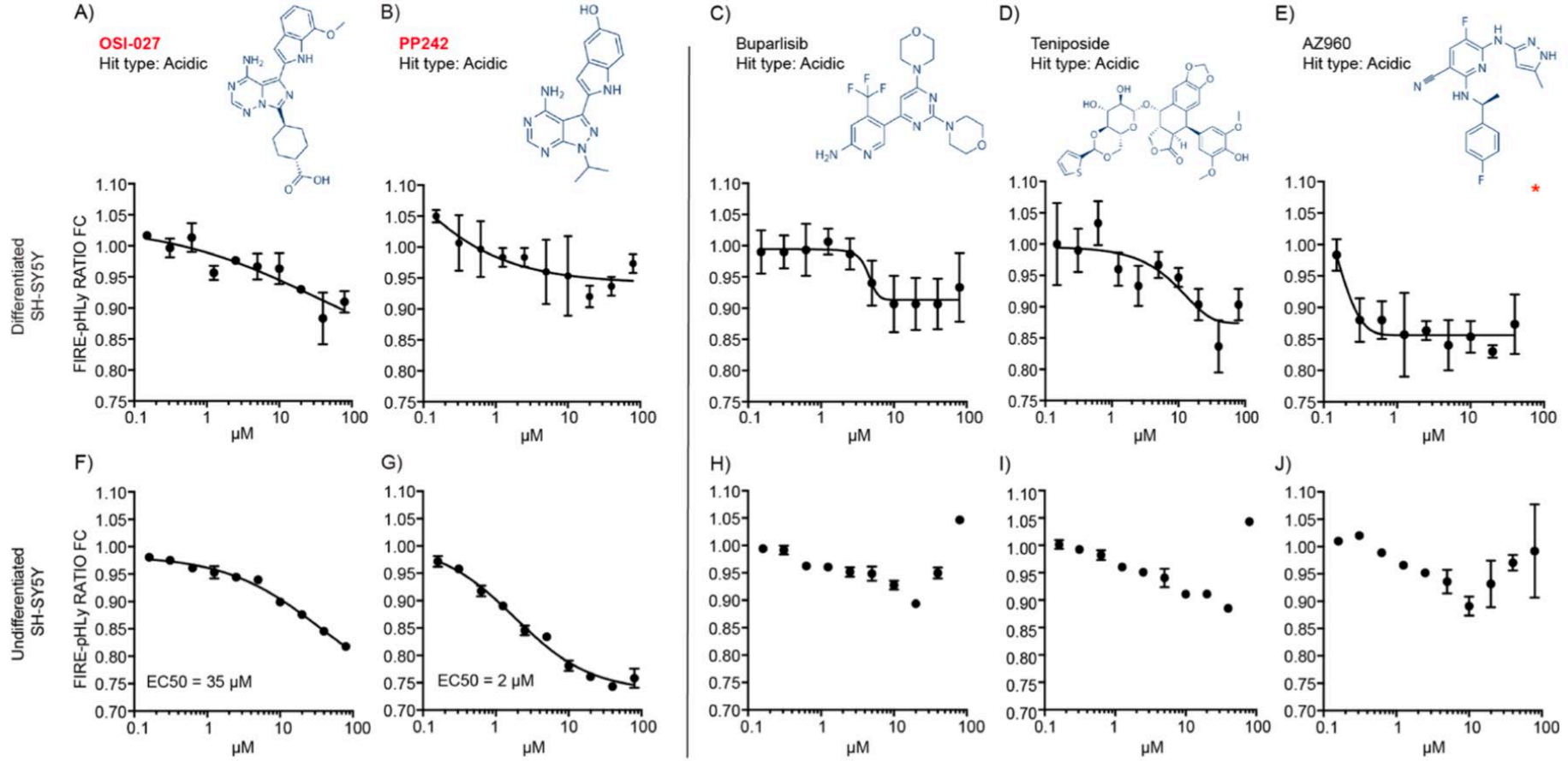
[a]OSI-027 was identified as a hit in both population- and object-based analysis. Compounds highlighted in red passed dose-response retesting in differentiated SH-SH5Y cells.

Table 2. Primary Filtered Acidifying Compounds Identified from Object-Based Analysis

Hit No.	SMDC ID	Compound	Bin Median	Bin Max
1	972531	OSI-027[a]	8.50	8
2	972562	WAY-600	8.51	8
3	972465	PP242	8.66	8
4	972508	Ibrutinib	8.64	8

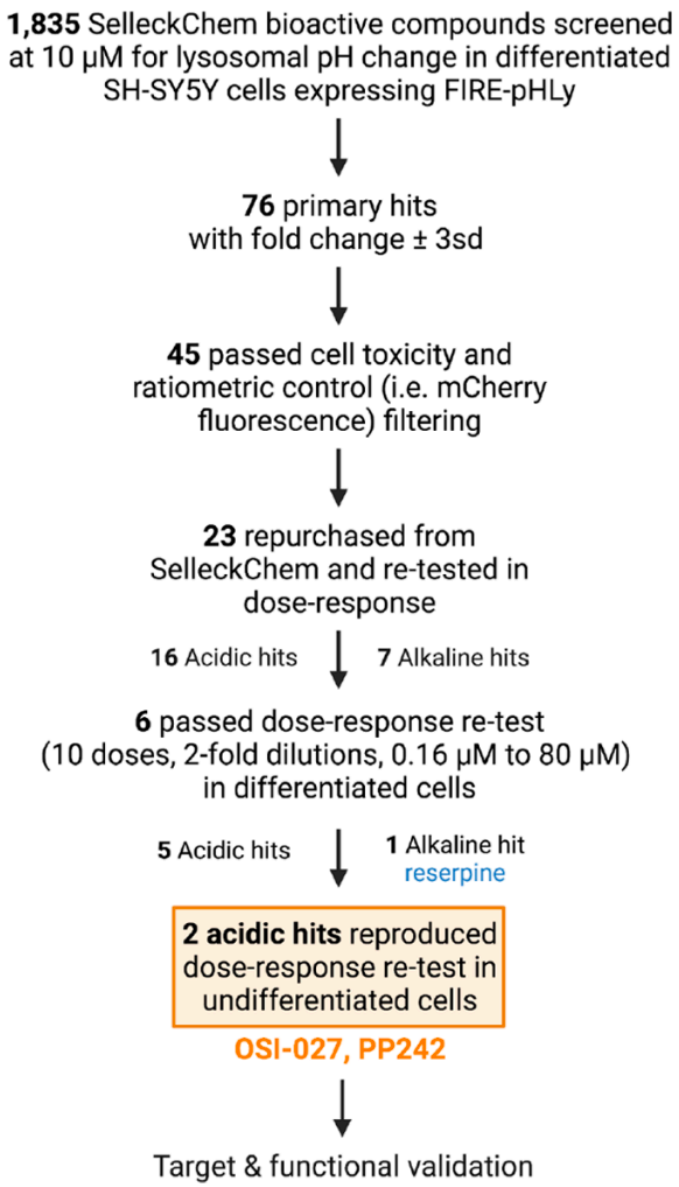
Hit Confirmation with Dose-Response Retesting

- all 16 acidic and 7 alkaline hits (identified in both population- and object-based analyses) retested
  - dose-response (0.15 -80 µM)
  - differentiated and undifferentiated SH-SY5Y cells (support hit confirmation in differing cellular states)
- Acidic hits: 5/16 show dose-dependent effect (2/5 in both cellular models)



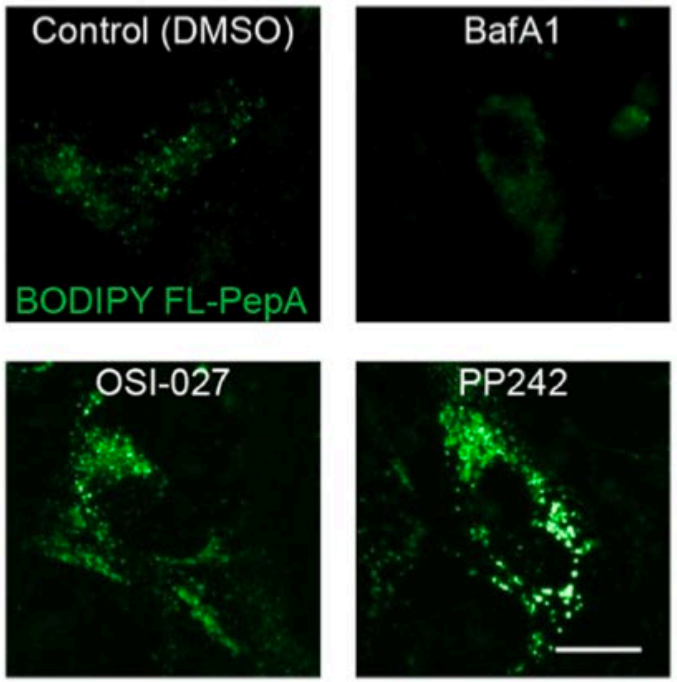
Ten-point dose-response curves (2-fold serial dilution) from 0.15 to 80 μM.
Treatment for 6 h before imaging. FIRE-pHLy ratios were taken from total mTFP1/mCherry fluorescence, displayed as a FC relative to control, and plotted according to dose.
Data points: mean ± SD, from 3 biological replicates; n = ~3000−5000 differentiated cells or ~15,000−20,000 undifferentiated cells per dose per time point.

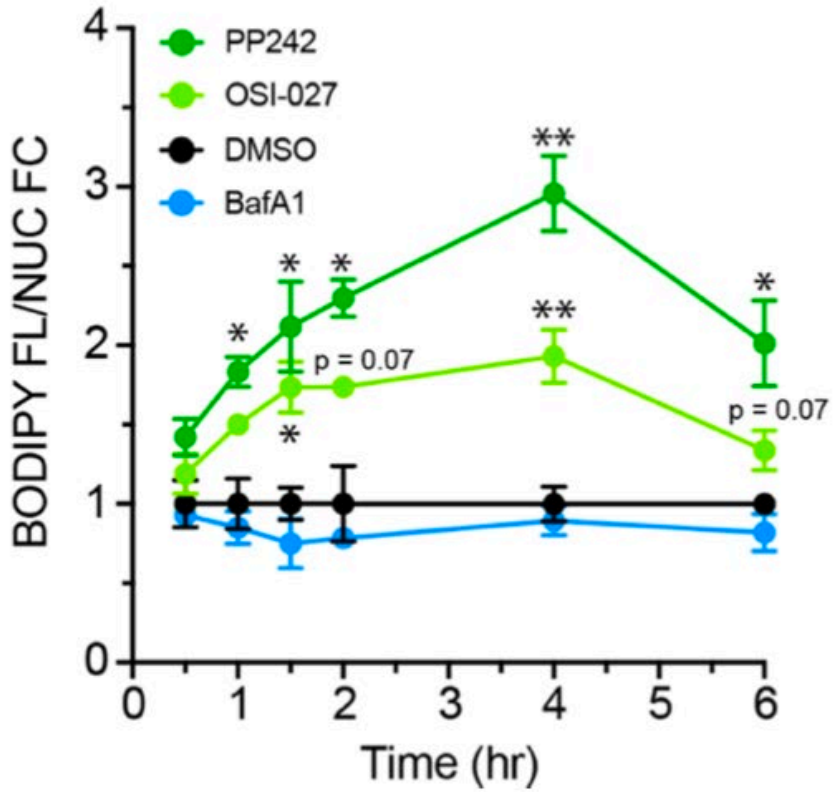
Summary


1,835 SelleckChem bioactive compounds screened at 10 µM for lysosomal pH change in differentiated SH-SY5Y cells expressing FIRE-pHLy
76 primary hits with fold change ± 3sd
45 passed cell toxicity and ratiometric control (i.e. mCherry fluorescence) filtering
23 repurchased from SelleckChem and re-tested in dose-response
16 Acidic hits
7 Alkaline hits
6 passed dose-response re-test (10 doses, 2-fold dilutions, 0.16 µM to 80 µM) in differentiated cells
5 Acidic hits
1 Alkaline hit
reserpine
2 acidic hits reproduced dose-response re-test in undifferentiated cells
OSI-027, PP242
Target & functional validation

Functional Validation of 2 Top Acidic Hits

- **Orthogonal method**: assess cathepsin D activity in undifferentiated SH-SY5Y cells (wt)
- **Cathepsin D**: auto-activated at acidic pH, activity can be used as a functional readout of lysosomal pH
- BODIPY FL-Pepstatin A: cathepsin D antagonist, binding to active form
- Treatment with OSI-027 and PP-242 at 10 µM for various times
  - Compare to DMSO control
  - negative control: BafA1 (inhibits V-ATPase proton pump)
- Read-out: fluorescent intensity





fluorescence normalized to cell number

mean ± SD, from 3 biological replicates;

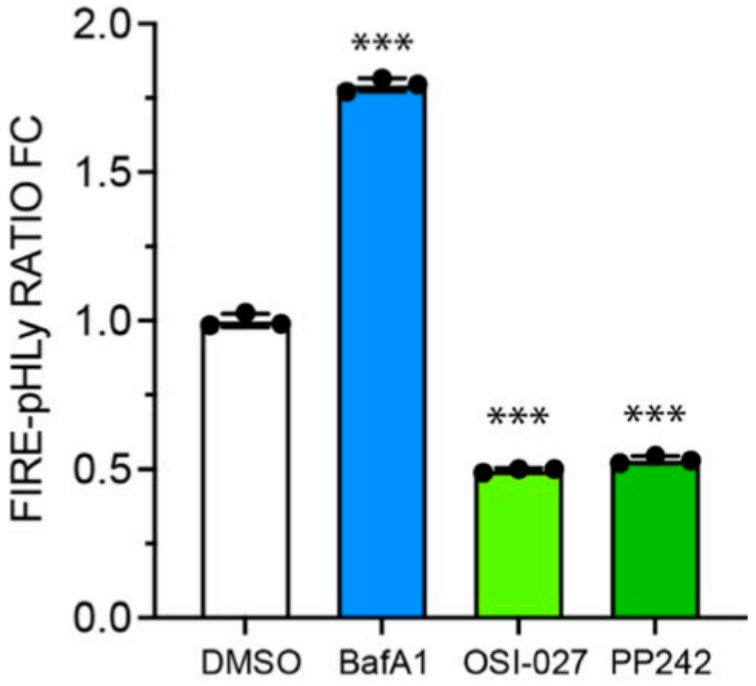
n = ~15,000– 20,000 cells per condition group per time point.

two-way ANOVA for multiple comparisons.

*p ≤ 0.05 and **p ≤ 0.01.

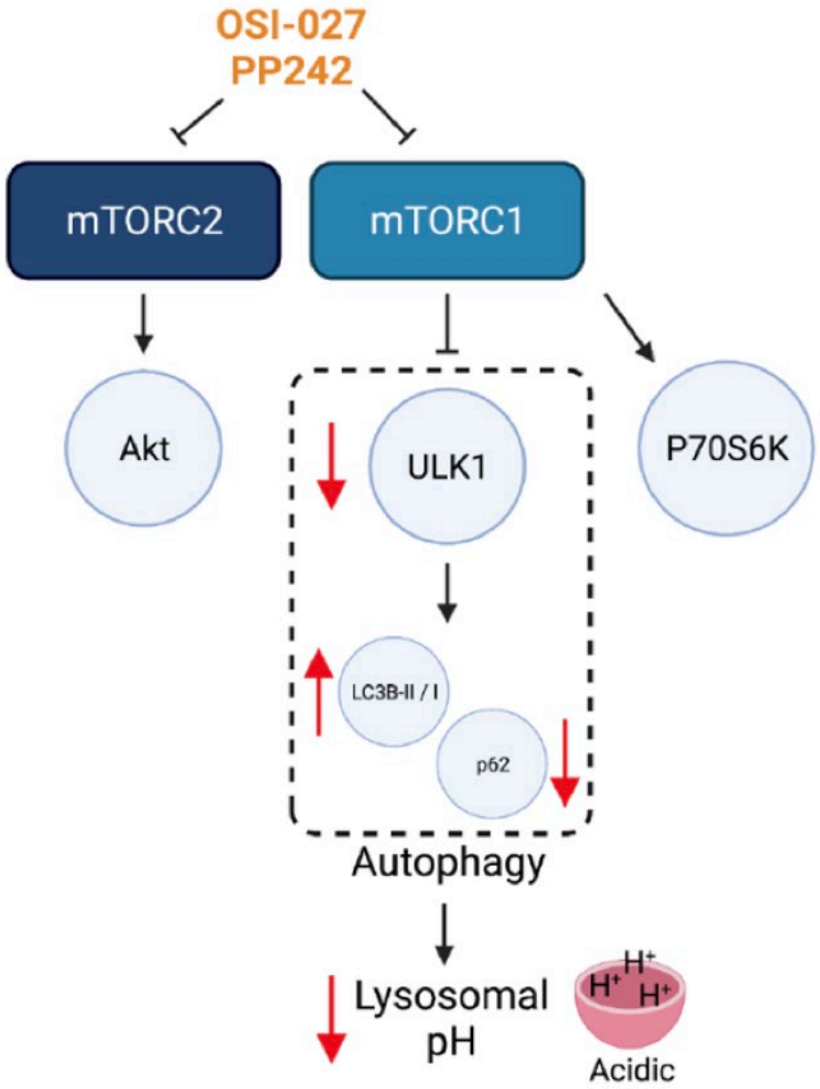
“Disease-relevant” model

- human-induced pluripotent stem cell (iPSC)-derived astrocytes stably expressing FIRE-pHLy
- treated for 24 h with OSI-027 and PP242
- Reactive astrocytes secrete neurotoxic factors
- Reactive astrocytes exhibit alkaline lysosomal pH
- implicated in the neuroinflammatory component of neurodegenerative diseases



Mechanism of action of OSI-027 and PP242

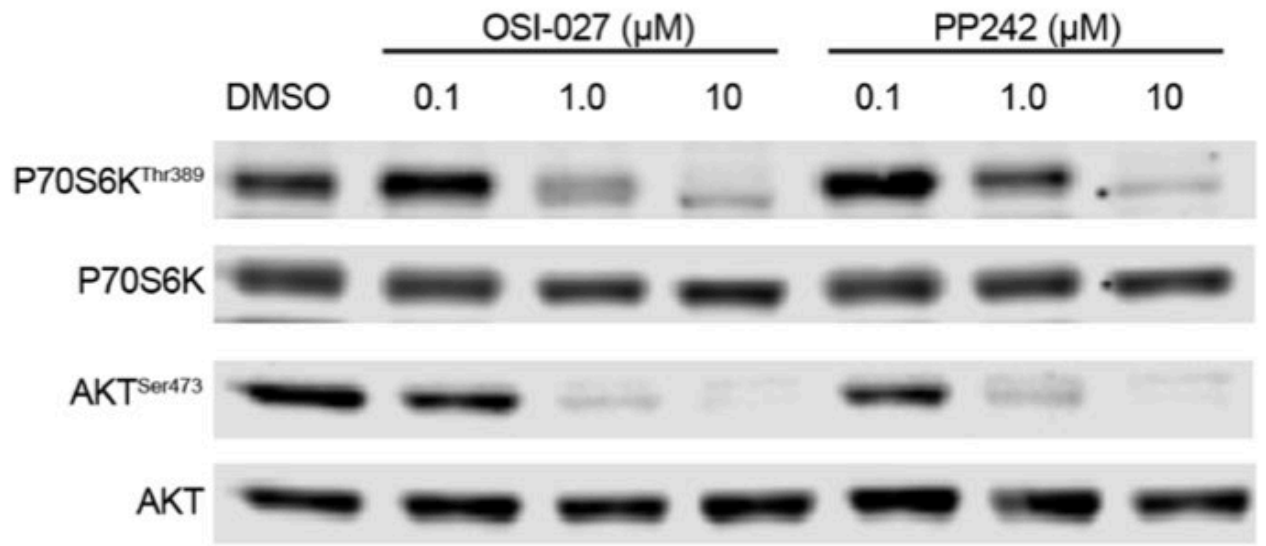
- potent and selective ATP-competitive inhibitors of mammalian target of rapamycin (mTOR)
- mTOR regulates cellular processes such as metabolism, growth, and proliferation
- mTOR inhibition coupled with autophagy induction
  - lysosomal activation and acidification



Is lysosomal acidification related to mTOR inhibition?

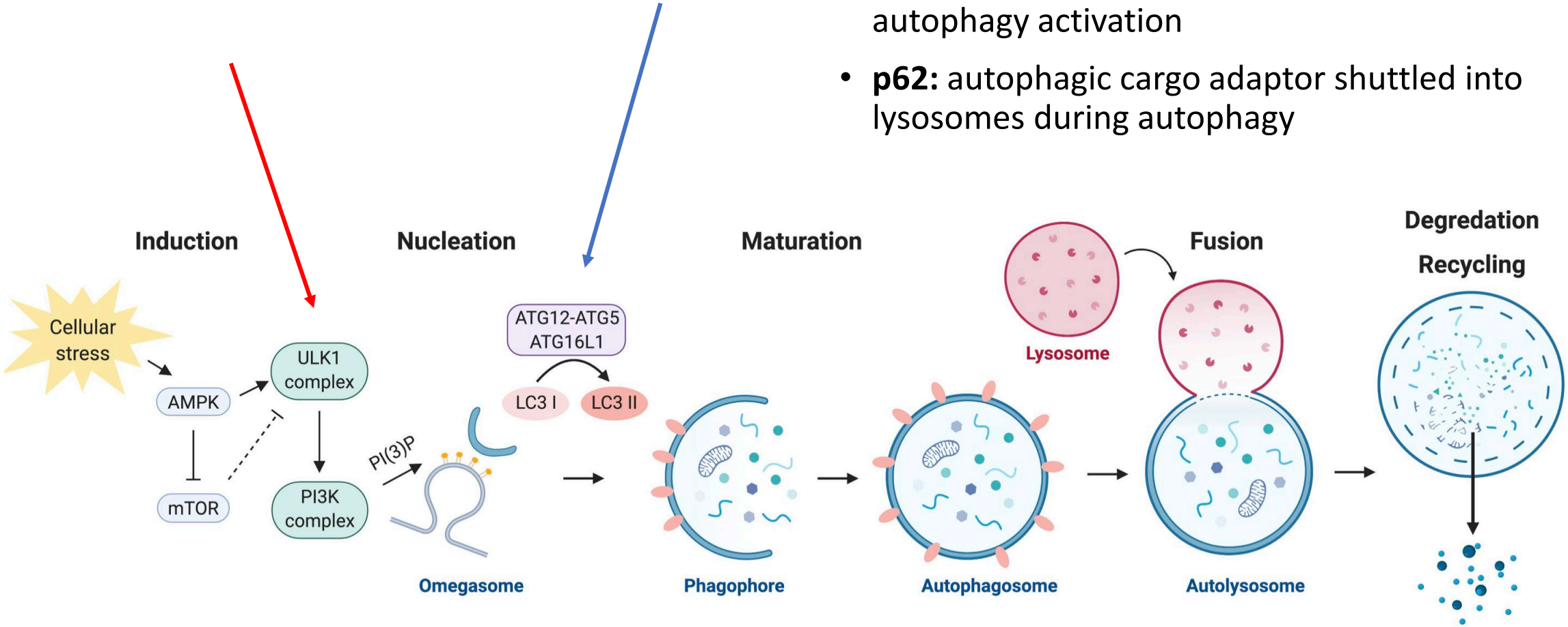
- **mTORC1 activity**:
  phosphorylation state of P70 S6 Kinase (P70S6K) at Thr389
- **mTORC2 activity**:
  phosphorylation of Akt at position Ser473

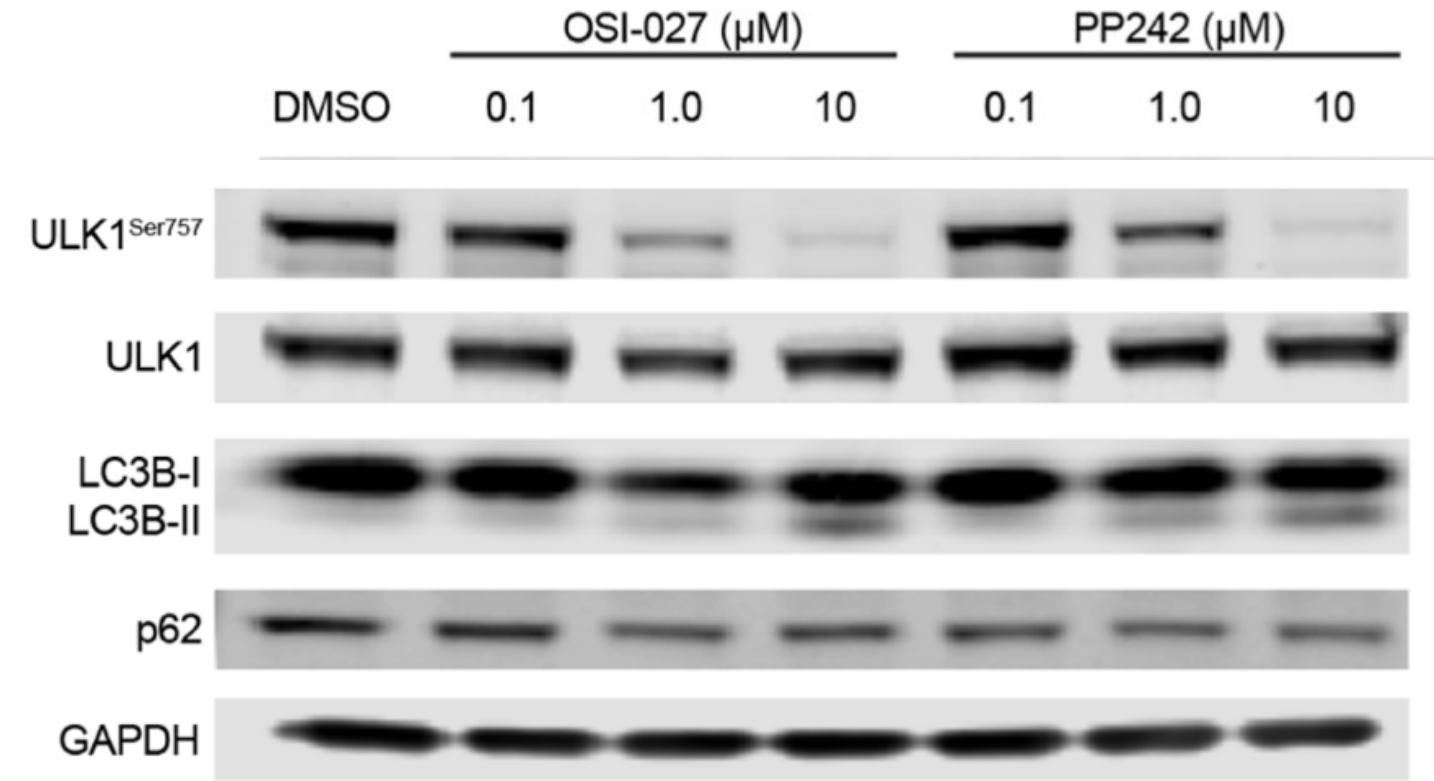
-> dose-dependent inhibition of downstream targets of mTORC1 and mTORC2

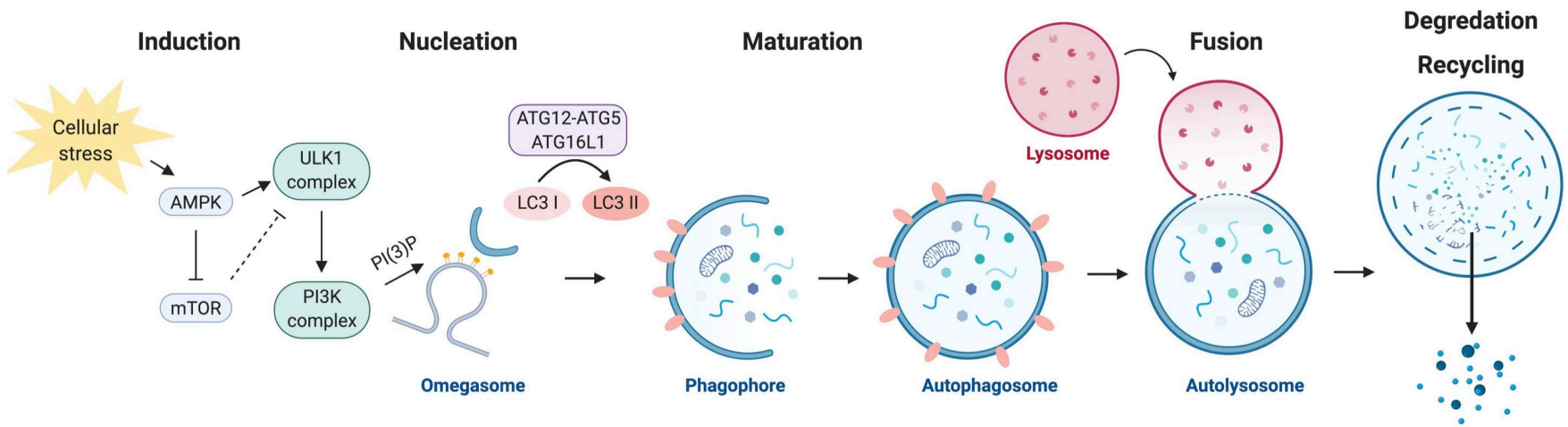


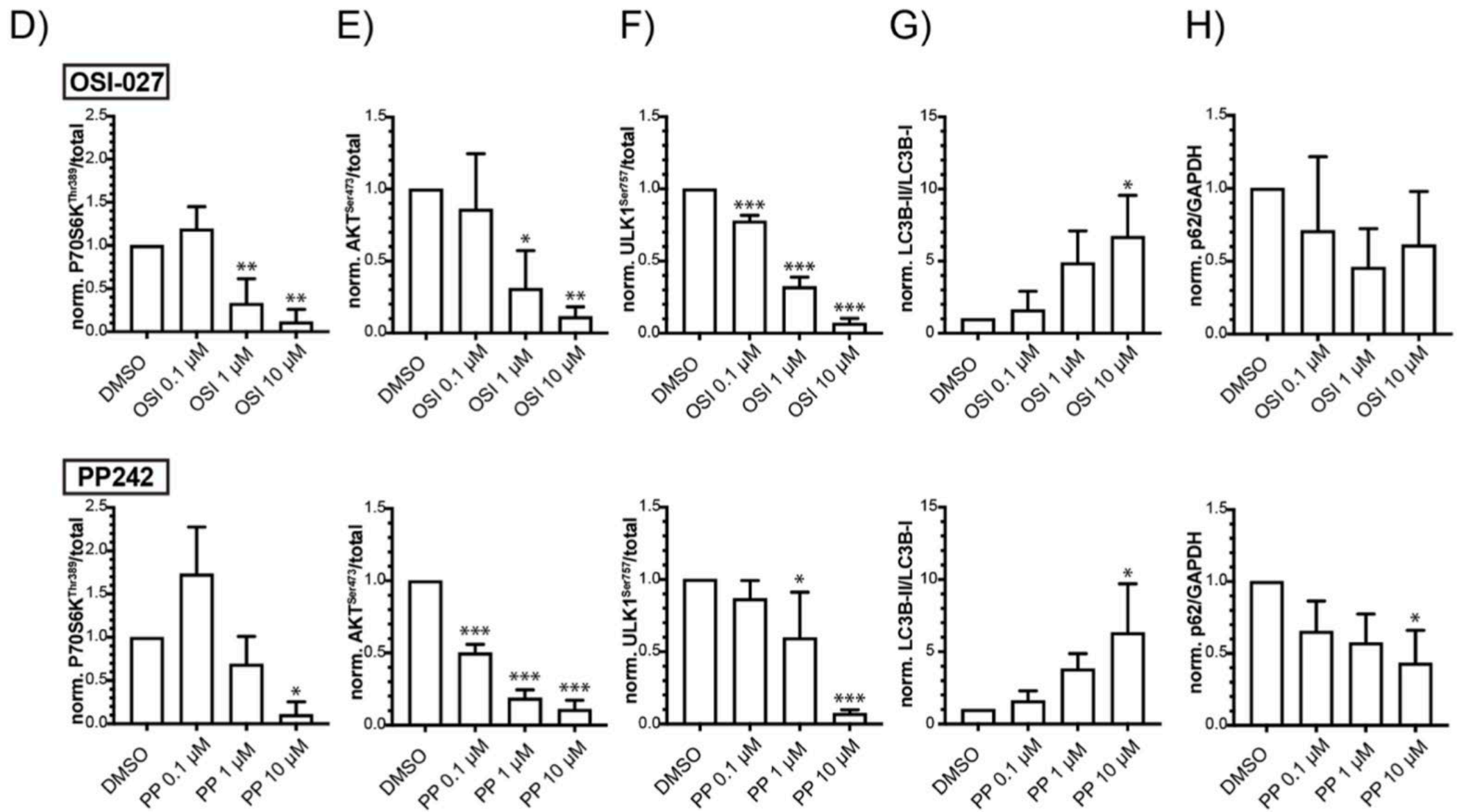
Do the compounds activate autophagy?

- mTORC1 negatively regulates autophagy through phosphorylation of Unc-51-like autophagy activating kinase (**ULK1**)
- Microtubule-associated protein light chain 3B (**LC3B**): conversion of LC3B-I to LC3B-II correlated with autophagosomes/reflects autophagy activation
- **p62:** autophagic cargo adaptor shuttled into lysosomes during autophagy




OSI-027 (μM)
PP242 (μM)
DMSO
0.1
1.0
10
0.1
1.0
10
ULK1Ser757
ULK1
LC3B-I
LC3B-II
p62
GAPDH


Induction
Nucleation
Maturation
Fusion
Degredation
Recycling
Cellular stress
AMPK
ULK1 complex
mTOR
PI3K complex
PI(3)P
ATG12-ATG5
ATG16L1
LC3 I
LC3 II
Lysosome
Omegasome
Phagophore
Autophagosome
Autolysosome


D)
E)
F)
G)
H)
OSI-027
PP242
norm. P70S6K Thr389/total
norm. AKT Ser473/total
norm. ULK1 Ser757/total
norm. LC3B-II/LC3B-I
norm. p62/GAPDH
DMSO
OSI 0.1 µM
OSI 1 µM
OSI 10 µM
PP 0.1 µM
PP 1 µM
PP 10 µM

OSI-027 and PP242 acidify Lysosomes more potently than other mTOR Inhibitors

- rapamycin and torin1: mTOR1 inhibitors
- No/minor effect on autophagy
- do not acidify lysosomes across the tested dose range up to 24 h

Conclusion

- OSI-027 and PP242 induce autophagy
- ability to acidify lysosomes may be secondary to the induction of autophagy rather than a direct action on the lysosome

Overall summary

- Population-based analysis used in standard plate-based HTS studies
- object-based analysis: novel technique, enables to dissect organelle **subpopulation phenotypes**
  - overall lower acidic hit rate
  - validation rate superior to population-based analysis

Overall summary II

- 2/16 primary acidic hits could be validated
- Possible reasons
  - biological factors (lysosomal pH dynamics)
  - screening limitations (library size, protein druggability of the targets)?
  - basal lumenal pH already highly acidic (~4.5): further acidification may be tightly regulated/detrimental
  - reduced dynamic range of assay exhibited by acidifiers compared to alkalinizing compounds

OSI-027 and PP242

- **OSI-027**: top hit in a mutant tau protein lowering screen in patient iPSC-derived neurons; effect much stronger than that of rapamycin
- **PP242**: rescued lysosomal acidity (LysoTracker) in a mouse PD cellular model system

Outlook

- Link between lysosomal acidification defect and clinical phenotypes?
- Why are OSI-027 and PP242 more effective in activating autophagy and decreasing lysosomal pH than other mTOR inhibitors?
- Undescribed targets independent of mTOR?
  - kinase inhibitors likely to have additional targets
  - KINOMEscan database: PP242 binds to multiple other kinases (PI3K, ABL proto-oncogene 1: regulate autophagy in cancer)

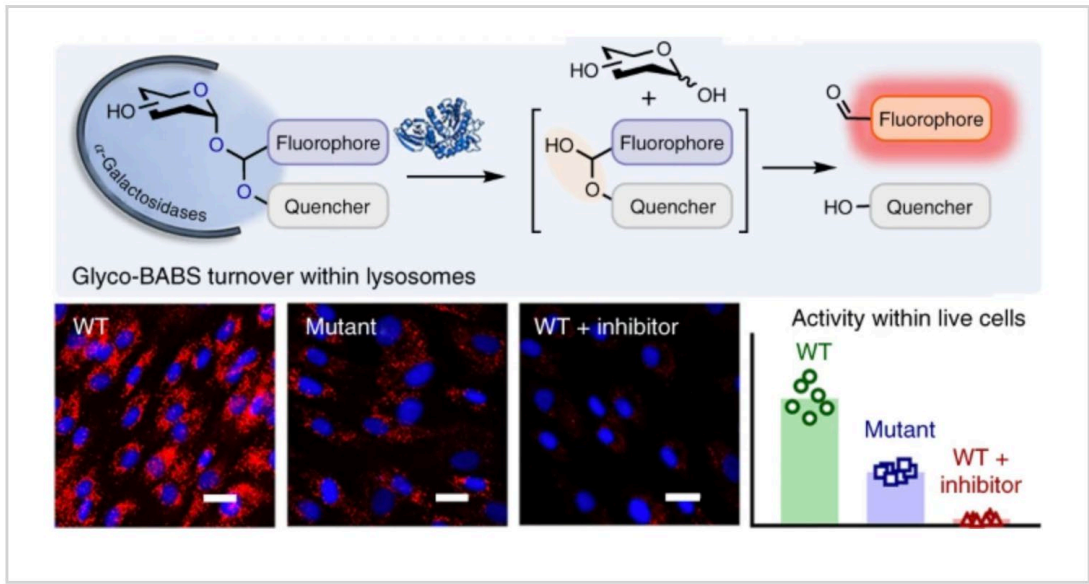
“OSI-027 and PP242 may serve as “tool” compounds to study mechanisms driving autophagy-mediated lysosomal activation in the context of neurodegenerative diseases."

ARTICLES
https://doi.org/10.1038/s41589-021-00960-x
nature chemical biology

Check for updates

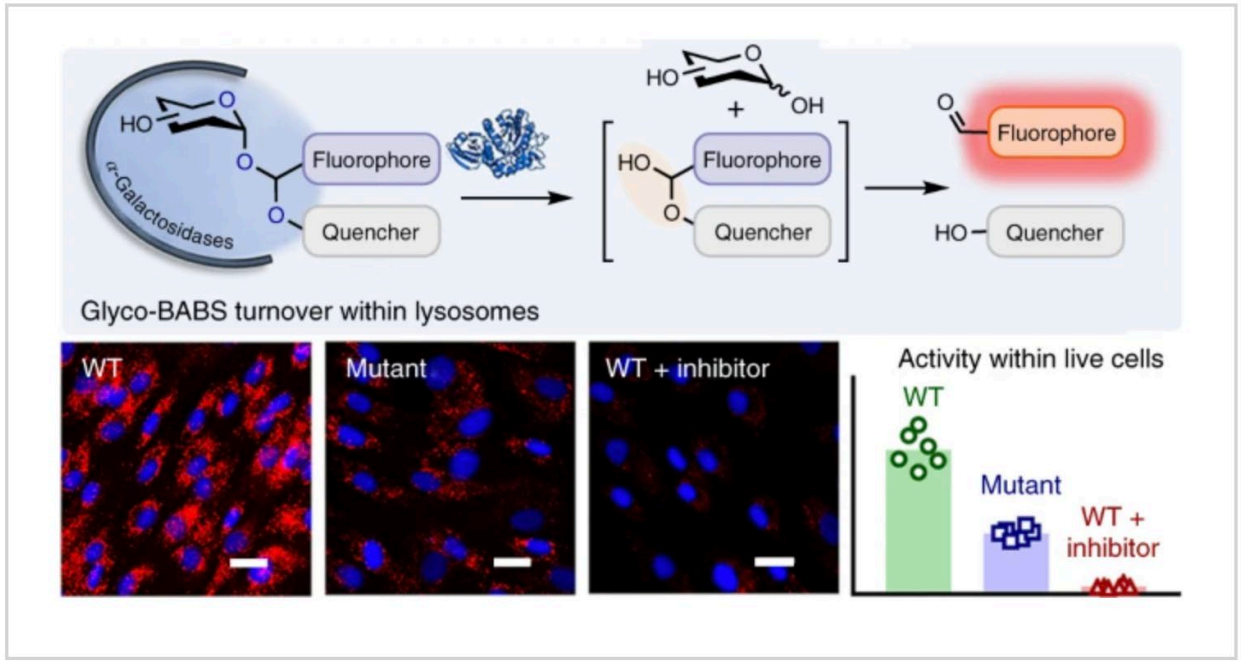
Quantifying lysosomal glycosidase activity within cells using bis-acetal substrates

Samy Cecioni[1,3,6], Roger A. Ashmus[1,6], Pierre-André Gilormini[1], Sha Zhu[1], Xi Chen[1], Xiaoyang Shan[1,2], Christina Gros[1], Matthew C. Deen[1], Yang Wang[1,4,5], Robert Britton[1] and David J. Vocadlo[1,2] ✉



Aim

- Design tools to quantitatively monitor (lysosomal) hydrolase activity within their physiological milieu
- Facilitate development of diagnostic tools and therapeutic approaches for LSDs

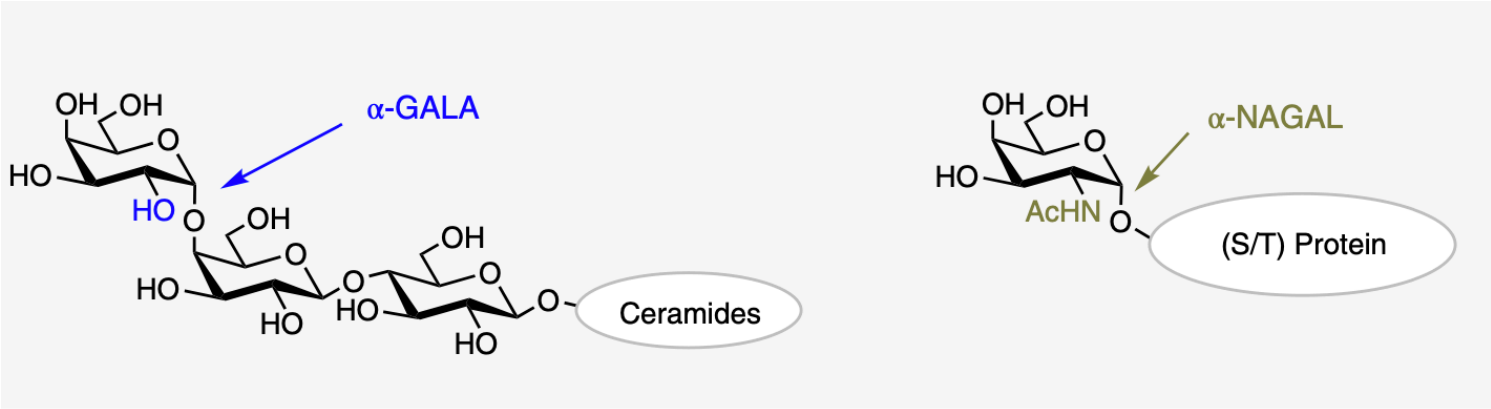


Theoretical background

- Mutations in glycosidases linked to many monogenic diseases
- Few small molecules targeting these enzymes reached clinics
- Most enzyme assays performed *in vitro* in the absence of cellular factors and/or in lysate-based assays

α-GALA and α-NAGAL

- **α-GALA** encoded by *GLA:* lysosomal hydrolase, cleaves terminal α-galactose (α-Gal) residues from globotriaosylceramide and di-galactosylceramides
- **α-NAGAL** (α-*N*-acetylgalactosaminidase) encoded by *NAGA:* closely related lysosomal enzyme (46% sequence homology), hydrolyzes α-linked *N*-acetylgalactosamine (α-GalNAc) residues from mucin glycoproteins and glycolipids



α-GALA and α-NAGAL

- Mutations (>900 describes in *GLA*) cause impairment in folding, trafficking and downstream lysosomal activity
- Substrate accumulation
- *GLA* mutations: X-linked, **Fabry disease**
- *NAGA* mutations: autosomal recessive, **Schindler/Kanzaki disease**
- Phenotypic variability (also in carriers of the same mutation)

Treatment options

- Fabry disease
  - lifelong iv injection of recombinant enzyme (antibody development possible)
  - Oral chaperone migalastat (DGJ)
- Schindler disease
  - DGJNAc (not on the market yet)

Diagnosis

- Often delayed
- current diagnostic path:
  - in vitro evaluation of enzymatic activity from fibroblast lysates
  - sequencing of the *GLA* gene
- **in vitro lysate assays**
  - Measure total enzymatic content in the absence of cellular factors
  - Do not reflect lysosomal α-GALA activity
- Overestimation of lysosomal α-GALA activity: contributes to incomplete correlation between specific mutations and disease severity

Goal

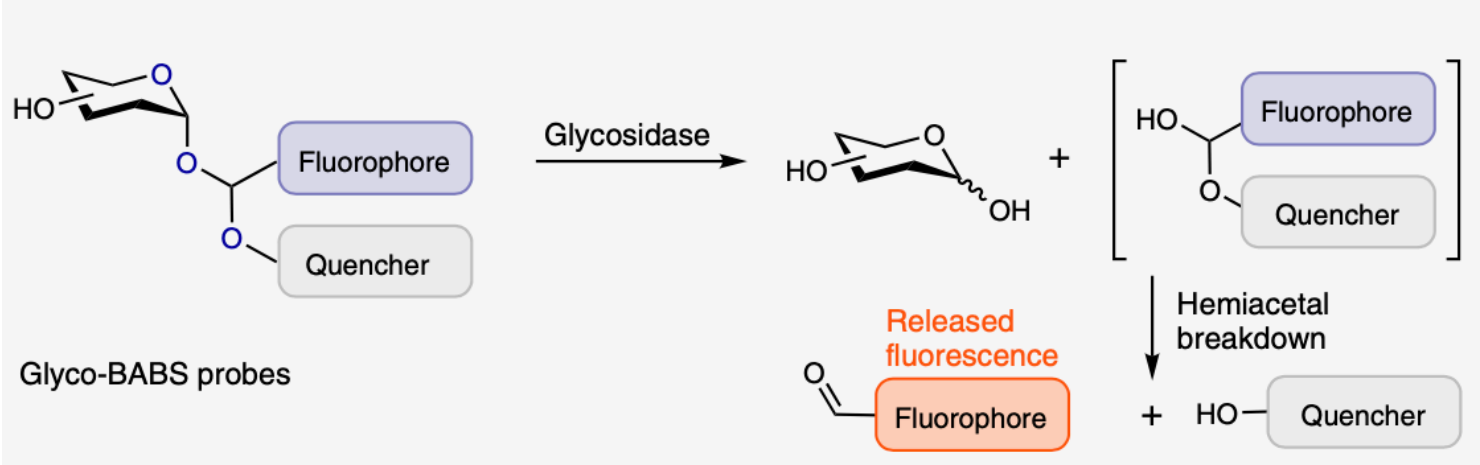
- develop a method to monitor α-GALA and α-NAGAL activity directly within the lysosomes of live cells
  - Monitor effect of candidate pharmacological chaperones within a relevant physiological milieu

Existing tools

- activity-based probes irreversibly labeling targeted enzyme with fluorophores
  - High sensitivity, but leads to enzyme inactivation (reports on active enzyme concentration rather than enzyme activity)
- substrate probes detecting enzyme turnover in live cells
  - Fluorophores should not be pH sensitive
  - Probe should be retained within cell/organelle

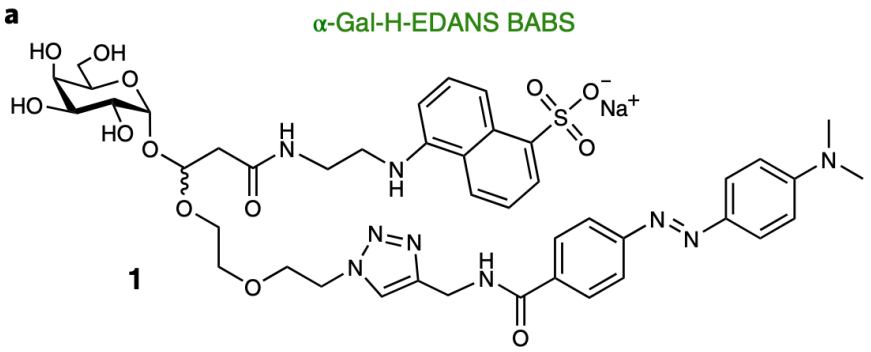
Fluorescence-quenched substrate probe suitable for live-cell imaging

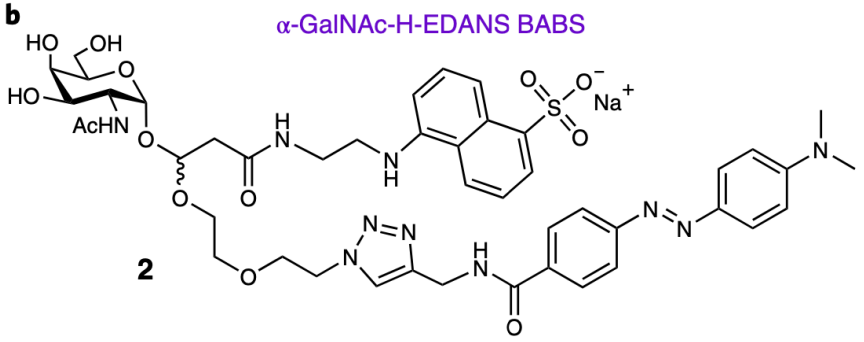
- “dark to bright” fluorescent probes
- enzymatic cleavage of the glycosidic bond liberates a hemiacetal
- spontaneous breakdown separates fluorophore from quencher



Design

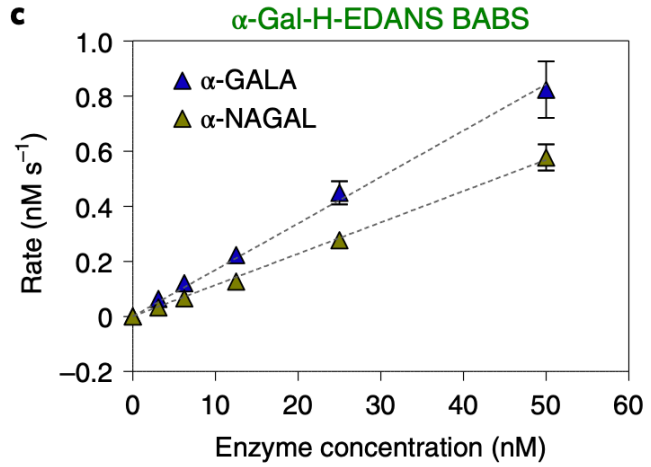
- **bis-acetal substrates**: fluorophore appended to the aldehyde-bearing product, polarity/interaction with imines should enhance retention of fluorescent moiety within cells
- EDANS/DABCYL pair for quenching

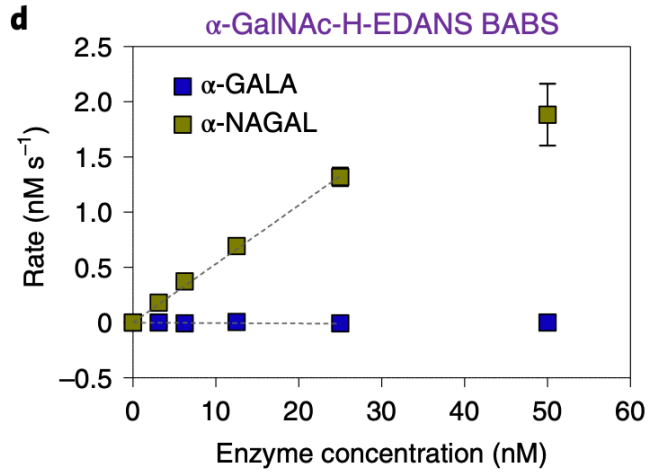




Proof of principle: Time-dependent changes in fluorescence

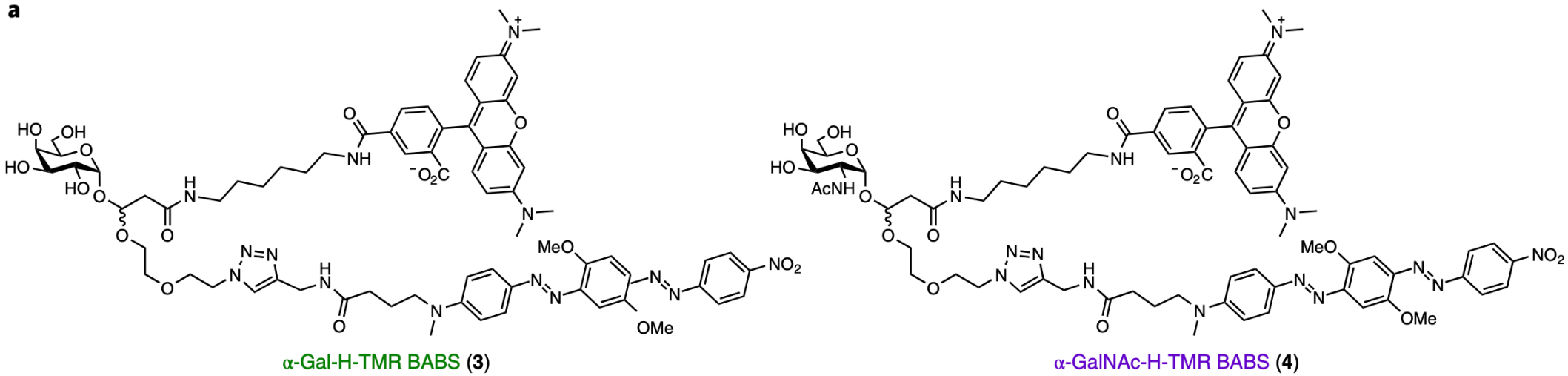
- Fixed substrate concentration, increasing concentrations of rec. enzymes (GALA, NAGAL)
- GalBABS: processed by both enzymes



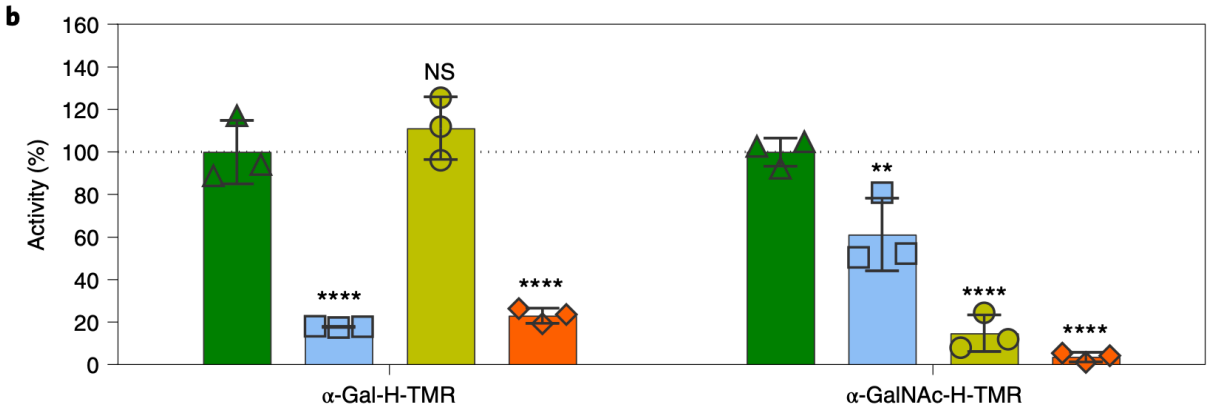


Monitoring of α-GALA and α-NAGAL activity in live cells

- Change to TAMRA (TMR) fluorophore and black hole quencher 2 (BHQ-2) more suitable for live-cell imaging
  - Excellent quenching
  - No spontaneous hydrolysis

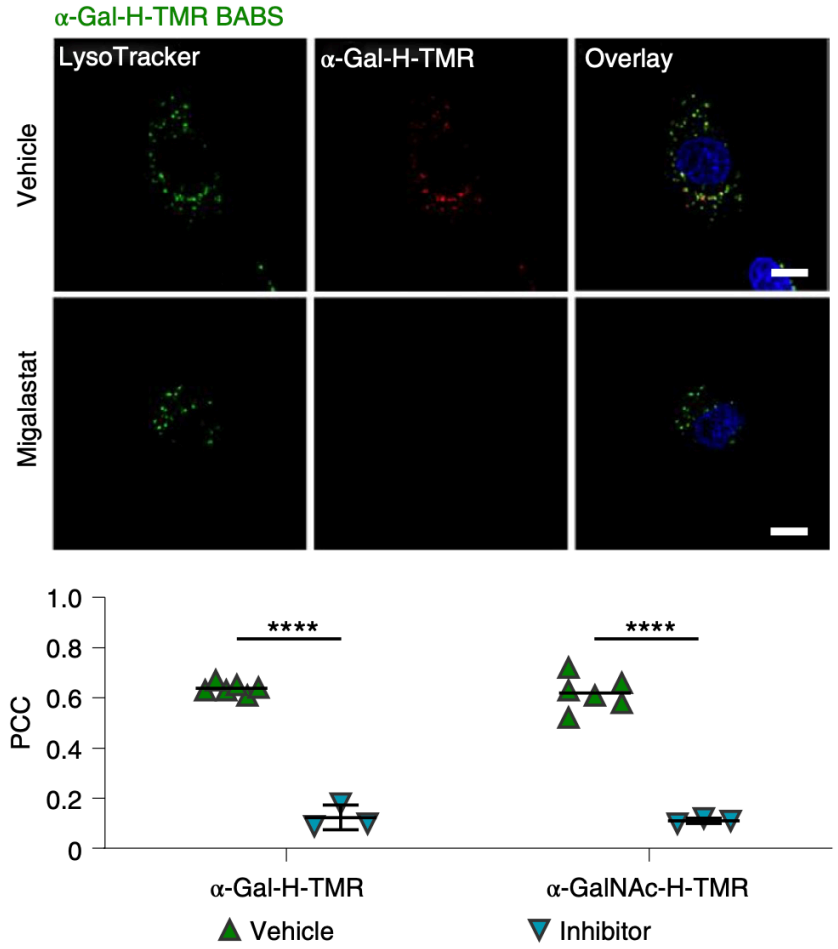


Monitoring of α-GALA and α-NAGAL activity in live cells



DMSO
Migalastat: GALA “inhibitor”
DGJNAc: NAGAL “inhibitor”

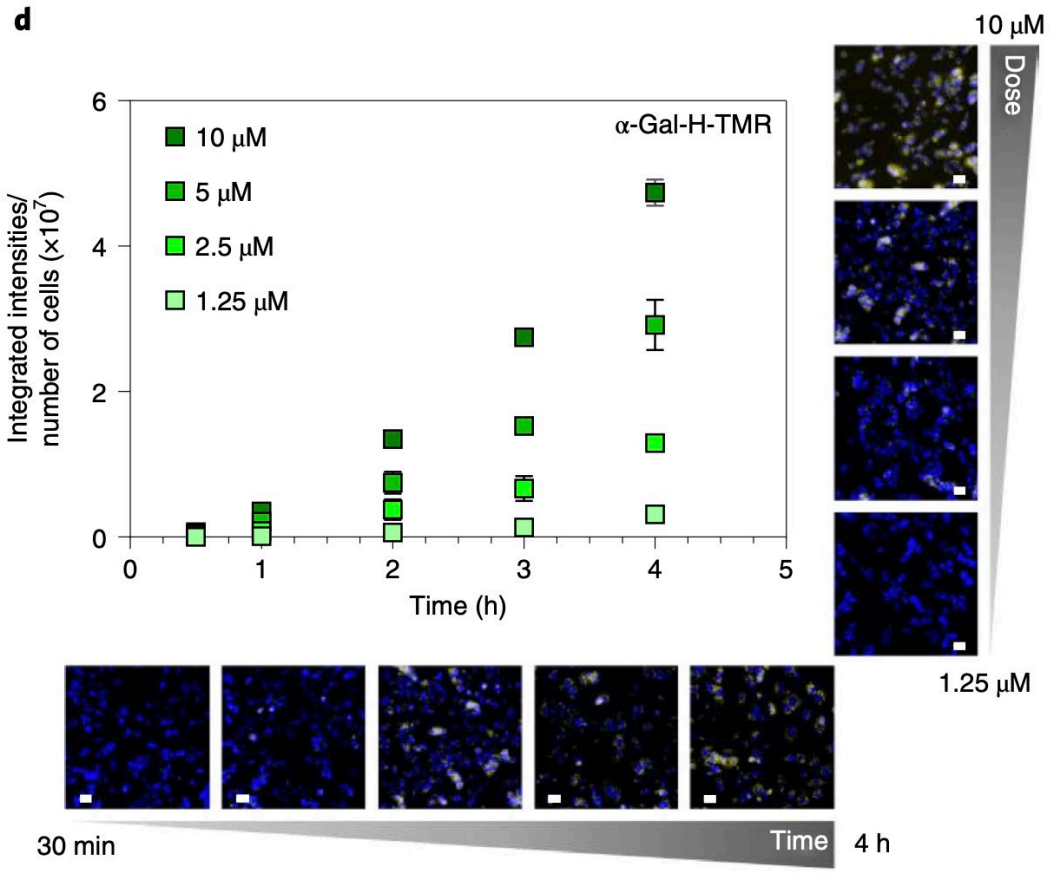
cross-inhibition of NAGAL by Migalastat previously reported in vitro



Co-localization with LysoTracker
Pearson’s correlation coefficient (PCC)

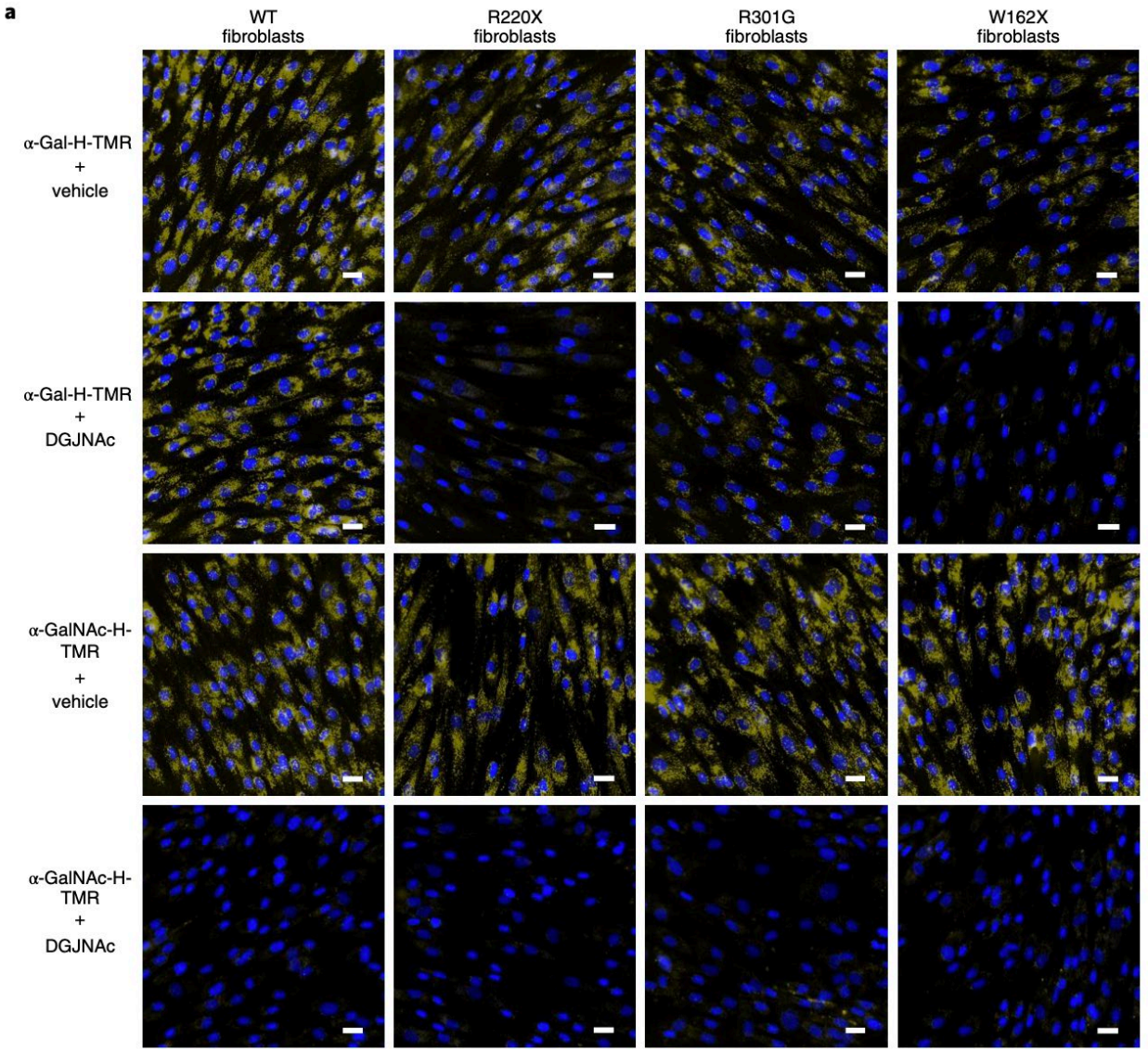
Monitoring of α-GALA and α-NAGAL activity in live cells

- **dose- and time-dependent linear turnover** of substrates in SK-N-SH cells
- short lag phase followed by a steady-state increase in fluorescence
- **No toxicity**, retention inside cells due to aldehyde group (stable signal over time)



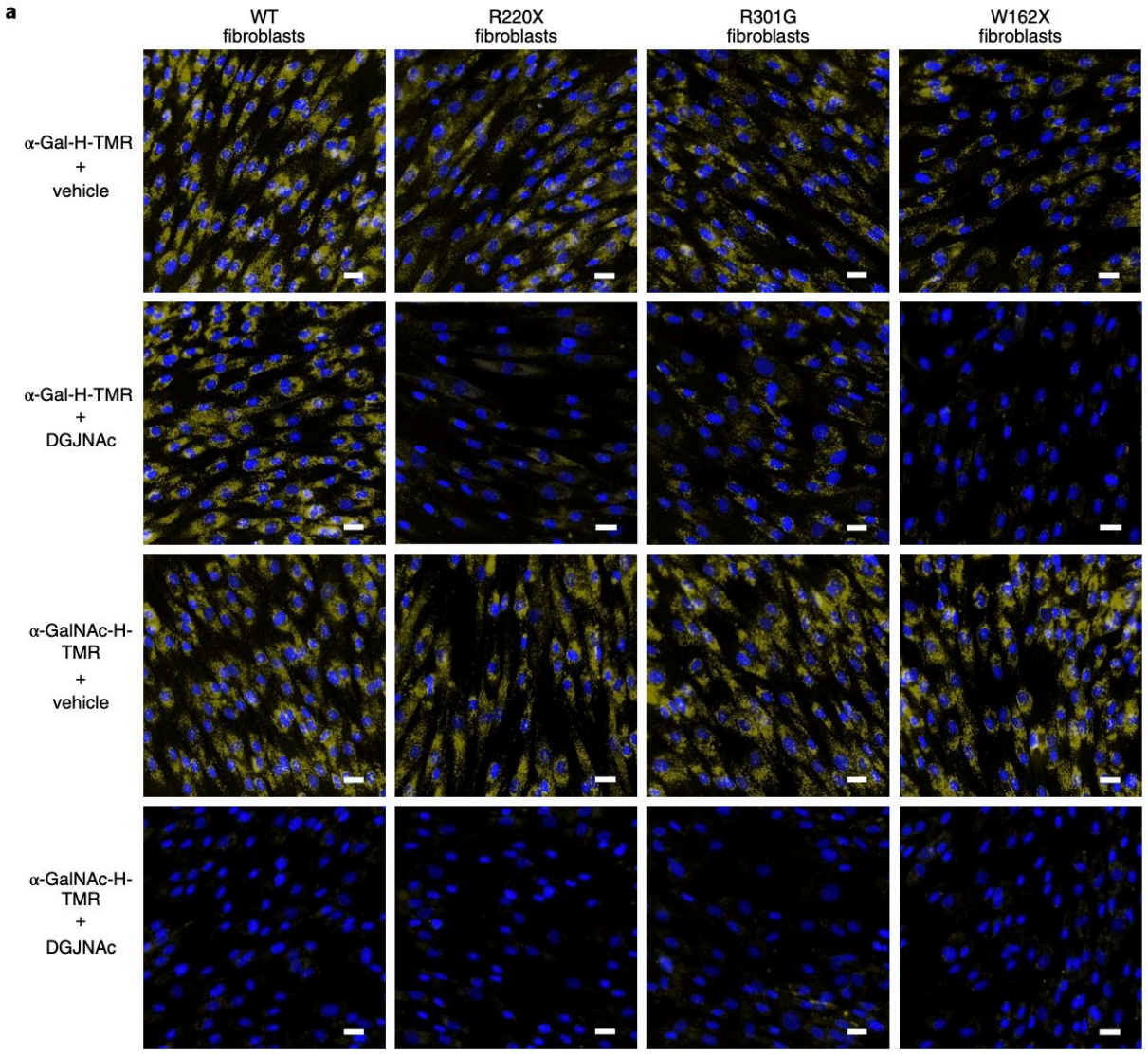
Glyco-BABSs quantify enzyme activity in patient fibroblasts

- Nonsense mutations in *GLA:* R220X and W162X
- Missense mutation: R301G reduced α-GALA activity
- Reduced activity (but still >20%) in all three mutant cell lines
- Reduced levels of α-GALA may manifest with increased α-NAGAL activity
- **Addition of DGJNAc**: no turnover of α-Gal-H-TMR in R220X or W162X cell lines



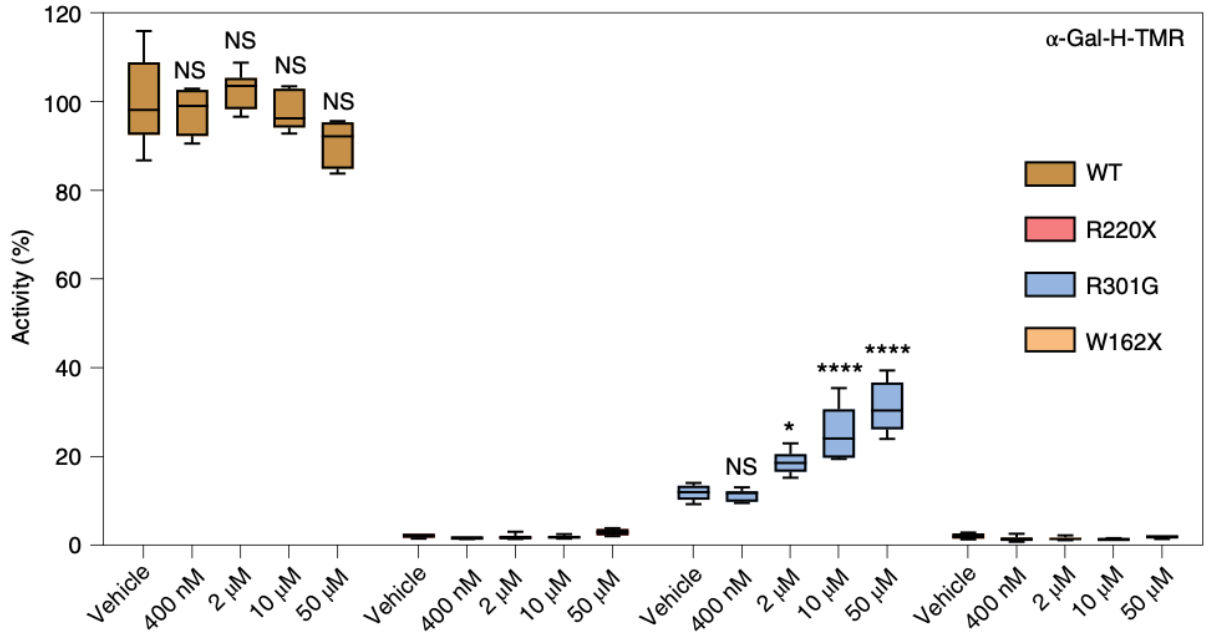
Glyco-BABSs quantify enzyme activity in patient fibroblasts

- DGJNAc can be used to selectively block α-NAGAL, ensures that substrate only reports on α-GALA activity
- treatment with migalastat and DGJNAc: complete blockade of substrate turnover in all fibroblast lines
  -> probes report only on α-GALA and α-NAGAL activity



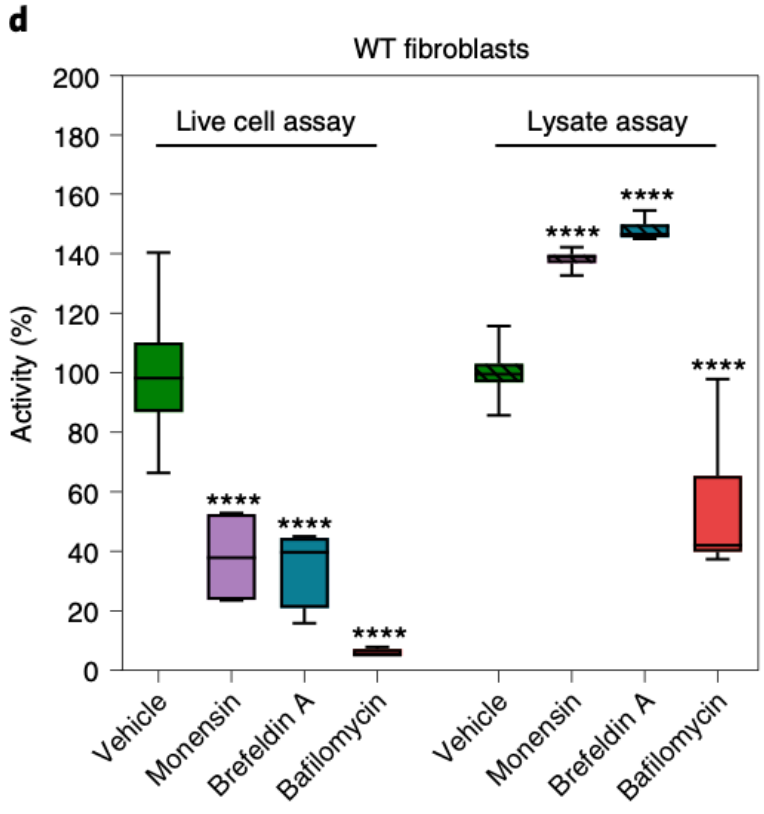
Measuring chaperoning in patient fibroblasts

- Treatment with migalastat for 4 days
- incubation with α-Gal-H-TMR and DGJNAc
- No effect in wt and nonsense mutation carrying fibroblasts
- **R301G fibroblasts**: dose-dependent increase in lysosomal α-GALA activity (31 ± 6% of WT for 50µM migalastat-treated cells)
- comparable to reports of chaperoning in R301G mutants using cell lysate assays



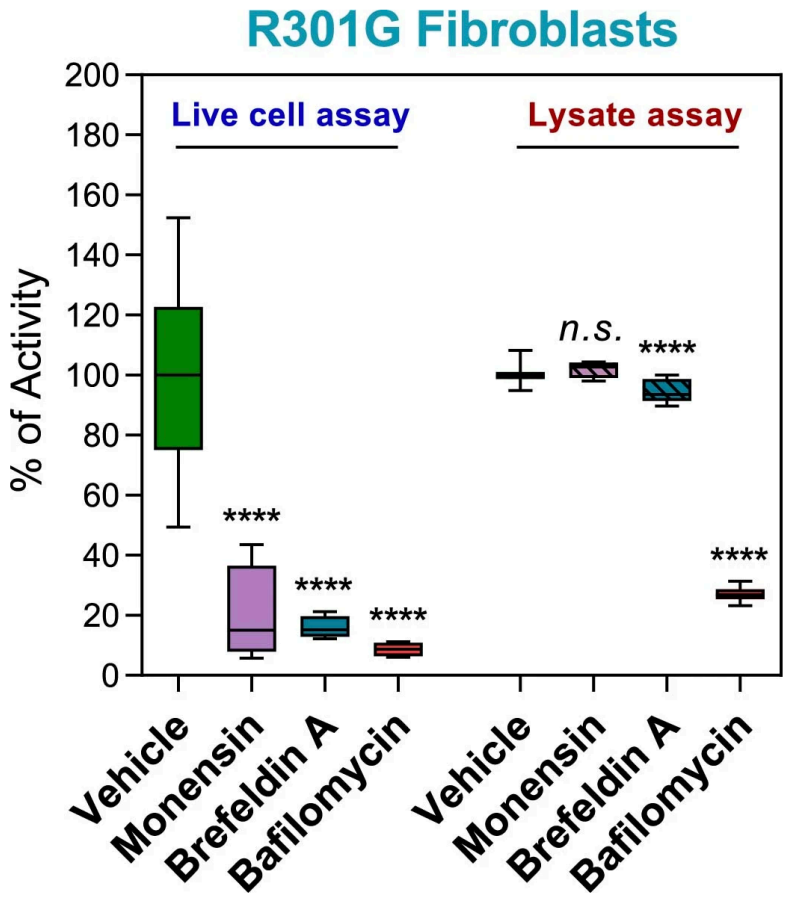
What about physiologically relevant measures of lysosomal enzyme activity that cannot be obtained using cell lysate assays?

- brefeldin A (BFA) and monensin (MON): inhibit trafficking through the secretory pathway to lysosomes
- bafilomycin (Baf): lysosomal deacidification
- **clear discrepancy** between lysate-based and in-vivo assay



What about physiologically relevant measures of lysosomal enzyme activity that cannot be obtained using cell lysate assays?

- lysate assays: no effect from treatment with BFA and MON
- decreased activity in response to Baf treatment, consistent with diminished activity due to deacidification



Conclusion

- standard lysate assays do not accurately reflect **impaired trafficking and decreased lysosomal activity** of α-GALA
- report on total cellular enzyme levels, including accumulated enzyme mislocalized within the secretory pathway due to mutations and perturbogens

Discussion

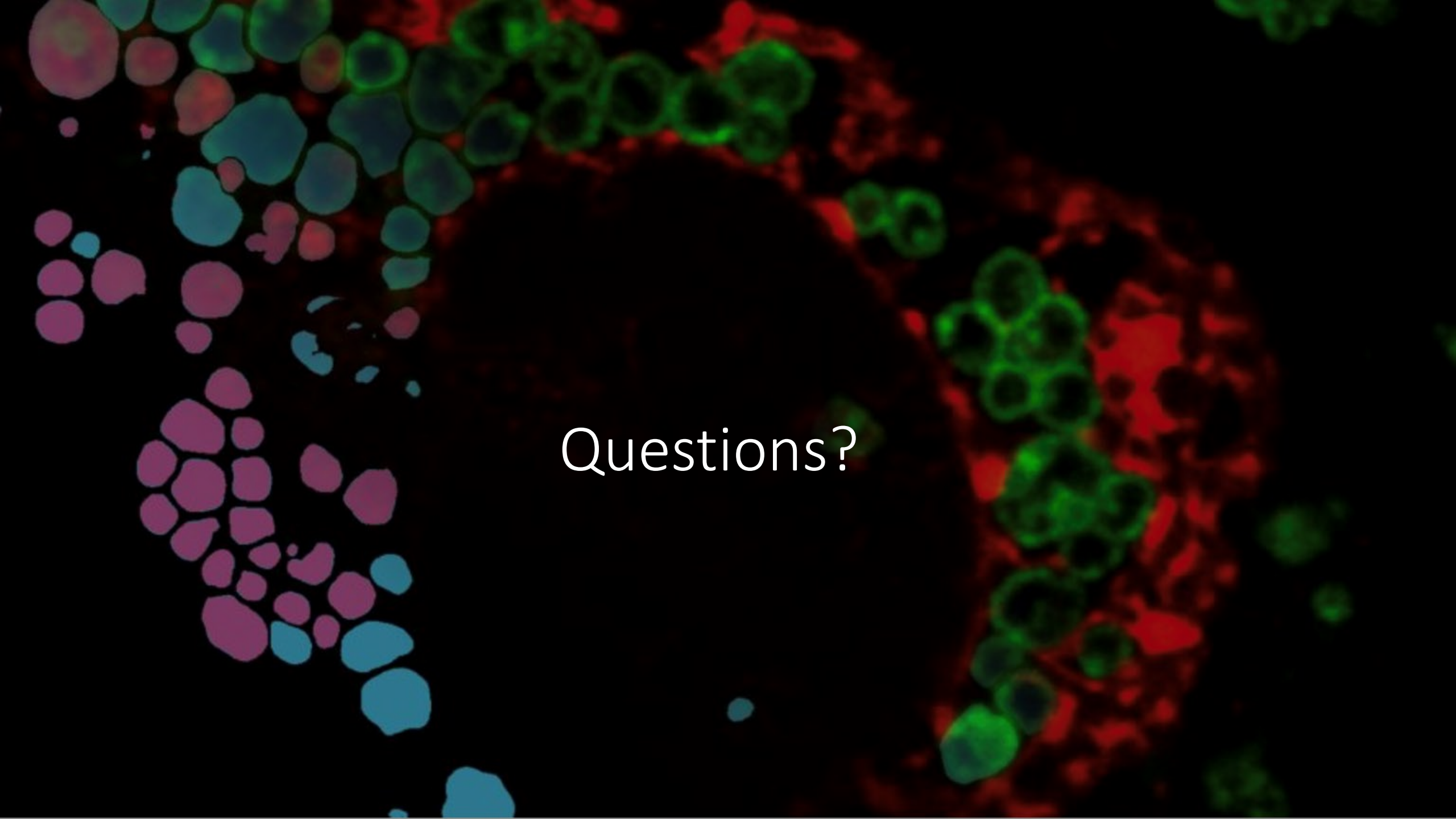
- BAB substrates enable linear **time- and dose-dependent** measurement of lysosomal enzymatic activity
- allow **quantitative studies** within cells
- permit the direct measurements of α-GALA and α-NAGAL activity **within the lysosomes of live cells**, capture effects of modifiers of enzymatic activity found within lysosomes

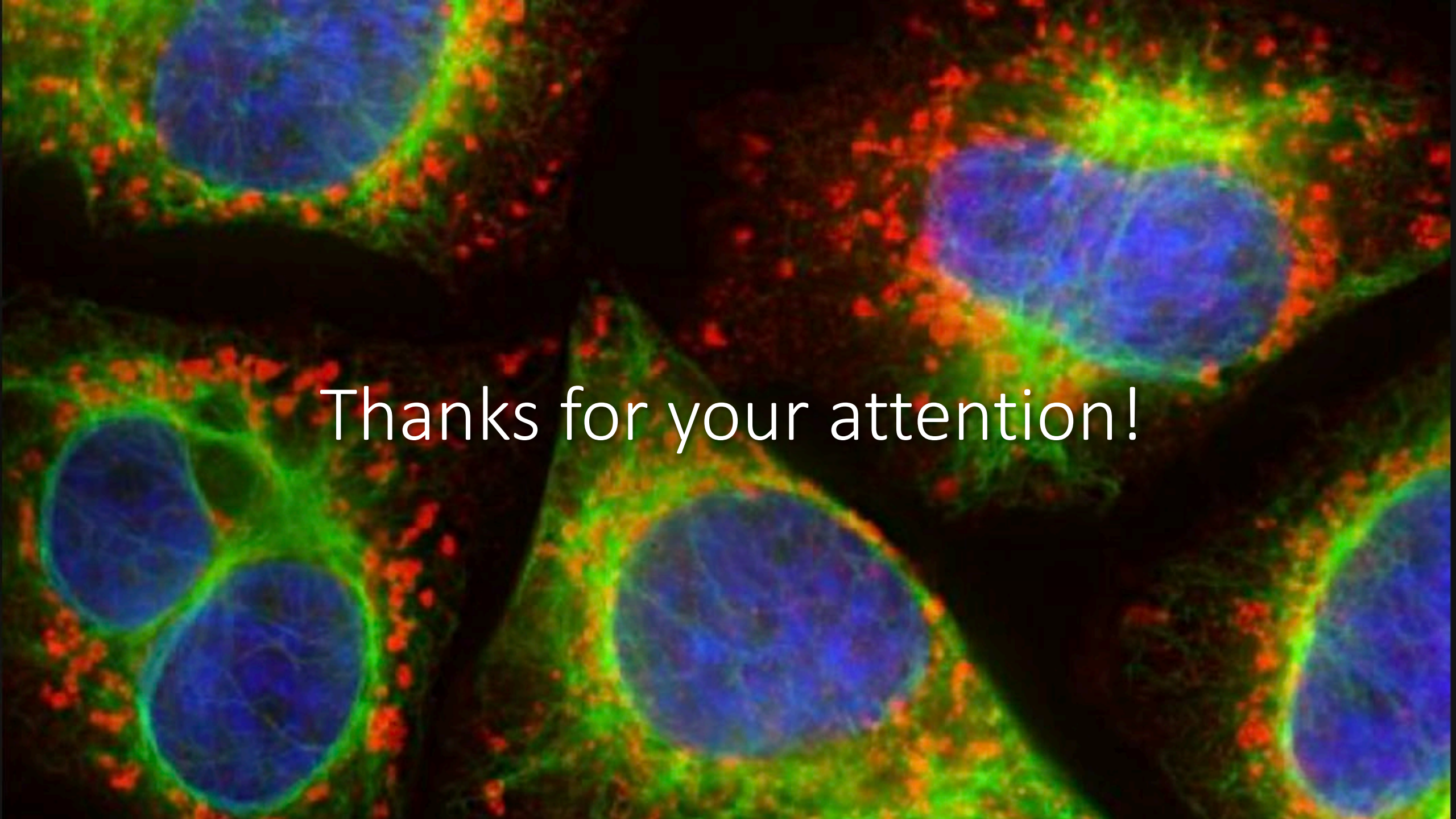
Discussion

- Cellular systems manifest considerable variability: collecting multiple replicates is necessary
  - Glyco-BABS substrates can be used in a 384-well microplate format
  - Extent of biological variability in cellular activity of enzymes needs further exploration
- products well **retained within lysosomes**: multicolor imaging (multiplexing) in conjunction with other small molecule/genetically encoded subcellular markers possible

Future Applications

- Might be used for **diagnostics and personalized medicine** approaches
  - Characterization of α-GALA activity in tissues from female heterozygous Fabry patients (random X inactivation: **mosaicism**)
  - Could enable clinicians to investigate cellular factors accounting for the poor correlation between *GLA* mutations and Fabry disease symptoms
  - aid development of new therapeutics: assess whether mutant enzymes are amenable to pharmacological chaperone therapies
- **allow high-throughput cell-based screening**
- development of additional BABS probes targeting other glycosidases

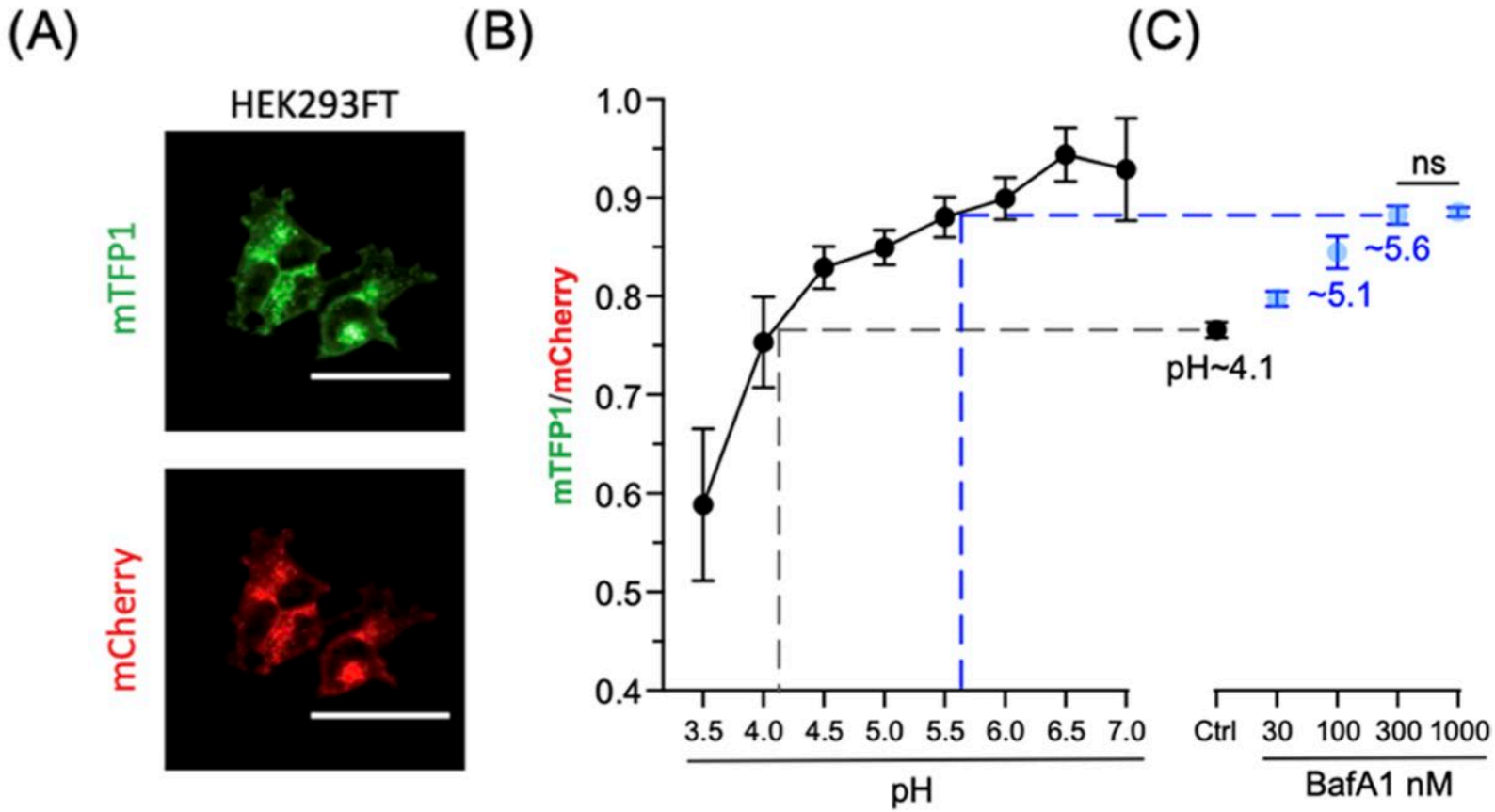

Questions?


Thanks for your attention!

Lysosomal characteristic	Methods	Comments
Total volume (number and size)	Fluorescence measurement (flow cytometry or fluorescence microscopy) of cellular staining of acidotropic dyes, such as LysoTracker dyes[92,215]	Simple to use but is not quantitative as stated by the manufacturer; can be adapted to clinical trial settings
	Western blot and fluorescence imaging of lysosomal markers such as LAMP1, LAMP2 etc.[216,217]	Simple but does not provide information on subcell populations[89]; can be adapted to clinical trial settings
	Electron microscopy[218]	Provides morphological information but laborious and semiquantitative
Biogenesis and activation status	Western blot and qPCR of TFEB (and also other family members)[219,220]	Simple but does not provide information on subcell populations; can be adapted to clinical trial settings
	Fluorescence imaging of the nuclear translocation of TFEB-GFP[219]	Limited usage in primary cells as they are hard to transfect
pH	Ratiometric fluorescence measurement with LysoSensor Yellow/Blue[92,221] or Oregon-Green 488 dye[222]	The dyes can have an alkalinizing effect on lysosomes and affect the accuracy of results[223]
Degradation ability	Fluorescence measurement of the degradation of labelled BSA (DQ-BSA Green/Red)[57]	Requires loading of BSA molecules to lysosomes by endocytosis and could potentially interfere with normal lysosomal function[224]
Protease expression	Western blot measurement of cathepsins[92], thiol reductase etc.	Simple but does not provide information on subcell populations; can be adapted to clinical trial settings
Protease activity	Fluorescence measurement of the cleavage of cathepsin substrates by Magic Red Cathepsin (B, K and L) kit[225]	N/A
Membrane stability	Membrane stability assay with acridine orange[226]	Phototoxic and stains nucleus as well[227]
Membrane integrity	Lysosomal galectin puncta assay[224]	N/A
	Cell fractionation to detect lysosomal content in cytosol[216]	Limited sensitivity as it fails to detect small amounts of lysosomal content[224]
Local calcium level	Live cell imaging of genetically encoded Ca^{2+} indicator: GCaMP3-ML1[34]	Limited usage in primary cells as they are hard to transfect

BSA, bovine serum albumin; LAMP, lysosome-associated membrane protein; N/A, not available; qPCR, quantitative PCR; TFEB, transcription factor EB.

Functional validation in different cell lines



PFA-fixed cells

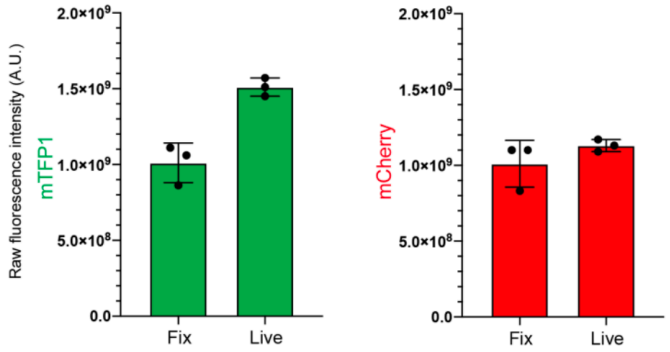


Fig S5. Fixed- and live-cell fluorescence measurements for mTFP1 and mCherry FPs. Raw mTFP1 and mCherry fluorescence intensities measured from FIRE-pHLy-expressing HEK293FT cells that were either imaged live (in culture media, pH 7.4) or post-PFA fixation (in PBS, pH 7.4). Data points are presented as mean ± S.D., from 3 independent wells; n = ~5,000 quantified cells per well.

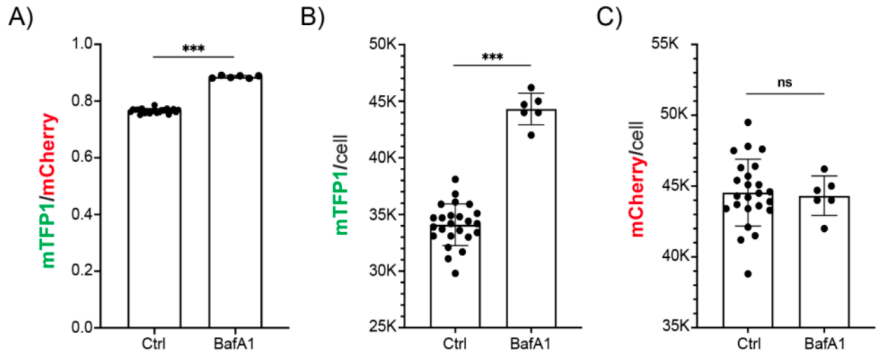
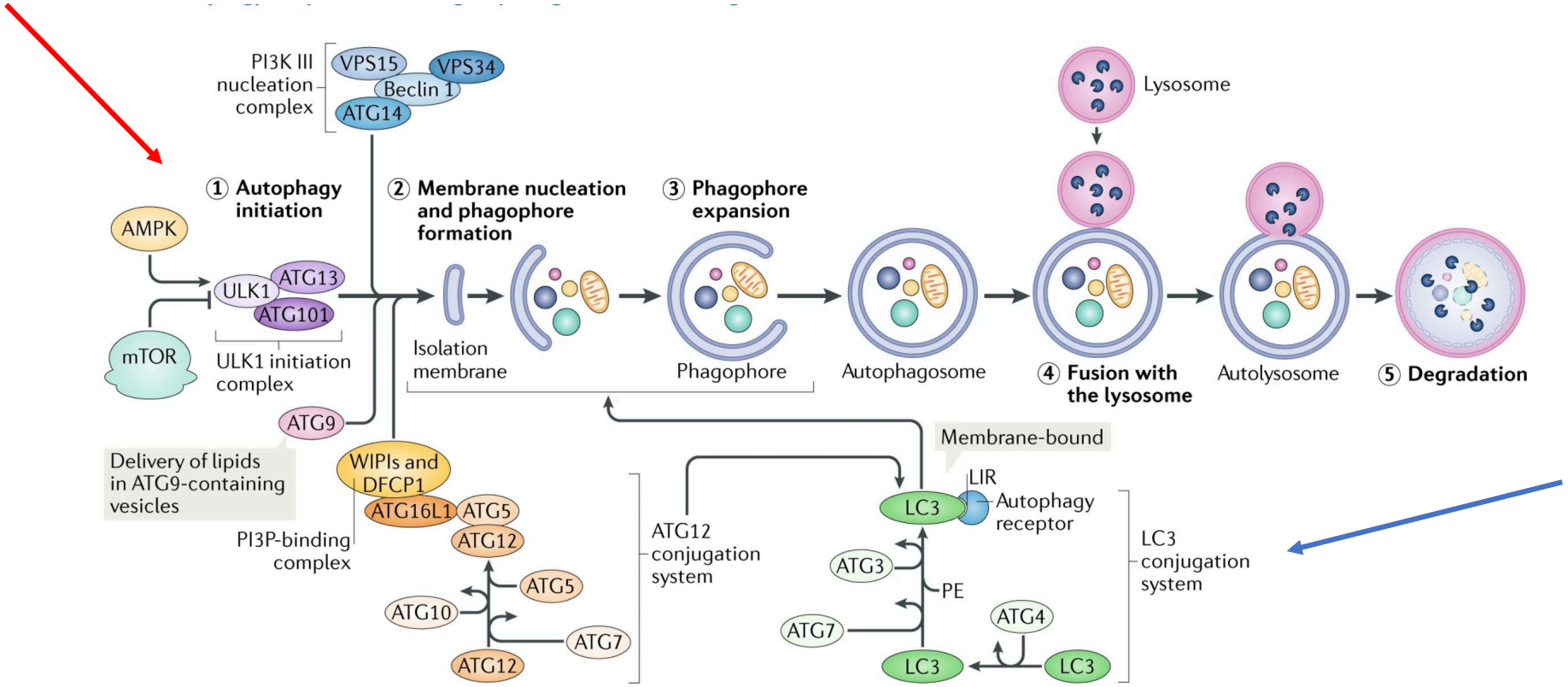


Fig S6. Ratiometric validation of individual FIRE-pHLy fluorophores under BafA1 conditions. (A) mTFP1/mCherry ratio quantified from FIRE-pHLy-expressing HEK293FT cells treated with 1 µM bafilomycin for 6 hours compared to 0.1% DMSO solvent control. (B) mTFP1 mean fluorescence intensity normalized by cell count. (C) mCherry mean fluorescence intensity normalized by cell count. Data points are presented as mean ± S.D., from 6 independent replicates; n=quantified 7,500 cells per replicate. Statistical analysis was performed using two-tailed, unpaired Welch's t-test for unequal variances. ***$p \leq 0.001$; ns = not significant.

Do the compounds activate autophagy?

- mTORC1 negatively regulates autophagy through phosphorylation of Unc-51-like autophagy activating kinase (**ULK1**)
- Microtubule-associated protein light chain 3B (**LC3B**): conversion of LC3B-I to LC3B-II correlated with autophagosomes/reflects autophagy activation
- **p62:** autophagic cargo adaptor shuttled into lysosomes during autophagy



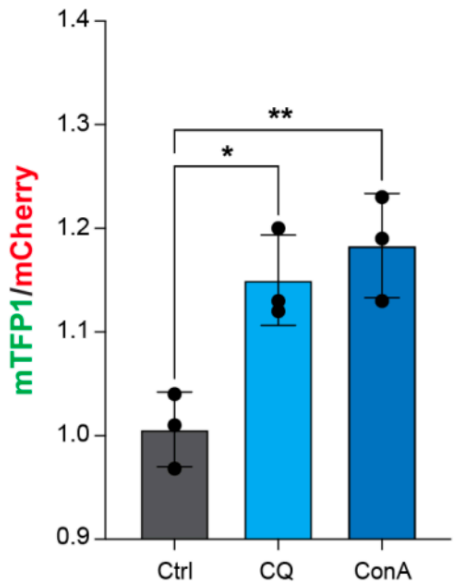


Fig S7. pH elevation with lysosomal pharmacological inhibitors.
Ratiometric measurements (mTFP1/mCherry) taken from FIRE-pHLy-expressing HEK293FT cells treated with 0.1% DMSO (Ctrl), 30 μM chloroquine (CQ) and 0.5 μM concanamycin A (ConA) for 6 hours before fixation. Data points are presented as mean ± S.D., from 3 independent wells; n = ~5,000 quantified cells per well. Statistics were conducted with one-way ANOVA for multiple comparisons. *$p \leq 0.05$; **$p \leq 0.01$.