# Identification of novel targets for autophagy inhibition in pancreatic ductal adenocarcinoma


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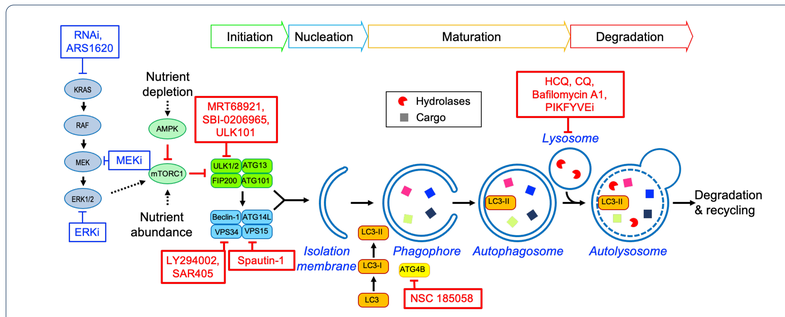
## Abstract

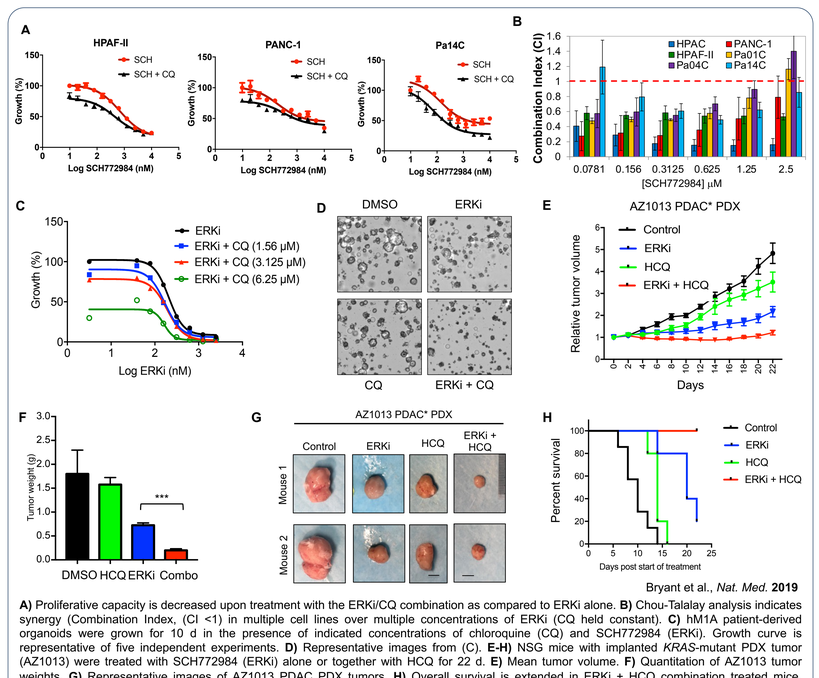

Pancreatic ductal adenocarcinoma (PDAC) is a deadly disease with a 5-year survival rate of 10%. There are multiple current directions for new PDAC treatments, one of which is perturbing the altered metabolic demands associated with tumorigenicity. One such metabolic process is autophagy, or "self-eating", which is a lysosomal-mediated process whereby cells degrade and recycle damaged organelles and macromolecules in order to sustain growth. Autophagy can be divided into four distinct phases: initiation, nucleation, maturation, and degradation. Our lab recently demonstrated that PDAC cells become more dependent on autophagy following inhibition of the RAF-MEK-ERK mitogen-activated protein kinase cascade. This dependency can be therapeutically targeted by combining autophagy inhibitors such as hydroxychloroquine (HCQ) with ERK and MEK inhibitors. Currently, HCQ is the only clinically used autophagy inhibitor; however, it is limited by low potency and lack of specificity. In order to better understand how to improve HCQ treatment, we performed a CRISPR/Cas9-mediated genetic loss-of-function screen in the presence of a related analog, chloroquine (CQ), to identify combination drug treatments that enhance anti-proliferative activity. Interestingly, several top hits were genes that encode proteins upstream in the autophagic pathway. To validate these results, I have begun treating PDAC cells with anti-autophagy combinations, using inhibitors against specific nodes of the autophagy pathway including ULK (initiation), VPS34 (nucleation), and lysosomal acidification (degradation). Preliminary data suggests that vertical inhibition of the autophagy pathway results in a further reduction of autophagic flux relative to inhibition of any single node and can also synergistically kill PDAC cells. Ongoing studies are aimed at understanding compensation to ULK monotherapy and elucidating the mechanism underlying the synergy observed with anti-autophagy inhibitor combinations.


## Pharmacologic inhibitors of autophagy




Multiple phases of autophagy are shown. ULK activation by AMPK triggers the first phase, initiation. In the nucleation phase, ULK phosphorylates Beclin-1, activating VPS34, a class II PI3K and stimulating phagophore formation. During maturation, LC3 is attached to autophagosome membranes, this is exploited to label autophagosomes in vitro. CQ inhibits lysosomal acidification and inhibition of PIKFYVE perturbs lysosomal dynamics. Blue/white boxes: perturbations that increase autophagy; red boxes: inhibitors of autophagy.

## ERKi and chloroquine combination synergistically inhibits PDAC tumor growth

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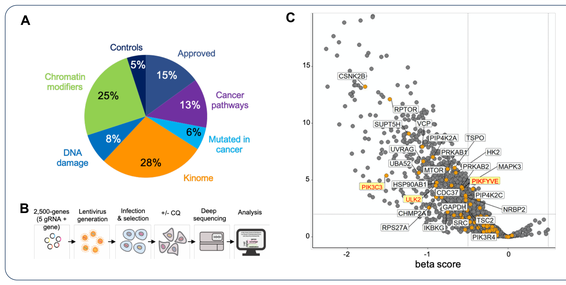
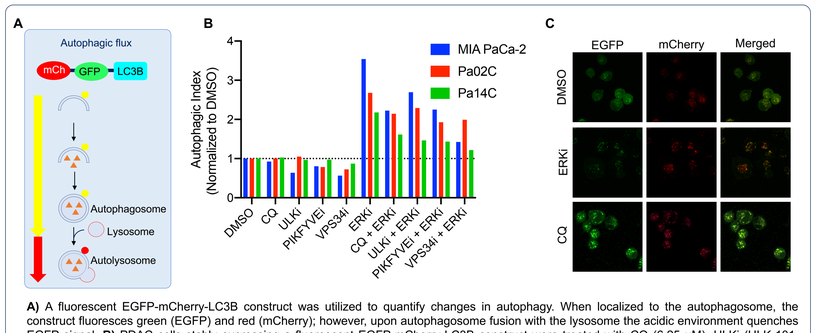
**A)** Proliferative capacity is decreased upon treatment with the ERKi/CQ combination as compared to ERKi alone. **B)** Chou-Talalay analysis indicates synergy (Combination Index, (CI <1) in multiple cell lines over multiple concentrations of ERKi (CQ held constant). **C)** hM1A patient-derived organoids were grown for 10 d in the presence of indicated concentrations of chloroquine (CQ) and SCH772984 (ERKi). Growth curve is representative of five independent experiments. **D)** Representative images from (C). **E-H)** NSG mice with implanted *KRAS*-mutant PDX tumor (AZ1013) were treated with SCH772984 (ERKi) alone or together with HCQ for 22 d. **E)** Mean tumor volume. **F)** Quantitation of AZ1013 tumor weights. **G)** Representative images of AZ1013 PDAC PDX tumors. **H)** Overall survival is extended in ERKi + HCQ combination treated mice.

## CRISPR/Cas9 genetic loss-of-function screen identifies autophagy-related genes whose loss sensitize PDAC cells to CQ treatment



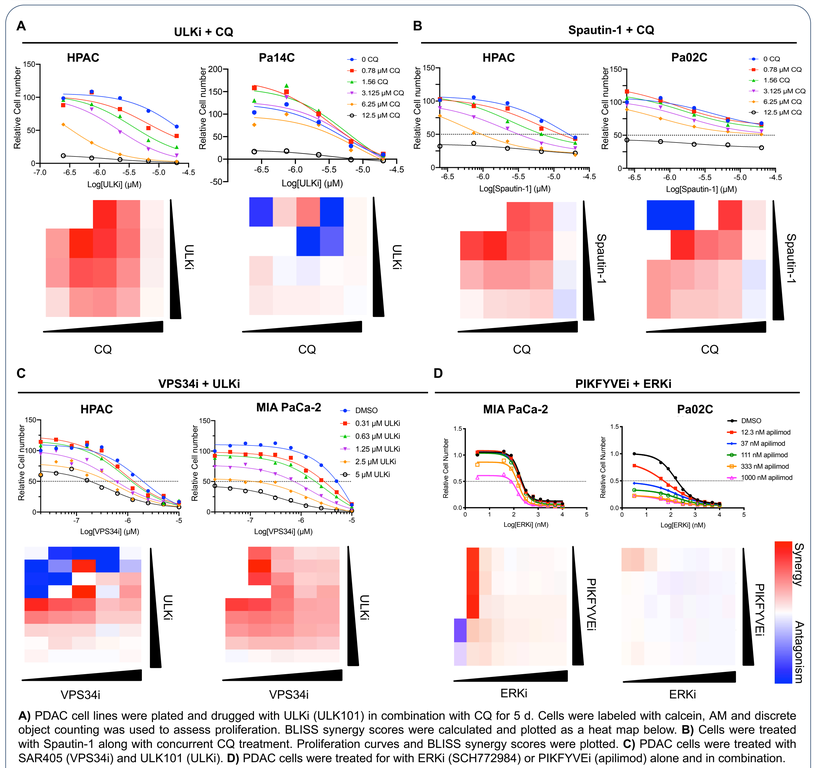

**A)** Compounds/pathways targeted in the Druggable Genome library (2,500 genes, 5 sgRNAs/gene; provided by K. Wood, Duke). **B)** Experimental workflow of CRISPR/Cas-9 mediated loss-of-function screen in presence of CQ. **C)** Volcano plot highlighting genes whose loss reduced growth of vehicle treated PDAC cells. Autophagy-related genes are labeled in orange; PIKFYVE is highlighted.

## Compounds targeting specific nodes of the autophagy pathway are capable of inhibiting ERKi-induced autophagic flux



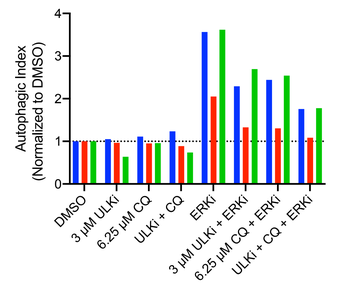

**A)** A fluorescent EGFP-mCherry-LC3B construct was utilized to quantify changes in autophagy. When localized to the autophagosome, the construct fluoresces green (EGFP) and red (mCherry); however, upon autophagosome fusion with the lysosome the acidic environment quenches EGFP signal. **B)** PDAC cells stably expressing a fluorescent EGFP-mCherry-LC3B construct were treated with CQ (6.25 μM), ULKi (ULK-101, 3μM) PIKFYVEi (apilimod, 100 nM), and VPS34i (SAR405, 1 μM) alone and in combination with ERKi (SCH772984, 1 μM). Following treatment, whole cell fluorescence was measured by flow cytometry and the autophagic index (mCherry/EGFP) was normalized to DMSO control and plotted. **C)** Representative images of cells quantified in **B**.

## Autophagy inhibitor combinations synergistically inhibit PDAC cell proliferation



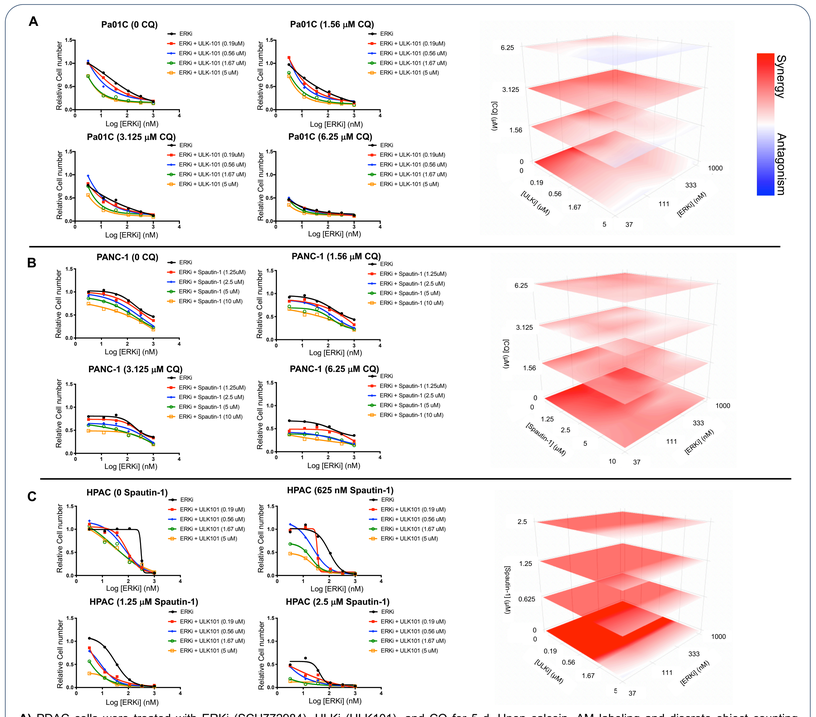

**A)** PDAC cell lines were plated and drugged with ULKi (ULK101) in combination with CQ for 5 d. Cells were labeled with calcein, AM and discrete object counting was used to assess proliferation. BLISS synergy scores were calculated and plotted as a heat map below. **B)** Cells were treated with Spautin-1 along with concurrent CQ treatment. Proliferation curves and BLISS synergy scores were plotted. **C)** PDAC cells were treated with SAR405 (VPS34i) and ULK101 (ULKi). **D)** PDAC cells were treated for with ERKi (SCH772984) or PIKFYVEi (apilimod) alone and in combination.

## Dual combination autophagy inhibitors can further reduce ERKi-induced flux relative to single agent treatment




PDAC cells stably expressing fluorescent EGFP-mCherry-LC3B were treated with ULKi (ULK101) and CQ alone and in combination. Following this, autophagic flux was induced with ERKi and inhibited with ULKi and CQ alone and in combination. Whole-cell fluorescence was measured with flow cytometry and autophagic index (mCherry/EGFP) was plotted.

## Vertical inhibition of the autophagy pathway synergizes with ERKi to further reduce PDAC cell proliferation




**A)** PDAC cells were treated with ERKi (SCH772984), ULKi (ULK101), and CQ for 5 d. Upon calcein, AM labeling and discrete object counting proliferation curves were plotted and triple combination BLISS synergy scores were calculated and plotted as heatmaps. Concentrations of ERKi are along the x-axis, ULKi along the y-axis, and CQ along the z-axis. **B)** PDAC cells were treated with combinations of ERKi, Spautin-1, and CQ and analyzed as in **A**. **C)** PDAC cells were treated for 5 d with ERKi, Spautin-1, and ULKi and analyzed as in **A**.

## Summary and Future Directions

- Inhibitors of the ERK MAPK cascade render KRAS-mutant PDAC addicted to autophagy; thus response to autophagy inhibitor treatment is enhanced.
- Vertical inhibition of the autophagy pathway results in enhanced anti-proliferative effects and an increased reduction in autophagic flux.
- Combined inhibition of multiple nodes of the autophagy pathway further reduces ERKi-induced autophagic flux, and synergistically enhances the anti-proliferative effects of the combination.
- Efforts to elucidate the mechanism underlying the synergy observed with these combinations are ongoing.

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