Regulation of Chaperone-Mediated Autophagy by PI3K Signaling

Zachary Ziemba

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Abstract

Chaperone-mediated autophagy (CMA) is a mechanism by which individual proteins are selectively bound to chaperones to be translocated across and degraded in the lysosome. We have previously reported that long-lived mouse models have been known to have constitutive upregulation of CMA and also increased AKT phosphorylation. GFAP is one of the only known regulators of chaperone-mediated autophagy, which itself is regulated by AKT. We have found that inhibiting the Insulin/PI3K/AKT pathway by using PI3K inhibitors and PDK-1 inhibitors decreases both AKT and subsequent GFAP phosphorylation *in vitro* and *in vivo*. We confirmed that this pathway stimulates chaperone-mediated autophagy independently of other autophagic pathways such as macroautophagy. Lastly, we also report that AMPK has been found to play a role of regulating CMA via the Insulin/PI3K/AKT pathway by phosphorylating IRS-1.

Introduction

One strain of mice with diminished growth hormone (GH) signaling is the Snell Dwarf mouse which has a drastic reduction in size. Changes to growth hormone lead to a cascade of further changes in an organism's endocrinology. These mutants have decreased insulin-like growth factor (IGF-1) signaling, lower rates of cancer & diabetes, and an increase in lifespan by approximately 40% [1]. We have previously reported that the Snell Dwarf and the GH receptor knockout mouse stocks have constitutive upregulation of a selective form of autophagy known as chaperone-mediated autophagy (CMA) [2]. Chaperone-mediated autophagy is a cellular process whereby individual proteins are selectively bound by chaperones and degraded in the lysosome after being translocated across the lysosomal membrane [3].

Chaperone-mediated autophagy has been found to play several important roles in the cell. It was first found to be upregulated during prolonged starvation to recycle amino acids [4], a role similar to other lysosomal degradation pathways like macroautophagy. Unlike macroautophagy, the selective nature of chaperone-mediated autophagy enables it to remove oxidized and toxic proteins without disturbing neighboring proteins [5]. It also helps modulate cellular processes by regulating the levels of enzymes and transcription factors [6]. As a result of the roles of CMA, its dysfunction and misregulation have been implicated in several pathogeneses that are associated with age. [7]. Neurodegeneration is a common pathology in old age that refers to the functional and physical loss of neurons. Alzheimer's disease is largely caused by the aggregation of mutant MAPT/Tau protein which forms neurofibrillary tangles and amyloid plaques. Normally, Tau protein is degraded by CMA while mutant Tau is not able to be entirely internalized for degradation [8]. The two most common proteins mutated in patients with Parkinson's disease, α-synuclein and leucine-rich repeat kinase 2 (LRRK2), have been found to be substrates of CMA [9]. Deficiencies in CMA have been linked to hepatosteatosis [10], Parkinson's disease, kidney hypertrophy, and several lysosomal storage disorders [11]. Because of the importance of CMA in cellular homeostasis and in aging-related disease pathogenesis, it is possible that it may play a role in longevity and healthspan.

The current model of chaperone-mediated autophagy starts with cytosolic heat-shock chaperone (HSC70) binding a pentapeptide motif of all substrate proteins, which is necessary [12] and sufficient [13] for CMA. This motif consists of glutamine (Q) followed by a sequence of a basic, an acidic, a bulky hydrophobic, and then a repeated basic or bulky hydrophobic residue which most often takes the form of KFERQ [14]. HSC70 and the substrate protein form a chaperone complex along with the cochaperones of HIP, HOP, HSP40, HSP90, and BAG-1 [15]. This chaperone complex brings substrate proteins to a splice variant of a heavily glycosylated type I integral membrane protein known as lysosomal-associated membrane protein type 2A (LAMP2A) [16]. The monomers of LAMP2A undergo a multimerization process after binding the chaperone-substrate complex to form a translocation complex that seems to involve at least two other proteins [17][18]. The LAMP2A complex works with lumenal HSC70 to unfold and translocate these proteins across the membrane for degradation by proteolytic enzymes [19].

Regulation of chaperone-mediated autophagy is currently understood to occur at the molecular level through one primary mechanism. CMA is inhibited by a lysosomal population of the mammalian target of rapamycin complex 2 (mTORC2) that phosphorylates AKT at threonine 308 (T308) in the activation loop and serine

473 (S473) in the C-terminal hydrophobic motif [20]. AKT is a kinase in the Insulin/PI3K/AKT growth signaling axis, which is activated by phosphorylation [21]. One of AKT's many targets is glial fibrillary acidic protein (GFAP), the primary positive regulator of CMA. Unphosphorylated GFAP binds LAMP2A to stabilize the multimerization into the translocation complex. When GFAP is phosphorylated by AKT, it dissociates from LAMP2A to instead form a complex with elongation factor 1 alpha (EF1α), which destabilizes the rate-limiting step of CMA in substrate translocation [22].[23]

Here we report that the use of class I PI3K inhibitors are able to activate chaperone-mediated autophagy in multiple cell lines via several markers of CMA. These markers include decreased GFAP phosphorylation and quantification of a fluorescent reporter of CMA activity. We also report that these results have been recapitulated in a heterogenous group of wildtype and Snell dwarf mice.

Results

Earlier work in the Miller lab has found that wild-type mice which are fed after being fasted show upregulation of AKT phosphorylation at the S473 residue while Snell dwarf mice do not show the typical robust increase in AKT phosphorlyation, when they are re-fed after a period of food deprivation (Figure 1A). Akt is activated by this phosphorylation, which implies that GFAP may be phosphorylated downstream. To directly measure the effect this has on CMA, we purified lysosomes from Snell and littermate control mice (Figure 1B). From there, we repeated the fasting and feeding procedures on both Snell dwarf and wild-type mice. In either the fasted or fed animals, Snell dwarf mice have reductions in lysosomal GFAP phosphorylation when compared to control mice (Figure 1C). This genotype effect is significant in the difference of phosphorylated GFAP levels unlike the fasting effect. We are unsure as to why the AKT was not found to be affected between Snell and wild-type as it should be upregulated with the increase in GFAP phosphorylation. Similarly, when we purified lysosomes from growth hormone receptor knockout (GHKRO) and liver-specific growth hormone receptor knockout (LiGHRKO) mice, we found that GFAP phosphorylation was decreased in the former (Figure 1D) and was unchanged in the latter (Figure 1E). This suggests that global reductions in growth hormone signaling are required to result in increases in CMA activity.

To further investigate the possibility of AKT regulating GFAP phosphorylation and CMA on the lysosome, we looked back at the Insulin/PI3K/AKT growth signaling axis to find ways of inhibiting AKT. Upstream in the pathway, class 1 phosphoinositide 3-kinases (PI3Ks) act to phosphorylate the 3'-hydroxyl groups of the phosphatidylinositol embedded in the plasma membrane, PTDINS(4,5)P_2, converting it to PTDINS(3,4,5)P_3 [24]. This membrane protein acts as a secondary messenger that allows binding of proteins with a pleckstrein homology (PH) domain. Specifically, PDK-1, AKT, and mTORC2 contain PH domains that bind to PTDINS(3,4,5)P_3 enabling phosphorylation of AKT [25].

To ensure that our inhibition of elements of the Insulin/PI3K/AKT pathway were genuine instead of due to other pathways those elements may be a part of, we decided to target both PI3K and PDK-1. We originally used three class I PI3K inhibitors (buparlisib, pictilisib, and copanlisib) and three PDK-1 inhibitors (BX795,

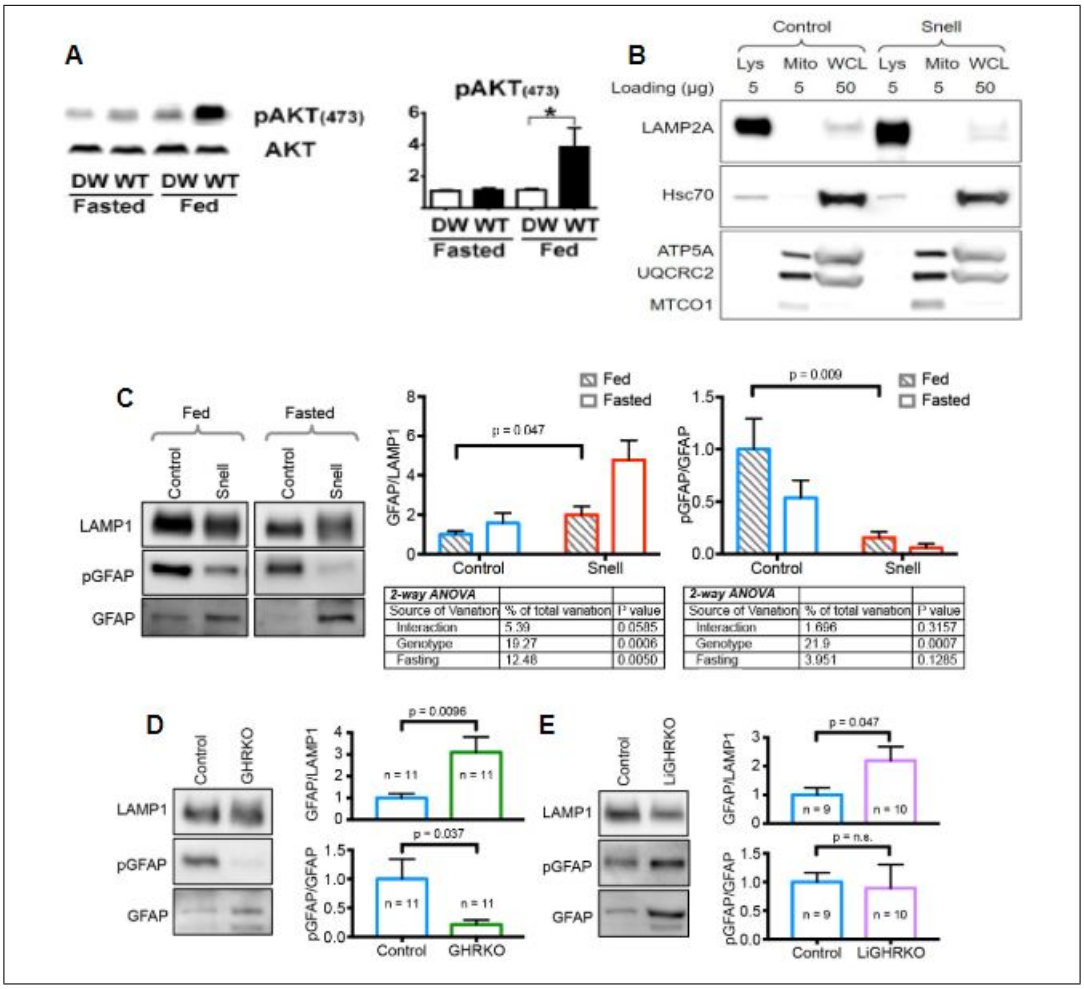

Figure 1: "AKT and GFAP phosphorylation in Snell Dwarf vs. WT mice"

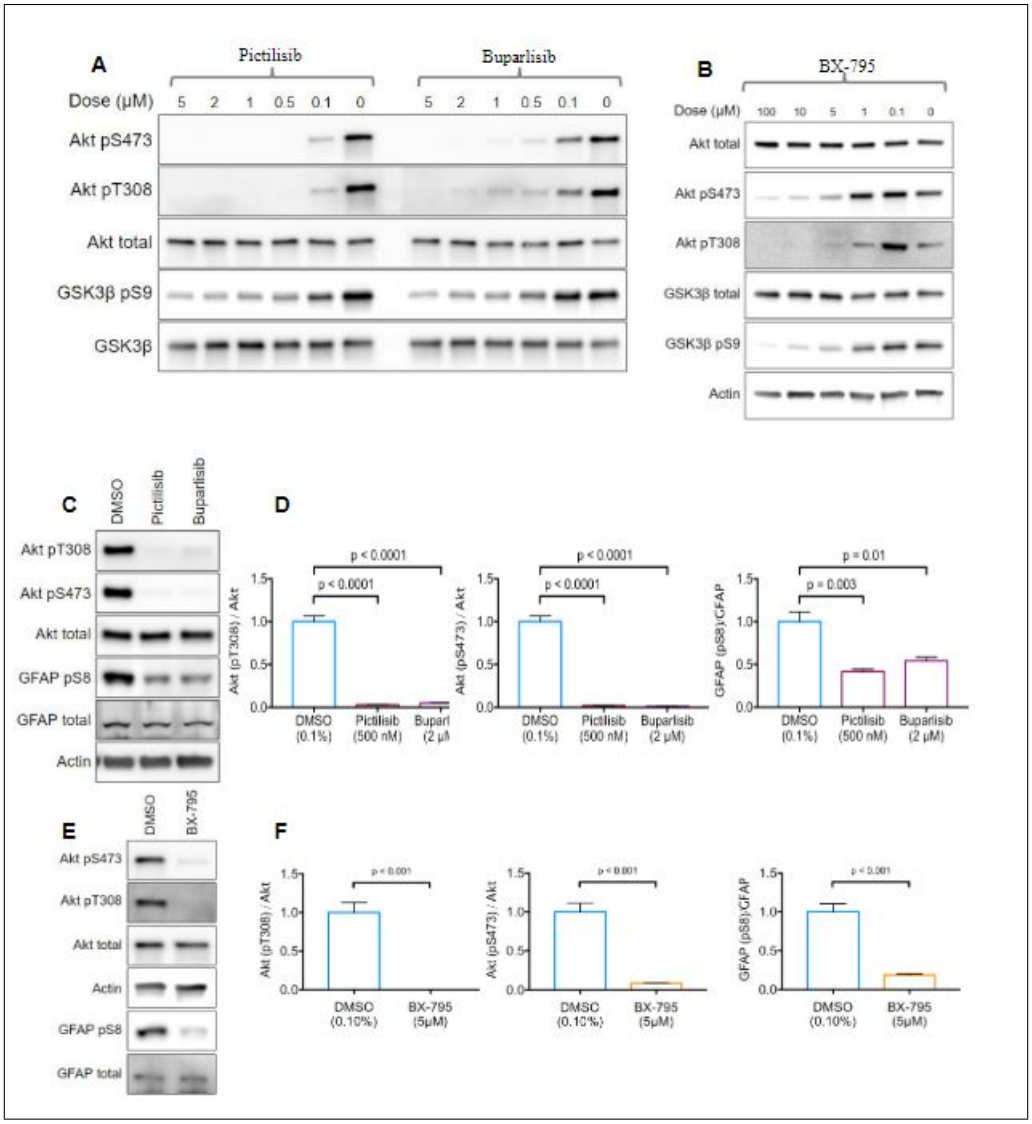

Figure 2: "Class 1 PI3K Inhibitors and PDK-1 Inhibitors Affect GFAP Phosphorylation"

BX912, and GSK2334470). We obtained similar results for all of these drugs, but only continued on with buparlisib and pictilisib in subsequent experiments. For those two as well as BX795, we began by finding the optimal dosage of our PI3K inhibitors (Figure 2A) and our PDK-1 inhibitor (Figure 2B) by looking for a dosage that completely ablated AKT phosphorylation. In the latter, we only saw complete ablation of phosphorylation at the T308 site, suggesting other kinases act at the S473 site which seem to still be dependent on PTDINS(3,4,5)P_3. We saw a decrease in AKT and GFAP phosphorylation in cells treated with buparlisib and pictilisib while total GFAP levels stayed constant (Figure 2C,D). Similarly, we saw decreased levels of AKT and GFAP phosphorylation in cells treated with BX795 (Figure 2E,F). This suggests that elements of the Insulin/PI3K/AKT signaling axis like PI3K and PDK-1 may be able to regulate chaperone-mediated autophagy on the lysosome.

To determine whether or not decreased GFAP phosphorylation indicated increased levels of CMA, we performed a pulse-chase experiment using a photo-switchable fluorescent reporter obtained from the Cuervo lab at Albert Einstein College of Medicine[26]. When CMA is active, the reporter accumulates on the lysosomal membrane due to having the KFERQ consensus sequence from RNAse fused to the reporter. The transfected reporter forms fluorescent punctae that are

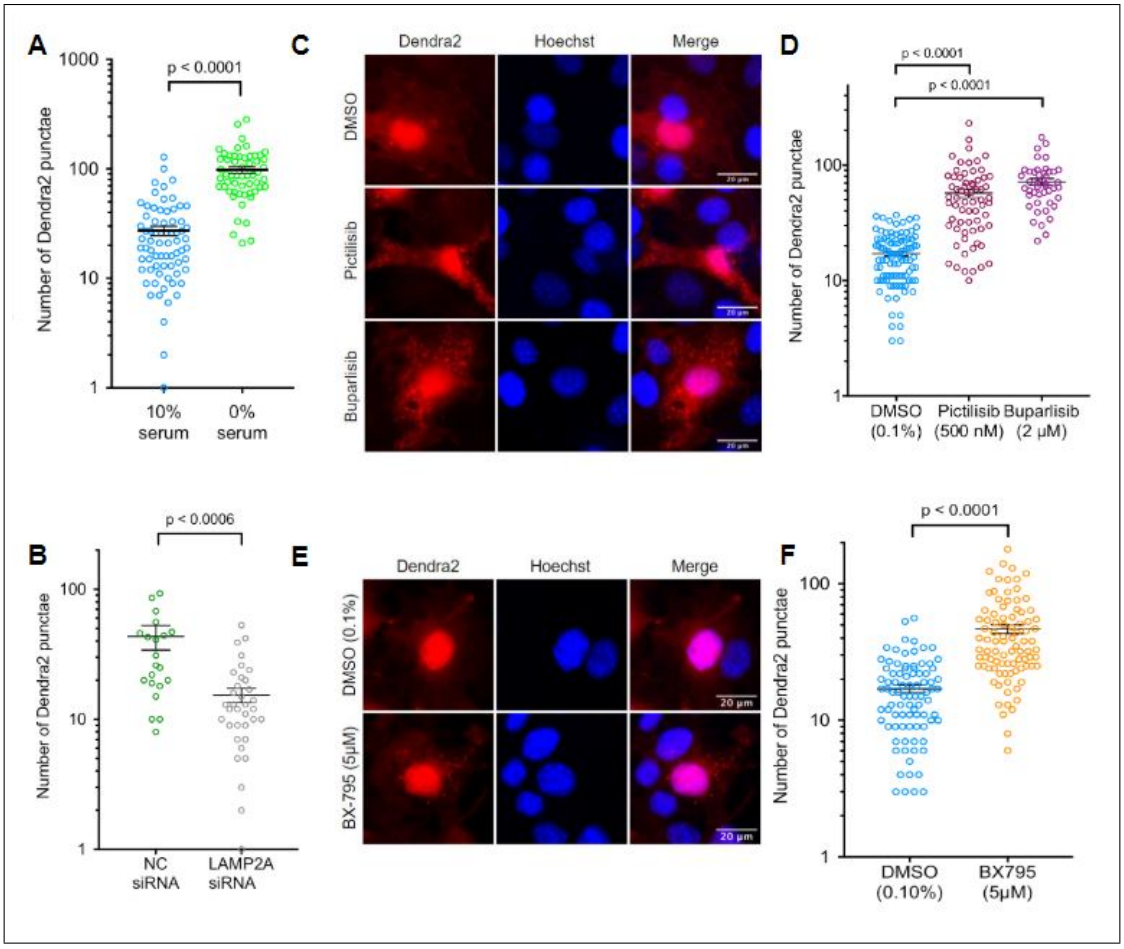

Figure 3: "Class I PI3K Inhibitors and PDK-1 Inhibitors Affect CMA Activity"

visible via microscopy. Blinded quantifications of punctae were then performed to relatively compare levels of CMA between the different conditions. We first verified that the reporter actually measured CMA activity by serum starvation (Figure 3A) and by knocking down lamp2a (Figure 3B). We observed a significant increase in the number of punctae when CMA is activated by serum deprivation and a significant decrease in the number of punctae when lamp2a is knocked down, indicating that the reporter is able to accurately determine conditions of either increased and decreased CMA activity. Next, we treated cells transfected with the reporter with our class I PI3K inhibitors, resulting in a significant increase in the number of lysosomal punctae in treated cells with no apparent effects to cell morphology or nuclear architecture (Figure 3C). There is a significant increase in the number of lysosomal punctae in treated cells (Figure 3D). The increase in CMA activity from PI3K inhibitors seems to be about the same as that of serum starvation. The PDK-1 inhibitor, BX795, has similar results in both the microscopic images (Figure 3E) and in the quantifications (Figure 3F). However, it does seem that the increase in CMA activity is not as apparent as with either pictilisib or buparlisib.

It is possible that our result of inhibiting the Insulin/PI3K/Akt signaling axis is cell-type specific, we performed the same procedure for class I PI3K inhibitors in two other cell lines. Specifically, we looked at AML12 hepatocytes and inter-medullary collecting duct (IMCD3) cells. We observed very similar results in both of these cell lines. In AML12, we saw decreases in GFAP phosphorylation with either buparlisib or pictilisib (Figure 4A). The higher doses that were required to completely ablate AKT phosphorylation are unexplained but it may be due to AML12 being

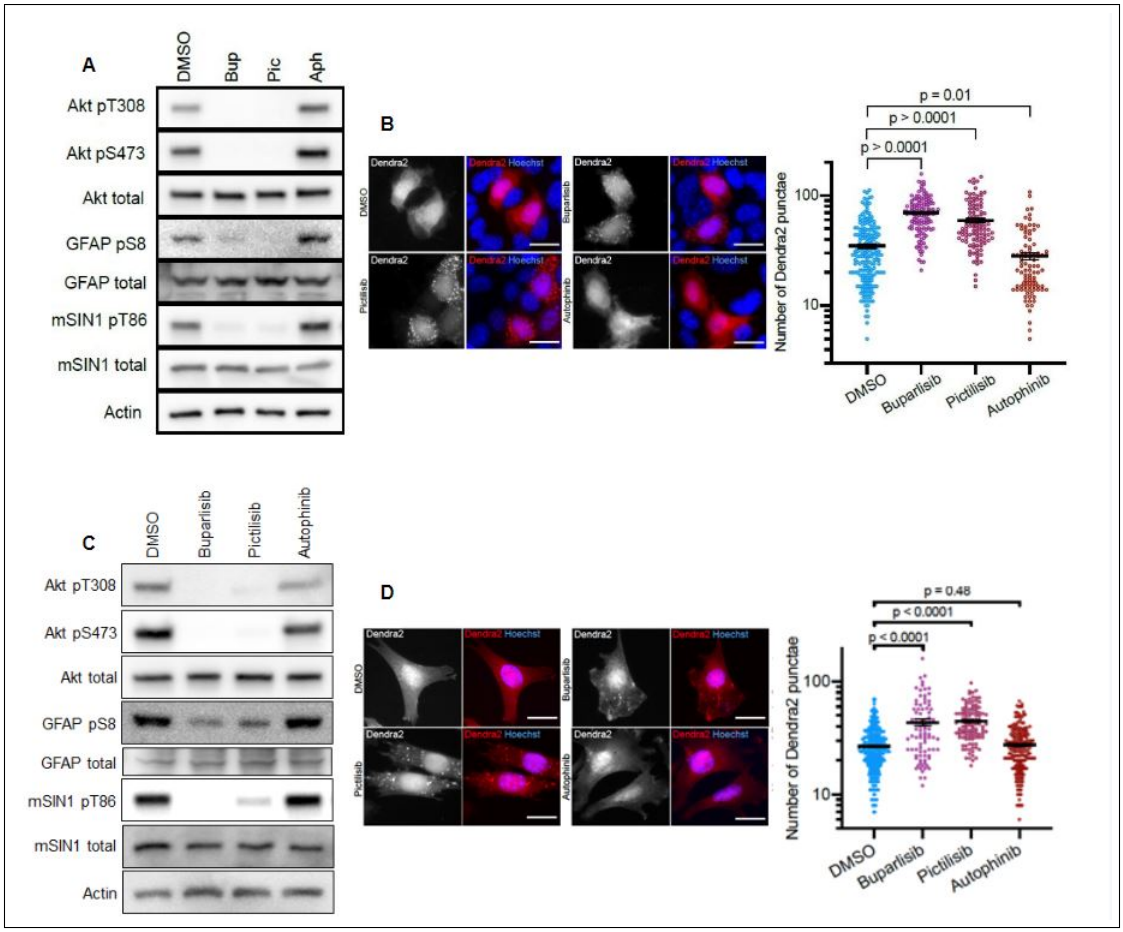

Figure 4: "The Effects of Class I PI3K Inhibitors is not cell-type specific"

hepatocytes which are capable of producing IGF-1 which can stimulate the pathway we are trying to inhibit. These drug treatments also resulted in increased number of punctae for both class I PI3K inhibitors (Figure 4B). When we performed the same experiments in IMCD3 cells, we again saw decreased GFAP phosphorylation (Figure 4C) and increased levels of punctae (Figure 4D).

Earlier work in the field had examined the potential of PI3K inhibitors in regulating chaperone-mediated autophagy and came to the conclusion that it did not have a potent, specific effect on CMA [27]. However, their assay only looked at general PI3K inhibitors, instead of class-specific ones. This is of importance as there is one known class III PI3K inhibitor known as vacuolar protein sorting 34 (VPS34) [28]. VPS34 produces PTDINS(3)P which controls membrane docking and fusion during the formation of autophagic vesicles required for macroautophagy [29][30]. To ensure that our results are due to properly targeting CMA instead of macroautophagy, we performed a macroautophagic flux assay to see if our drugs had any effect of macroautophagy in mIMCD3 cells. By using bafilomycin A, a vacuolar type H^+-ATPase inhibitor[31], we can see that the interaction term between our drugs are not significant suggesting that class I PI3K inhibitors (Figure 5A,B) do not have an effect on macroautophagy. However, the class 3 PI3K inhibitor, autophinib, does have an effect on (Figure 5C). Since then, we have carried out the same procedures in mice with two of our class I PI3K inhibitors (pictilisib and buparlisib). Lysosomes isolated from the livers of treated mice show increased binding and uptake of known CMA substrates. Collectively, this work suggests that insulin signaling plays a role in regulating CMA.

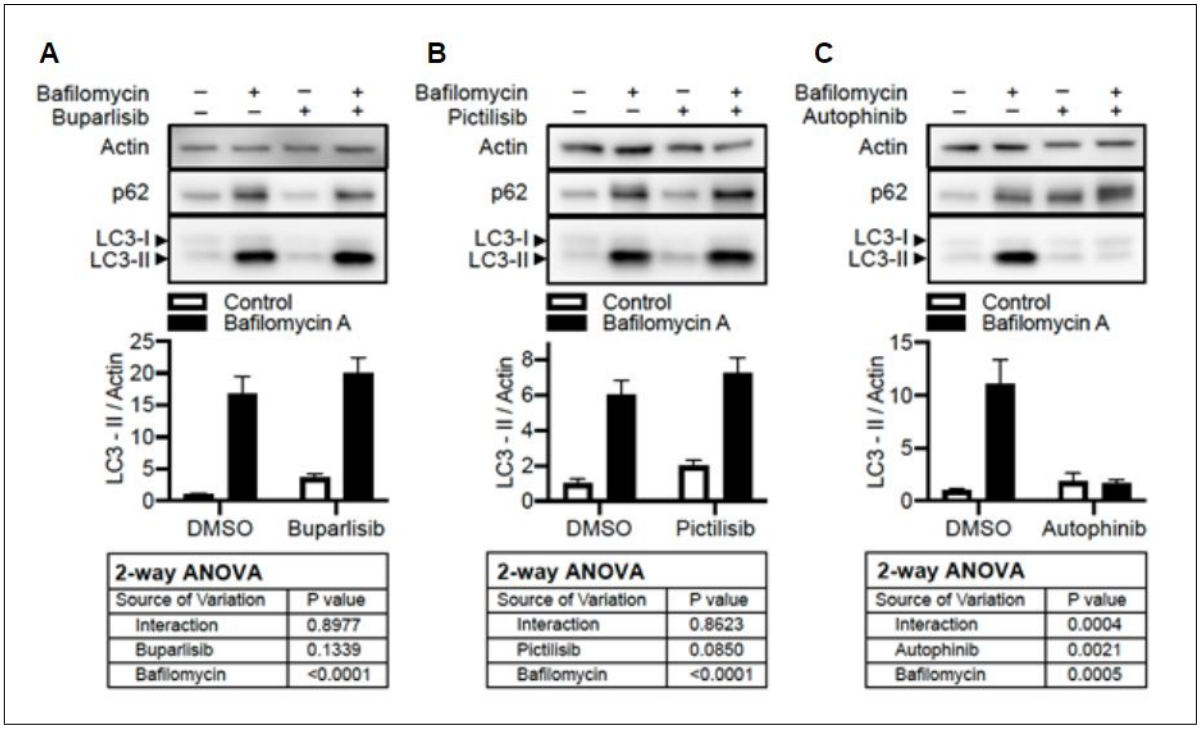

Figure 5: "Class I PI3K Inhibitors don't have an effect on macroautophagy"

Beyond this story, we wanted to look for other potential regulators of CMA that may act to inhibit or activate the Insulin/PI3K/Akt signaling axis. We decided to look into 5'-AMP-activated protein kinase (AMPK) given its function as a conserved master regulator of energy homeostasis [32] and of several prolongevity pathways [33] which is also known to inhibit the Insulin/PI3K/Akt signaling axis. [34]. AMPK is a heterotrimeric with a catalytic α subunit and two regulatory subunits in β and γ [35]. The β and γ subunits are regulated by the allosteric binding of AMP to activate AMPK and also promote phosphorylation of the α subunit at T172 [36]. The catalytic α subunit of AMPK acts on the Insulin/PI3K/Akt signaling axis through two hypothetical mechanisms. AMPK is known to phosphorylate insulin receptor substrate 1 (IRS-1) at its S789 site. AMPK may also regulate CMA by phosphorylating protein phosphotase 2A (PP2A) at both S298 and S336 to activate PP2A [37] such that it can dephosphorylate and inhibit Akt [38]. To determine the role that these pathways had on CMA, we treated NIH3T3 cells with the Akt inhibitor, afuresertib [39], and the allosteric AMPK activator, A-769662 [40] (Figure 6A,B). ACC1 is a substrate of AMPK which we can see has increased phosphorylation in the presence of A-769662. Both afuresertib and A-769662 inhibit the phosphorylation of the Akt substrate, GSK3β, and the phosphorylation of GFAP. To confirm that this implied increased levels of CMA, we used our assay from earlier to see that both afuresertib (Figure 6C) and A-769662 (Figure 6D) result in an increase in the number of punctae compared to the DMSO control (Figure 6E). When we transfected cells with an siRNA for PP2A, A-769662 was still able to inhibit Akt phosphorylation (Figure 6F,G) which implies that AMPK acts to inhibit Insulin/PI3K/Akt signaling instead through IRS-1. When the procedure was repeated in primary fibroblasts of mouse tails, we found similar results in terms of IRS-1 and GFAP phosphorylation (Figure 6H). These results imply that AMPK can upregulate CMA activity by phosphorylating IRS-1. This provides another target for regulation of chaperone-mediated autophagy besides class I PI3K on the plasma membrane.

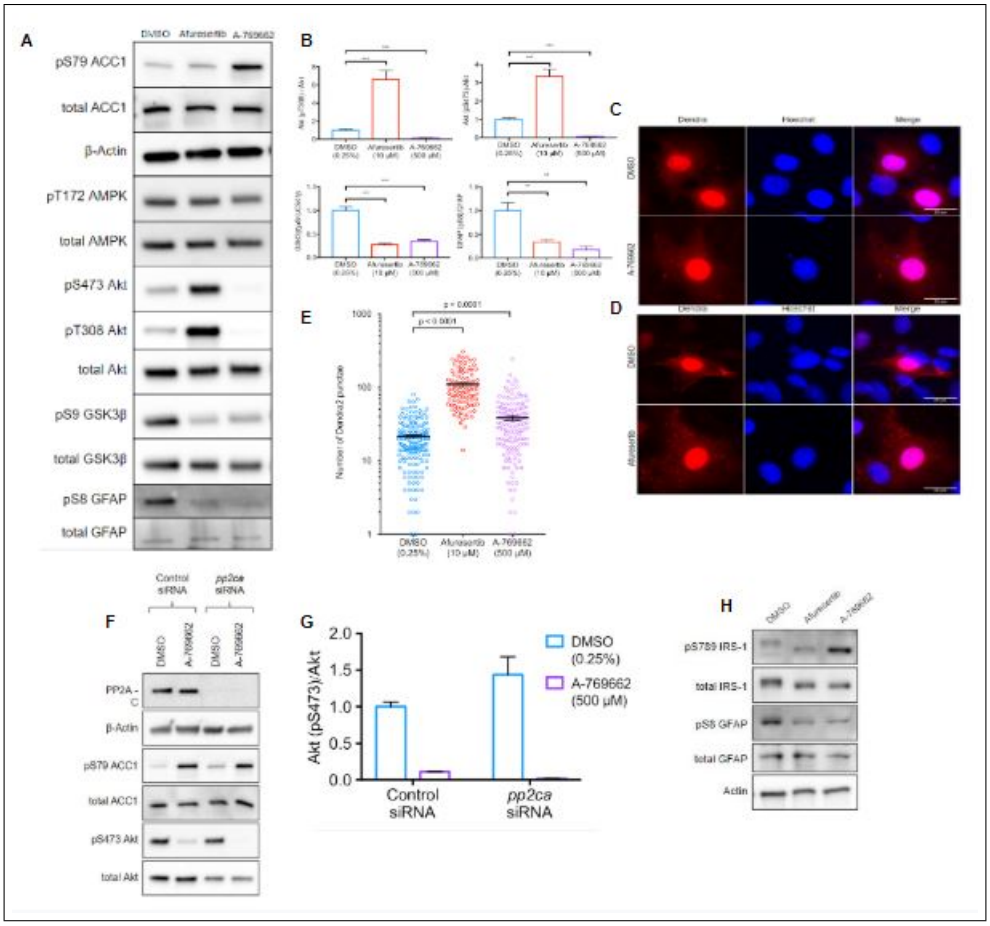

Figure 6: "AMPK regulates CMA through IRS-1, and not PP2A"

Materials and Methods

Mouse Husbandry

All mice were housed and cared for in our facilities according to established protocols that were approved by the University of Michigan's Animal Care and Use Program.

Cell culture

Mouse NIH3T3 cells were cultured in DMEM with 10% FBS under standard conditions. Mouse AML12 cells were cultured in DMEM:F12 supplemented with 10% FBS, 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone. Mouse IMCD3 cells were cultured in DMEM:F12 with 10% FBS under standard conditions. Cells were transfected using Lipofectamine 2000 following instructions of the manufacturer.

Western Blot Analysis

Tissues were frozen with liquid nitrogen and stored at -70 degrees Celsius. Tissues or cell culture were processed, cell lysates were obtained, and equal amounts of protein were loaded for Western blot analysis. Antibodies were purchased from Cell Signaling Technologies (Akt #2920S, pAkt-T308 #4056S, pAkt-S473 #4060S, GSK3β #12456S, pGSK3β-S9 #5558S, Actin #3700S, mSIN1 #12860S, pSIN1-T86 #14716S, LC3A/B #12741S, ACC1 #4190S, pACC1-S79 #11818S, AMPKα #2532S, and pAMPK-T172 #2535S) and from Abcam (LAMP2A ab125068, HSC70 ab51052, ATP5A ab14748, UQCRC2 ab14745, MTCO1 ab14705, GFAP ab7260, pGFAP-S8 ab3562A, and p62 ab109012).

Fluorescence Quantification of Punctae

Photoconversion of cells grown on coverslips was carried out with a 405/20 nm LED array (Norlux) for 5 min using 50 mW/cm^2 light intensity. More than 90% of the cells were viable after the photoconversion. For immunofluorescence, cells grown on coverslips were fixed in 3% formaldehyde in phosphate buffer saline (PBS), blocked with 10% FBS, permeabilized with 0.01% of TritonX-100 for 30min and then incubated with the primary and corresponding secondary antibodies diluted in 5% FBS in PBS.

Statistical Analysis

Unless indicated otherwise, results are presented as mean ± standard error of the mean (SEM). Statistical tests for genotype, fasting and fed conditions (nutritional), or interaction effects were assessed by two-way analysis of variance (ANOVA) using $P = 0.05$ as the threshold for significant effects. To compare specific effects between groups, an unpaired t-test was used with $P = 0.05$ as the criterion for significance.

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