cdc-Like/Dual-Specificity Tyrosine Phosphorylation–Regulated Kinases Inhibitor Leucettine L41 Induces mTOR-Dependent Autophagy: Implication for Alzheimer’s Disease^[S]

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ABSTRACT

Leucettines, a family of pharmacological inhibitors of dual-specificity tyrosine phosphorylation regulated kinases and cdc-like kinases (CLKs), are currently under investigation for their potential therapeutic application to Down syndrome and Alzheimer’s disease. We here report that leucettine L41 triggers bona fide autophagy in osteosarcoma U-2 OS cells and immortalized mouse hippocampal HT22 cells, characterized by microtubule-associated protein light chain 3 membrane translocation and foci formation. Leucettine L41-triggered autophagy requires the Unc-51-like kinase and is sensitive to the phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and 3-methyladenine,

suggesting that it acts through the mammalian target of rapamycin (mTOR)/PI3K-dependent pathway. Leucettine L41 does not act by modifying the autophagic flux of vesicles. Leucettine L41-induced autophagy correlates best with inhibition of CLKs. Leucettine L41 modestly inhibited phosphatidylinositol-3-phosphate 5-kinase, FYVE domain-containing activity as tested both in vitro and in vivo, which may also contribute to autophagy induction. Altogether these results demonstrate that leucettines can activate the autophagic mTOR/PI3K pathway, a characteristic that may turn advantageous in the context of Alzheimer’s disease treatment.

Introduction

Macroautophagy (hereafter referred to as autophagy) is a mechanism conserved among eukaryotes with essential roles in homeostasis and development (Mizushima and Levine,

2010). Defects in autophagy have been associated with many human diseases, including cancers, myopathy, and neurodegenerative, infectious, and cardiovascular diseases (see Yang and Klionsky, 2010, for review). Autophagy is a “self-digesting” process by which the cell engulfs cytoplasmic components in vesicles (autophagosomes) that fuse with lysosomes into autolysosomes and have their content degraded by lysosomal hydrolases (Yang and Klionsky, 2010). Autophagy allows cells to degrade long-lived, aggregated, or damaged proteins and organelles, catabolizing existing cytoplasmic components to generate nutrients and energy to maintain essential cellular activities. Many protein kinases are involved in the control of autophagy, including Unc-51-like kinases 1 and 2 (ULK1 and

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ABBREVIATIONS: BiP, immunoglobulin heavy chain binding protein; CLKs, cdc-like kinases; DMSO, dimethylsulfoxide; DYRKs, dual-specificity tyrosine phosphorylation-regulated kinases; EBSS, Earle’s balanced salt solution; ER, endoplasmic reticulum; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; GroPIns, glycerophosphorylinositol; HPLC, high-performance liquid chromatography; IP3, inositol-1,4,5-trisphosphate; LC3, microtubule-associated protein light chain 3; mRFP, monomeric red fluorescent protein; mTOR, mammalian target of rapamycin; PE, L- α -phosphatidylethanolamine; PI3K, phosphatidylinositol 3-kinase; PIKfyve, phosphatidylinositol-3-phosphate 5-kinase, FYVE domain-containing; PI, phosphatidylinositol; PtdIns(3,5)P₂, phosphatidylinositol-3,5-bisphosphate; PtdIns3P, phosphatidylinositol-3-monophosphate; PtdIns5P, phosphatidylinositol-5-monophosphate; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SB600125, anthra[1-9-cd]pyrazol-6(2H)-one; S-CR8, 2-(S)-(1-ethyl-2-hydroxyethylamino)-6-(4-(2-pyridyl)benzyl)-9-isopropylpurine; siRNA, small interfering RNA; SR proteins, serine-arginine-rich proteins; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene; ULK, Unc-51-like kinase; YM201636, 6-amino-N-[3-[4-(4-morpholinyl)pyrido[3',2':4,5]furo[3,2-d]pyrimidin-2-yl]phenyl]-3-pyridinecarboxamide; Z-PP-CHO, prolyl endopeptidase inhibitor II.

ULK2), AMP-activated protein kinase, and mammalian target of rapamycin (mTOR), which monitors cellular nutrient status and negatively regulates autophagy (Wullschleger et al., 2006; Chan et al., 2007; Hoyer-Hansen and Jaattela, 2007). Phosphatidylinositol 3-kinase (PI3K/Vps34), which leads to the formation of phosphatidylinositol-3-monophosphate (PtdIns3P), is a key inducer of the mTOR autophagic pathway (He and Klionsky, 2009). A few years ago, it emerged that autophagy can also be induced through an mTOR-independent pathway by a decrease in intracellular levels of inositol and inositol-1,4,5-trisphosphate (IP3) through inhibition of inositol monophosphatase by lithium (Sarkar et al., 2005).

Splicing involves the removal of introns from pre-mRNA and the joining of exons. Almost all human genes undergo alternative splicing. Alternative splicing is a highly regulated process that produces multiple mRNA variants from a single pre-mRNA. This influences gene expression, mRNA localization or stability, and generates several protein isoforms from a single pre-mRNA (Blencowe, 2006). The phosphorylation state of serine-arginine-rich (SR) proteins, a family of splicing factors, plays an important role in splicing regulation (Long and Caceres, 2009). SR proteins are phosphorylated by several protein kinases, including SR protein kinases (Gui et al., 1994) and the dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs; de Graaf et al., 2004) and cdc-like kinase 1 (CLK1; Duncan et al., 1997), the activity of which is required to maintain the phosphorylation state of SR proteins (Yomoda et al., 2008). Given the growing number of examples where splicing is abnormally regulated in human disease (review in Singh and Cooper, 2012), it is no surprise that there is increasing interest in pharmacological inhibitors of SR protein kinases, DYRKs, and CLKs as potential therapeutic drugs (review in Hagiwara, 2005; Smith et al., 2012). In this context we recently described leucettines, a family of low molecular weight inhibitors of DYRKs and CLKs derived from the marine natural product leucettamine B (Debdab et al., 2011; Tahtouh et al., 2012).

Leucettines were recently found to interact with phosphatidylinositol-3-phosphate 5-kinase, FYVE domain-containing (PIKfyve), the lipid kinase that catalyzes the phosphorylation of phosphatidylinositol-3-monophosphate (PtdIns3P) to phosphatidylinositol-3,5-bisphosphate [PtdIns(3,5)P₂] and the phosphorylation of phosphatidylinositol (PI) to phosphatidylinositol-5-monophosphate (PtdIns5P) (Tahtouh et al., 2012). PIKfyve is part of the PtdIns(3,5)P₂-regulatory complex, comprising the scaffolding protein ArPIKfyve and the lipid phosphatase Sac3 (reviewed in Shisheva, 2012). It was demonstrated that PIKfyve is involved in autophagy, although its mechanism of action is still unclear (Jefferies et al., 2008; de Lartigue et al., 2009).

We show here that leucettines trigger autophagy in cultured HT22 and U-2 OS cells due to their ability to inhibit CLKs and PIKfyve rather than DYRKs. Moreover we show that reduction of CLK1 activity is sufficient to induce autophagy in U-2 OS cells. Leucettine-induced autophagy occurs through the mTOR-, ULK-, and PI3K-dependent pathway. This unexpected property of leucettines may turn out to be of particular relevance in view of their therapeutic potential for the treatment of cancers and Alzheimer's disease.

Materials and Methods

Reagents. Rapamycin, wortmannin, and 3-methyladenine were purchased from Sigma-Aldrich (St. Louis, MO). SB600125 (anthra[1-9-

cd]pyrazol-6(2H)-one), SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole], tunicamycin, and prolyl endopeptidase inhibitor II (Z-PP-CHO) were from Calbiochem (EMD Millipore, Billerica, MA); UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Gleevec was from Cayman Chemical (Ann Arbor, MI). YM201636 (6-amino-N-[3-[4-(4-morpholinyl)pyrido[3',2':4,5]furo[3,2-d]pyrimidin-2-yl]phenyl]-3-pyridinecarboxamide) was purchased from Symansis (Timaru, New Zealand). Olomoucine, roscovitine, S-CR8 [2-(S)-(1-ethyl-2-hydroxyethylamino)-6-(4-(2-pyridyl)benzyl)-9-isopropylpurine], aloisine, alsterpaullone, leucettines L41, L14, L33, and L38 were synthesized in the laboratory as previously described (Leost et al., 2000; Mettey et al., 2003; Bettayeb et al., 2008; Debdab et al., 2011).

Cell Culture Experiments. U-2 OS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 IU of penicillin and streptomycin. In the cases of cotreatment with wortmannin, 3-methyladenine, or Z-PP-CHO, cells were pretreated for 1 hour before addition of leucettine L41, rapamycin, or LiCl depending on the experiments. Cytotoxicity was assayed using the CytoTox 96 Non-Radioactive Cytotoxicity Test from Promega (Madison, WI) according to manufacturer's instructions. For Earle's balanced salt solution (EBSS)-induced autophagy, cells were washed four times with prewarmed EBSS then allowed to grow in EBSS for another 6 hours.

Cloning and Transfection Procedures. Human microtubule-associated protein light chain 3 (LC3)B image clone (IOH13267) was purchased from ImaGene (Source Bioscience Lifesciences, Nottingham, UK). Monomeric red fluorescent protein (mRFP):enhanced green fluorescent protein (EGFP):LC3 were generated as previously described (Kimura et al., 2007). Green fluorescent protein (GFP) constructs were transfected using FuGene-6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Stably expressing mRFP:EGFP:LC3 and EGFP:LC3 clones were selected with G418 treatment. Double-stranded small interfering RNA (siRNA) oligomers were transfected into U-2 OS cells using Dharmafect 1 (Dharmacon/ThermoScientific, Pittsburgh, PA), according to the manufacturer's instructions. Validated siRNAs targeting DYRK1A (SI00605241), CLK1 (SI00059332), PIKfyve (SI00155120), and ULK1 (SI0223237) were purchased from Qiagen (Valencia, CA). Control depletion was carried out using All Star Negative Control siRNA oligomers from Qiagen.

Antibodies, Immunoblotting, and Indirect Immunofluorescence. Gel electrophoresis and immunoblotting were performed using standard protocols. For immunofluorescence, cells were grown on glass coverslips, fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized by 50 μ M digitonin, and processed using standard protocols. Images were acquired with a Leica DFC360 FX CCD camera on a Leica AF6000 microscope (Leica Microsystems, Wetzlar, Germany) using a 40 $\times$ numerical aperture 0.85 objective. Image processing was performed by ImageJ (NIH, Bethesda, MD). Quantification of LC3 foci was performed using ImageJ threshold function and automated counting. Primary antibodies used in this study included anti- α -tubulin (DM1A; Sigma-Aldrich), anti-LC3 [4E12 (MBL International, Nagoya, Japan) for immunofluorescence, or 2G6 (Enzo Life Sciences, Farmingdale, NY) for immunoblots], anti-DYRK1A (Abnova, Taipei City, Taiwan), anti-phospho-Thr70-4E-BP1 (Cell Signaling Technology), anti-immunoglobulin heavy chain binding protein (BiP) (Cell Signaling Technology), and anti-CLK1 (ab74044; Abcam, Cambridge, MA).

Reverse-Transcription Polymerase Chain Reaction and Polymerase Chain Reaction. Total cellular RNAs were extracted using Trizol (Gibco/Life Technologies, Grand Island, NY) according to the manufacturer's instructions. One microgram was used to perform cDNA synthesis using SuperScript Reverse Transcriptase III (Invitrogen/Life Technologies, Carlsbad, CA) and random decamers (Ambion/Life Technologies, Austin, TX). cDNAs were amplified using Taq polymerase (Roche Diagnostics) and appropriate primers for CLK1 (GCTGCAAATACAATCACTCT and GCTTTATGATCGATG CACT). Products were separated by agarose gel electrophoresis and visualized using ethidium bromide.

Protein Kinase Assays. Recombinant DYRK1A, B, 2, 3, and CLK1, 2, 3, 4 were expressed in *Escherichia coli*, purified, and assayed with appropriate substrates with 15 μ M radiolabeled [γ - 32 P]ATP in the presence of a range of concentrations of drugs as described previously (Debdab et al., 2011; Tahtouh et al., 2012). IC₅₀ values were determined from the dose-response curves.

Recombinant PIKfyve Activity Assay. Silica gel G60 thin-layer chromatography plates were from Merck (Darmstadt, Germany), [γ - 32 P]ATP from PerkinElmer (Waltham, MA), brain L- α -phosphatidylethanolamine (PE) from Avanti Polar Lipids (Alabaster, AL), and C16:0/C16:0-PtdIns3P from Echelon (Salt Lake City, UT). All other compounds used were from Sigma-Aldrich. Recombinant GST-PIKfyve protein corresponding to the short form of murine PIKfyve was produced in SF9 infected cells, purified, and its activity was measured as described previously (Chicanne et al., 2012). Briefly, increasing amounts of L41 or YM201636 were added to the samples containing recombinant PIKfyve (0.2 μ g), and PtdIns3P (50 pmol)/PE (20 nmol) vesicles, and lipid kinase buffer. After 10 minutes at room temperature, the phosphorylation reactions were initiated by addition of a mixture of ATP (50 μ M) and [γ - 32 P]ATP (20 μ Ci). After incubation at 37°C for 30 minutes, phosphorylated lipids were extracted, separated by thin-layer chromatography, and the amount of 32 P-PtdIns(3,5)P₂ generated was quantified. To assess the effect of L41 on both PtdIns(3,5)P₂- and PtdIns5P-generating activity of PIKfyve, we conducted a similar in vitro lipid kinase activity assay using the native PtdIns substrate from Avanti Polar Lipids (100 μ M PtdIns, sonicated prior to use in 20 mM HEPES, pH 7.5, 1 mM EDTA), and adenovirally produced PIKfyve as an enzyme source, which was immunopurified on anti-PIKfyve antibodies as we described elsewhere (Sbrissa et al., 1999, 2002).

In Vivo PIKfyve Activity Inhibition Assay: myo-[2- 3 H]inositol Labeling, Lipid Extraction, and High-Performance Liquid Chromatography. The effect of L41 on phosphoinositide steady-state levels was assessed in 3T3 L1 adipocytes labeled with myo-[2- 3 H]inositol (PerkinElmer) following our protocols for adipocytes in vivo labeling (Sbrissa et al., 2012) with a slight modification. Briefly, cells (35-mm dishes) maintained for 18 hours in glucose- and inositol-free Dulbecco's modified Eagle's medium (DMEM) containing 5% dialyzed fetal bovine serum, 0.5% bovine serum albumin, 5 μ g/ml insulin, 5 μ g/ml transferrin, 2 mM pyruvate, 25 mM HEPES, pH 7.4, 100 IU/ml penicillin, and 100 μ g/ml streptomycin were labeled for 40 hours with 22.5 μ Ci/ml myo-[2- 3 H]inositol in glucose- and inositol-free DMEM containing 5 μ g/ml transferrin, 5 μ g/ml insulin, 2 mM pyruvate, and 25 mM HEPES, pH 7.4, at 37°C in a 5% CO₂/95% air humidified incubator. Treatment with 0 [vehicle, final dimethylsulfoxide (DMSO), 0.2% v/v], 1, 5, or 20 μ M L41 inhibitor was for the last 75 minutes of the labeling period. Lipids were extracted in the presence of EDTA and tetrabutylammonium sulphate (5 mM each), deacylated, and analyzed by high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA) on a 5- μ m Partisphere SAX column (4.6 mm $\times$ 250 mm; Whatman/GE Healthcare Life Sciences, Pittsburgh, PA) as detailed previously (Sbrissa et al., 2012). [32 P]Glycerophosphorylinositol 5P ([32 P]GroPIns5P), [32 P]GroPIns3P, [32 P]GroPIns(4,5)P₂, and [32 P]GroPIns(3,5)P₂ prepared by enzymatic synthesis with [γ - 32 P]ATP as described elsewhere (Sbrissa et al., 1999) were coinjected as internal HPLC standards. Fractions were collected every 0.25 minutes and analyzed for 3 H and 32 P radioactivity after addition of the scintillation mixture. Data evaluation and documentation were performed by Microsoft Excel. Individual peak radioactivity was quantified by area integration and presented as a percentage of the summed radioactivity from the [3 H]GroPIns3P, -4P, -5P, -(3,5)P₂, and -(4,5)P₂ peaks ("total radioactivity").

Results

Leucettine L41 Triggers Accumulation of LC3 Foci. We first tested the ability of a variety of protein kinases inhibitors to induce autophagy. U-2 OS, a human osteosarcoma cell line, and HT22, a mouse immortalized hippocampal

cell line, were treated with a small panel of inhibitors at 10 μ M and autophagy was assayed after 24 hours by counting LC3 foci, a hallmark of autophagy. With the exception of alsterpaullone, a glycogen synthase kinase-3 β inhibitor, similar results were obtained for both cell lines, and U-2 OS results are described below (Fig. 1; Supplemental Fig. 1). Rapamycin, an mTOR inhibitor, and Gleevec, a c-Abl tyrosine kinase inhibitor, were used as positive controls (10 $\pm$ 0.3 and 17 $\pm$ 1 LC3 foci per cell, respectively; Ertmer et al., 2007). Inhibition of cyclin-dependent kinases with olomoucine, roscovitine, or S-CR8 did not induce an increase of autophagy compared with cells treated with DMSO (5.1 $\pm$ 0.4, 5.9 $\pm$ 0.3, and 6.6 $\pm$ 0.24, respectively, versus 4.1 $\pm$ 0.5 LC3 foci per cell; Fig. 1A). Inhibition of glycogen synthase kinase-3 β with aloisine (4.7 $\pm$ 0.2 foci per cell) or alsterpaullone (6.6 $\pm$ 0.2 foci per cell) or inhibition of c-Jun N-terminal kinases (4.3 $\pm$ 0.4 foci per cell) and p38 (5.9 $\pm$ 0.5 foci per cell) with SB600125 and SB203580, respectively, did not induce an accumulation of LC3 foci in cells (Fig. 1A). On the other hand, treatment with 10 μ M leucettine L41 (L41), an inhibitor of both DYRKs and CLKs recently developed in our laboratory (Debdab et al., 2011; Tahtouh et al., 2012; Fig. 1D), induced LC3 foci formation (11 $\pm$ 1 foci per cell) in a dose-dependent manner (Figs. 1A and 2A), suggesting that inhibition of DYRKs, CLKs, and/or other targets could induce autophagy. A low level of toxicity of L41 was confirmed by measuring amounts of lactate dehydrogenase released in the medium after 24 hours of treatment with 10 μ M L41 (Fig. 1B). Autophagy and LC3 foci accumulation can result from endoplasmic reticulum (ER) stress (see Deegan et al., 2013, for review). To determine whether L41 treatment was triggering ER stress, the expression levels of BiP, a protein upregulated upon ER stress, was estimated. Tunicamycin was used as a positive control. As shown in Fig. 1C, BiP protein levels were unaffected by L41 treatment, demonstrating that L41 does not induce ER stress. Because under certain circumstances LC3 foci can be dissociated from autophagy (Kuma et al., 2007), we further confirmed autophagy induction upon L41 treatment by detecting the conversion of the soluble LC3-I form to the membrane-bound LC3-II form by immunoblotting (Fig. 2B). Moreover, treatment with 3-methyladenine, an inhibitor of autophagy, prevented L41-induced autophagy in U-2 OS cells (3.2 $\pm$ 0.6 foci per cell; Fig. 2C), further confirming that the observed accumulation of LC3 foci upon L41 treatment is genuine autophagy. Taken together our data show that L41 is a new inducer of autophagy.

Leucettine L41 Triggers Autophagy through a ULK1- and mTOR-Dependent Pathway. Several signaling pathways are known to regulate autophagy. One is controlled by mTOR and PI3K and requires the activation of ULK1 (Chan et al., 2007). Another pathway is mTOR-independent that depends on IP3 levels and can be activated by lithium (Sarkar et al., 2005). To determine whether leucettine L41 is inducing autophagy through the mTOR-dependent or mTOR-independent pathway we used siRNA and chemical inhibition approaches. As shown in Fig. 2D, ULK1-depleted cells treated with L41 were unable to undergo autophagy (6.0 $\pm$ 0.3 foci per cell versus 16.2 $\pm$ 4.9 in L41 control siRNA-treated cells) suggesting that ULK1 is required for L41 to induce autophagy (Fig. 2D). The PI3K inhibitor wortmannin is a well known inhibitor of mTOR-dependent autophagy. Loss of autophagy (5.3 $\pm$ 0.6 foci per cell) upon cotreatment with leucettine L41 and wortmannin further confirmed that L41 induces autophagy

Fig. 1. Leucettine L41 induces accumulation of LC3 foci in U-2 OS cells. (A) Average number of LC3 foci per cell after 24-hour treatment at 10 μ M of the indicated drugs (white bar, DMSO; light gray, compounds; black, positive control). The main target of each drug is indicated. Mean of at least three independent experiments, more than 150 cells were analyzed per experiment. (B) Percentage of cytotoxicity as measured by lactate dehydrogenase (LDH) release in the cytoplasm (% of maximum LDH release induced by staurosporine). Cells were treated for 24 hours (mean of three independent experiments; error bars represent S.E. M.; ** $P < 0.01$). (C) Whole lysates of cells treated with either DMSO, 20 μ M L41, or 0.5 μ g/ml tunicamycin (Tunic) were analyzed by immunoblotting with an anti-BiP antibody. α -Tubulin (Tub) was used as loading control. Numbers indicate BiP/ α -tubulin signal-intensity ratio. (D) Structure of leucettine L41. CDKs, cyclin-dependent kinases; GSK3, glycogen synthase kinase-3; MEK, mitogen-activated protein kinase.

through the mTOR and PI3K-dependent pathway (Fig. 2C). U-2 OS cells treated with leucettine L41, rapamycin or DMSO were analyzed for mTOR activation. mTOR phosphorylates 4E-BP1 at Thr70 (see Tchekina and Komelkov, 2012, for review). 4E-BP1 phosphorylation was inhibited by rapamycin, as shown by the complete phospho-Thr70 signal disappearance (Fig. 2E). Leucettine L41 treatment caused significant reduction of 4E-BP1 phosphorylation further confirming that L41-induced autophagy is mediated by the mTOR-dependent pathway (Fig. 2E). Next, we assessed if L41-induced autophagy was also dependent on IP3 levels. Intracellular levels of inositol were raised using an inhibitor of prolyl-endopeptidase (Z-PP-CHO) or myo-inositol (Sarkar et al., 2005) and cells were cotreated with either rapamycin, LiCl, or L41. As shown in Fig. 2F, Z-PP-CHO was able to reduce autophagic response to LiCl (5.0 ± 0.1 foci per cell versus 11.8 ± 0.7) and had no effect on mTOR-dependent autophagy (10.8 ± 0.7 foci per cell versus 11.0 ± 0.3). Cells treated with L41 and Z-PP-CHO or L41 and myo-inositol (Fig. 2F; Supplemental Fig. 2) for 24 hours still underwent autophagy as evidenced by accumulation of LC3 foci (12.1 ± 0.9 and 11.5 foci per cell respectively). Taken together these results strongly suggest that autophagy observed upon L41 treatment is mediated solely through the mTOR/PI3K-dependent pathway.

Accumulation of LC3 foci in L41-treated cells could result from the perturbation of autophagic flux rather than from induction of autophagy per se. To investigate this possibility we established a stable U-2 OS cell line expressing the human isoform of LC3B fused to both mRFP and EGFP. This strategy allows differentiation of pre- and postfusion vesicles through observation of red-only foci (Fig. 3A; Kimura et al., 2007). At the phagophore and autophagosome stages mRFP:GFP:LC3 is exposed to cytosolic pH: both GFP and mRFP are correctly folded and are able to fluoresce, giving red and green signals. Upon fusion of autophagosomes with lysosomes, internal pH decreases and GFP, but not mRFP, folding is affected preventing the green fluorescence and leading to red-only foci. Counting of green and red versus red-only foci allowed determination of leucettines' effect, if any, on autophagic flux (Fig. 3A). Cells starved in EBSS medium underwent autophagy

as demonstrated by the appearance of green- and red-only foci. Treatment with leucettine L41 induced both an increase in the number of prefusion vesicles (4.75 ± 0.03 versus 0.88 ± 0.28 in DMSO-treated cells) and of vesicles fused to lysosomes (11.07 ± 1.88 versus 2.02 ± 0.5), demonstrating that the inhibitor does not induce a perturbation of the autophagic flux (Fig. 3, B and C) but is a true autophagy inducer.

Inhibition of CLKs Rather Than DYRKs Is Involved in Leucettine L41-Triggered Autophagy. L41 inhibits DYRKs and CLKs kinases with equal potency (Debdab et al., 2011; Tahtouh et al., 2012). It has been reported that CLK1 regulates its own splicing and that CLK1 inhibition promotes inclusion of exon 4 in the mRNA, thereby increasing the level of the full-length kinase isoform. Changes in abundance of this splice variant thus acts as a marker of CLK1 activity (Duncan et al., 1997; Muraki et al., 2004). Reverse-transcription polymerase chain reaction analysis of mRNA from L41-treated U-2 OS cells shows that L41 treatment induces a 150-fold increase of the exon 4-containing CLK1 mRNA (Supplemental Fig. 3B; Muraki et al., 2004). Similarly to L41, treatment with TG003, a widely used CLK inhibitor (Supplemental Fig. 3A; Muraki et al., 2004) also had inhibitory effects on DYRK1A and CLKs and induced autophagy in U-2 OS cells, further confirming that inhibition of either DYRKs or CLKs leads to autophagy (Supplemental Fig. 3C). We used leucettines that are more specific to either one or the other kinase family (Fig. 4A; Debdab et al., 2011) to determine which of the two kinase families is involved in autophagy. Treatment of U-2 OS cells with leucettine L38, an inhibitor showing more potency toward DYRKs, did not induce autophagy (Fig. 4B; 4.5 ± 0.2 LC3 foci per cell compared with 4.9 ± 1.9 in DMSO-treated cells), whereas treatment with leucettine L14, an inhibitor more specific to CLKs (Fig. 4B), induced autophagy to levels similar to those of rapamycin-induced autophagy (15.4 ± 1.5 LC3 foci per cell compared with 14.2 ± 4.2 in rapamycin-treated cells). Leucettine L33, inactive on both families of kinases, was unable to trigger autophagy (4.6 ± 0.4 LC3 foci per cell). These results suggest that inhibition of CLKs contributes more to autophagy induction by leucettines than inhibition of DYRKs. To further confirm these results, we used siRNA against either

Fig. 2. Leucettine L41 induces autophagy. (A) Dose-dependent induction of autophagy. Average number of LC3 foci per cell after 24-hour treatment with L41 at the indicated doses. Immunofluorescence detection of LC3 (green) and DNA (blue) in U-2 OS cells treated with various doses of L41. Rapamycin (Rapa) was used as a reference compound. (B) L41 triggers LC3 conversion from soluble (LC3-I) to membrane-bound form (LC3-II). Whole lysates of cells treated with either DMSO (C), 0.5 μ M rapamycin, or 20 μ M L41 were analyzed by immunoblotting with an anti-LC3 antibody. Numbers indicate LC3-II/LC3-I signal-intensity ratio. (C) L41-induced autophagy is inhibited by 3-methyladenine and wortmannin. (Left) Immunofluorescence detection of LC3 and DNA in U-2 OS cells treated with either DMSO (Control), 0.5 μ M rapamycin, 20 μ M L41, alone or in combination with 10 μ M wortmannin (Wort) or 5 mM 3-methyladenine (3MA). (Right) Average number of LC3 foci per cell. (D) L41-induced autophagy requires ULK1. (Left) Immunofluorescence detection of LC3 and DNA in U-2 OS cells treated with control siRNA, ULK1 siRNA (60 hours), L41 (20 μ M, 24 hours), or a combination of drug (for 24 hours) after 36 hours siRNA (60 hours total). (Right) Average number of LC3 foci per cell. (E) Whole lysates of cells treated with either DMSO (C), 20 μ M L41, or 0.5 μ M rapamycin were analyzed by immunoblotting with an anti-phospho-Thr70-4E-BP1 antibody (Ph-4E-BP1). α -Tubulin (Tub) is used as loading control. Numbers indicate Ph-4E-BP1/ α -Tubulin signal-intensity ratio. (F) L41-induced autophagy is not inhibited by increased intracellular inositol. (Left) Immunofluorescence detection of LC3 (green) and DNA (blue) in U-2 OS cells treated 24 hours with either DMSO (Control), 0.5 μ M rapamycin, 10 mM LiCl, 20 μ M L41, alone or in combination with 25 μ M of Z-PP-CHO (ZPP). (Right) Average number of LC3 foci per cell under the various conditions. (Mean of three independent experiments; more than 150 cells were analyzed per experiment. * P < 0.05; ** P < 0.01; *** P < 0.001; error bars represent S.E.M.; scale bar, 10 μ m.)

DYRK1A or CLK1. A partial depletion of CLK1 was sufficient to trigger autophagy (50% depletion, 14.4 ± 1.3 foci per cell versus 6.4 ± 0.4 in control siRNA-treated cells; Fig. 4, C and D), whereas depletion of DYRK1A was not ($\sim 95\%$ depletion; 5.9 ± 0.7 foci per cell). Taken together our data suggest that induction of autophagy by leucettines likely results from inhibition of CLKs, rather than from inhibition of DYRKs. This is further confirmed by the lack of an additive effect upon combined depletion of CLK1 and DYRK1A (Fig. 4D).

PIKfyve Kinase Is Inhibited by Leucettine L41 and Is Implicated in Leucettine L41-Induced Autophagy. Surprisingly, we noticed that addition of L41 to CLK1 siRNA increased autophagy even higher (30.2 ± 3.3 LC3 foci per cells versus 14.4 ± 1.3 in CLK1 siRNA-treated cells), suggesting that L41 could also affect other proteins involved in autophagy. Other CLKs are obvious candidates, but PIKfyve (Fig. 5D) is also interesting in this context. Indeed a recent study demonstrated that L41 could bind the lipid kinase PIKfyve from HT22

Fig. 3. Leucettine L41 does not modify the autophagic flux. (A) Scheme showing how pH influences color detected from mRFP:GFP:LC3 construct. In phagophores (Phago) and autophagosomes (Autophag) pH is elevated and GFP is correctly folded. mRFP:GFP:LC3 internal and external to the vesicle fluoresce in red and green; merged vesicles give a yellow signal. After fusion with lysosomes (Lyso), only internal LC3 remains in autophagolysosomes (Autolyso), where pH decreases, leading to GFP misfolding and loss of green fluorescence. mRFP is not affected by the pH decrease. Only red signal is visible; merged vesicles give a red signal. (B) Fluorescence pictures of U-2 OS cells stably expressing mRFP:EGFP:LC3 treated with either DMSO (Control), EBSS, or 20 μ M L41. DNA is stained with 4',6-diamidino-2-phenylindole (blue). Scale bar, 10 μ m. (C) Quantification of LC3 foci observed in B. Green columns show amounts of green foci, red columns of red foci, black columns represent the ratio between red and green labeling (R/G ratio). (Mean of three independent experiments; more than 150 cells were analyzed per experiment; error bars show S.E.M.)

cell line extracts and from mouse brain tissue (Tahtouh et al., 2012). Moreover, treatment with L41 induces vacuole formation in U-2 OS cells ($18.1\% \pm 0.8$ versus $2.1\% \pm 0.2$ in untreated cells; Fig. 5A), a phenotype reminiscent of PIKfyve inhibition by the YM201636 compound (Jefferies et al., 2008; Ikononov et al., 2009; Fig. 5A; $30.8\% \pm 0.6$). Additionally, inhibition of PIKfyve by YM201636 triggers autophagy (de Lartigue et al., 2009; Martin et al., 2013; Fig. 5B). Taken together these data made PIKfyve a strong potential candidate that could explain the increased autophagy observed upon CLK1 siRNA and L41 cotreatment, prompting us to test whether L41 could inhibit PIKfyve activity. As shown in Fig. 5C, L41 is able to reduce recombinant PIKfyve in vitro activity for PtdIns(3,5)P₂ synthesis ($IC_{50} = 3.3 \mu$ M). We also verified the effect of L41 on PtdIns5P generation with adenovirally produced PIKfyve and native PtdIns substrate preparation (Shisheva, 2013), which data demonstrated that both PtdIns5P and PtdIns(3,5)P₂ synthesis were similarly inhibited, with an IC_{50} value of 0.7–1 μ M (data not shown). These data suggest that the synergy observed upon CLK siRNA and L41 treatment could result from PIKfyve inhibition. The reference PIKfyve inhibitor, YM201636, efficiently inhibited both the PtdIns(3,5)P₂- and PtdIns-5P-producing activities of recombinant PIKfyve, with an IC_{50} of 0.04 μ M, very close to the previously reported value (Fig. 5C and data not shown; Jefferies et al., 2008; Ikononov et al., 2009). L41 also modestly inhibited PIKfyve in vivo as evidenced by HPLC analyses of steady-state levels of phosphoinositides in differentiated 3T3L1 adipocytes (Fig. 5, D and E). We observed a decline in PtdIns5P levels and an increase in PtdIns3P levels upon exposure of 3T3L1 adipocytes to the L41 compound (Fig. 5E). The degree of autophagy observed in CLK1 knocked-down cells treated with L41 (38.9 ± 0.7 foci per cells), PIKfyve knocked-down cells treated with L41 (44.2 ± 0.3 foci per cells), or cells knocked down for both CLK1 and PIKfyve (43.1 ± 5.9 foci per cells) were all similar, further underscoring the potential involvement of PIKfyve in L41-induced autophagy (Fig. 5F). In this latter case addition of leucettine L41 did not increase autophagy levels (38.9 ± 3.5 foci per cells; Fig. 5F) suggesting that other potential L41 targets (Tahtouh et al., 2012) are not involved in autophagy. Taken together our results suggest that autophagy induced by L41 treatment likely results from combined inhibition of both CLK1 and PIKfyve kinases,

albeit single reduction of the activity of any of these two kinases is sufficient to trigger autophagy. Thus leucettines are new, nontoxic autophagy inducers acting through the mTOR-dependent pathway at least partly through PIKfyve and CLK inhibition.

Discussion

CLKs/DYRKs Inhibitory Leucettines Induce Autophagy. Leucettines have been described as a novel class of low molecular weight pharmacological inhibitors essentially selective for the DYRK and CLK kinases (Debdab et al., 2011; Tahtouh et al., 2012). The discovery of their ability to induce autophagy was rather unexpected. We here indeed demonstrate that leucettine L41 induces autophagy in HT22 and U-2 OS cells in a dose-dependent manner without affecting the autophagic flux. This autophagy is characterized by the classic appearance of LC3 foci associated with a shift of its electrophoretic mobility linked to its conversion from the LC3-I to the LC3-II form. Leucettine-induced autophagy reaches levels similar to those seen by classic autophagy inducers, rapamycin and Gleevec. It is also sensitive to classic inhibitors of autophagy such as the PI3K inhibitor wortmannin and 3-methyladenine. We thus establish leucettines as a new class of autophagy-inducing drugs.

Molecular Mechanisms of Leucettines in Their Autophagy-Inducing Activity. By employing siRNA-mediated protein silencing (ULK1) and pharmacological inhibition (rapamycin, wortmannin, 3-methyladenine, Z-PP-CHO, myo-inositol, LiCl) (Fig. 2), we demonstrate herein that L41-induced autophagy is mediated through the mTOR/PI3K, ULK1-requiring pathway rather than through the mTOR-independent, IP3-requiring pathway. Besides the well described autophagy inducers, rapamycin (mTOR), Gleevec (c-Abl), and staurosporine (unselective kinase inhibitor), all other kinase inhibitors tested were unable to trigger autophagy in U-2 OS cells (Fig. 1); therefore, we suggest that inhibition of the specific kinase targets of leucettines might be involved in triggering autophagy. Our data from protein silencing and pharmacological inhibition (Figs. 4 and 5) support the implication of CLK1 inhibition in leucettine-induced autophagy. Furthermore, we show that interaction with PIKfyve may contribute to autophagy induced by leucettines. Although L41 is not a potent inhibitor of PIKfyve catalytic

Fig. 4. Inhibition of CLKs rather than DYRKs is sufficient to induce autophagy. (A) Structure of leucettines used in this study. (B) LC3 foci induction by various leucettines correlates with their ability to inhibit CLKs. (Left) Average number of LC3 foci per U-2 OS cell after 24-hour treatment at 0.5 μ M of rapamycin or 20 μ M of the indicated drugs. (Right) IC_{50} (μ M) on DYRK1A and CLK1 of leucettines L14, L33, L38, and L41. (C) siRNA depletion of CLK1, but not DYRK1A, triggers autophagy. (Top) Whole lysates of cells treated for 68 hours with control siRNA (cntrl) or siRNA directed against DYRK1A or CLK1 were analyzed by immunoblotting with an anti-DYRK1A or CLK1 antibody; α -tubulin was used as a loading control. (Bottom) Immunofluorescence detection of LC3 (green) and DNA (blue) in U-2 OS cells following treatment with the indicated siRNA. Scale bar, 10 μ m. Right, average number of LC3 foci in treated cells. (D) L41 potentiates CLK1 siRNA effects. Cells were treated with the indicated siRNA and rapamycin (Rapa) or leucettine L41 as indicated, and the number of LC3 foci was assayed. (Mean of at least three independent experiments; more than 150 cells were analyzed per experiment; error bars show S.E.M.; * P < 0.05; ** P < 0.01.)

activity, binding of PIKfyve to L41 may also alter its intracellular distribution and ability to interact with other regulators. Interference with PIKfyve activity indeed appears to be sufficient to induce autophagy. L41 potentiates CLK1 siRNA-induced autophagy to levels reached by CLK1/PIKfyve double siRNA treatment, suggesting the coexistence of two independent pathways implicating CLK1 and PIKfyve. Inducing autophagy by inhibiting these two kinases could, therefore, be additive.

Downstream Events of Leucettines' Action Leading to Autophagy. CLKs and DYRKs are key regulators of pre-mRNA splicing as they phosphorylate serine-arginine-rich splicing factors (see Debdab et al., 2011, and references therein). Splicing is a major source of gene diversification, allowing the generation of several proteins from a single gene. It is estimated that, on average, more than 100,000 proteins are generated from the 25,000 human genes. We have shown that, indeed, leucettines modify the splicing pattern of a number of pre-mRNAs, thus leading to changes in the proportion of splice variants (Debdab et al., 2011; Tahtouh et al., 2012; T. Tahtouh, personal communication). Global transcriptomics studies confirm this alternative splicing induced by leucettines (T. Tahtouh, personal

communication). It is thus quite likely that splicing of pre-mRNAs encoding regulators of autophagy is altered by leucettines. There are a few examples linking splicing and autophagy. For instance, siRNA-mediated depletion of core spliceosome components reduces mTOR mRNA and protein levels leading to autophagy (Quidville et al., 2013). Splicing of X-box-binding protein 1 mRNA leads to an autophagic response in endothelial cells through transcriptional activation of Beclin-1 (Margariti et al., 2013). We can thus envisage that inhibition of CLKs may trigger autophagy by modulating the splicing of pre-mRNAs of specific autophagy regulators. These remain to be identified.

CLK1 inhibition-induced autophagy could also be mediated by p53, a transcription factor critical to cellular stress responses. It was recently demonstrated that p53 plays an important and complex role in autophagy (Maiuri et al., 2010, for review) and is activated upon disruption of the splicing machinery (Allende-Vega et al., 2013). The p53 pre-mRNA itself undergoes different splicing events, leading to the generation of at least 11 isoforms (review by Marcel et al., 2013). Transcriptomic studies could help us to understand the transcriptional events leading to autophagy induced by CLK1 knockdown and L41 treatment.

Fig. 5. Leucettine L41 potentiates CLK1 siRNA-induced autophagy through PIKfyve inhibition. (A) L41 treatment induces vacuole formation. (Left) Differential interference contrast picture of untreated U-2 OS cells (Cntrl) or treated for 24 hours with 20 μ M L41 (L41) or with PIKfyve siRNA for 68 hours. Arrowheads point to vacuoles. (Right) Quantification of phenotype observed on the left panels. (B) Inhibition of PIKfyve triggers autophagy. Quantification of LC3 foci in cells treated with DMSO (Cntrl), rapamycin or the PIKfyve inhibitor YM201636. (C) Inhibition of recombinant PIKfyve activity by increasing doses of L41 (red line, open circles) and YM201636 (black line, black squares). The PIKfyve assay was performed in the presence of its substrate PtdIns3P and [γ -³²P]ATP. The lipid substrate and product were separated by thin-layer chromatography. The amount of ³²P-PtdIns(3,5)P₂ produced was quantified and the results are expressed as percentage of maximal, uninhibited activity, and are mean of two to three independent experiments. (D) Schematic simplified representation of the phosphoinositide metabolism. Note that PIKfyve catalyzes the conversion of PtdIns(3)P to PtdIns(3,5)P₂ and in certain conditions PtdIns to PtdIns5P. (E) Effect of various doses of L41 on the steady-state levels of the phosphoinositides, as measured in vivo in myo-[2-³H]inositol labeled differentiated 3T3L1 adipocytes by HPLC analyses. Individual peak radioactivity was quantified by area integration and presented as a percentage of the summed radioactivity from the [³H]GroPIns3P, -4P, -5P, -(3,5)P₂, and -(4,5)P₂ peaks ("total radioactivity"); mean of three independent experiments. (F, left) Cells were subjected to control (Cntrl), CLK1, PIKfyve siRNA, and L41 (co)treatment as indicated. The number of LC3 foci was scored. (Right) Immunofluorescence images showing representative examples of the phenotype quantified on the leftmost panel. DNA is stained with 4',6-diamidino-2-phenylindole (blue), LC3 is in green. (Means of at least three independent experiments; more than 150 cells were analyzed per experiment; error bars show S.E.M.; * P < 0.05; ** P < 0.01; **** P < 0.0001; scale bar, 10 μ M.)

For instance are p53- or autophagy-specific genes upregulated? This could be of importance for cancer and neurodegenerative diseases treatment.

Induction of Autophagy, a Contribution to Neurodegeneration Therapeutic Properties of Leucettines. Splicing is regulated by CLK1 through phosphorylation of SR proteins and it is influenced by environmental stresses (Biamonti and Caceres, 2009). Aberrant splicing is implicated in the development of many pathologies (Faustino and Cooper, 2003), including cancer (Kaida et al., 2012) and type 1 myotonic dystrophy (Romeo, 2012) and neurodegenerative diseases (Mills and Janitz, 2012). New drugs targeting splicing are currently under development (Bonnal et al., 2012) and could be of great benefit to fight these diseases.

Defective autophagy is linked to neurodegenerative diseases. Restoring defective autophagy provides protection against neurodegeneration (see Marino et al., 2011, and Hochfeld et al., 2013, for reviews on autophagy and neuroprotection). For instance, defective clearance of autophagosomes induces their accumulation in brain samples from patients suffering from Alzheimer's or Parkinson's diseases and is linked to neurodegeneration (Boland et al., 2008; Dehay et al., 2010; Vila et al., 2011). Moreover, lowering mTOR activity (thereby inducing autophagy) reduced amyloid- β and tau hyperphosphorylation, rescuing cognitive defects through

autophagy in mice models of Alzheimer's disease (Caccamo et al., 2010; Spilman et al., 2010). Rapamycin reduces cognitive impairments linked to plaques and tangles (Majumder et al., 2011). Autophagy downregulates amyloid- β levels (Tian et al., 2011). Recently, latrepirdine, a potential Alzheimer's disease drug, was shown to trigger autophagy, to reduce amyloid- β neuropathology, and to prevent behavioral deficits in Alzheimer's disease mouse models (Steele and Gandy, 2013). Stimulation of autophagy by trehalose in P301S tau transgenic mouse improves the tau pathology (Schaeffer and Goedert, 2012). Methylene blue also activates autophagy and reduces taupathy (Congdon et al., 2012). Altogether these data support the idea that increasing the basal level of autophagy should be beneficial in the treatment of neurodegenerative diseases like Alzheimer's disease.

By inducing autophagy through the mTOR/PI3K pathway leucettines could be used to target neurodegenerative diseases, the low toxicity of leucettine L41 being an asset. Our laboratory recently showed that leucettines protect against glutamate-induced neurotoxicity in HT22 cells (Tahtouh et al., 2012). Glutamate-mediated excitotoxicity contributes to acute and chronic central nervous system diseases. Leucettines' protective effects could thus be mediated by their proautophagic properties. Inhibition of DYRK1A is also seen as a promising approach for the treatment of Alzheimer's

disease. Thus, the ability of leucettines to inhibit both DYRK1A and CLKs and to trigger autophagy while not inducing cell death promotes this class of molecules as promising therapeutic drug candidates for Alzheimer's disease that deserve more investigation.

Authorship Contributions

Participated in research design: Fant, Meijer.

Conducted experiments: Fant, Durieu, Chicanne, Sbrissa.

Contributed new reagents or analytic tools: Limanton, Carreaux, Bazureau.

Performed data analysis: Fant, Chicanne, Payrastre, Shisheva, Meijer.

Wrote or contributed to the writing of the manuscript: Fant, Payrastre, Shisheva, Meijer.

References

- Allende-Vega N, Dayal S, Agarwala U, Sparks A, Bourdon JC, and Saville MK (2013) p53 is activated in response to disruption of the pre-mRNA splicing machinery. *Oncogene* **32**:1–14.
- Bettayeb K, Oumata N, Echalié A, Ferandin Y, Endicott JA, Galons H, and Meijer L (2008) CR8, a potent and selective, roscovitine-derived inhibitor of cyclin-dependent kinases. *Oncogene* **27**:5797–5807.
- Biamonti G and Caceres JF (2009) Cellular stress and RNA splicing. *Trends Biochem Sci* **34**:146–153.
- Blencowe BJ (2006) Alternative splicing: new insights from global analyses. *Cell* **126**:37–47.
- Boland B, Kumar A, Lee S, Platt FM, Wegiel J, Yu WH, and Nixon RA (2008) Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *J Neurosci* **28**:6926–6937.
- Bonnal S, Vigevari L, and Valcárcel J (2012) The spliceosome as a target of novel antitumor drugs. *Nat Rev Drug Discov* **11**:847–859.
- Caccamo A, Majumder S, Richardson A, Strong R, and Oddo S (2010) Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and Tau: effects on cognitive impairments. *J Biol Chem* **285**:13107–13120.
- Chan EY, Kir S, and Tooze SA (2007) siRNA screening of the kinome identifies ULK1 as a multidomain modulator of autophagy. *J Biol Chem* **282**:25464–25474.
- Chicanne G, Severin S, Boscheron C, Terrisse AD, Gratacap MP, Gaits-Iacovoni F, Tronchère H, and Payrastre B (2012) A novel mass assay to quantify the bioactive lipid PtdIns3P in various biological samples. *Biochem J* **447**:17–23.
- Congdon EE, Wu JW, Myeku N, Figueroa YH, Herman M, Marinac PS, Gestwicki JE, Dickey CA, Yu WH, and Duff KE (2012) Methylthioninium chloride (methylene blue) induces autophagy and attenuates tauopathy in vitro and in vivo. *Autophagy* **8**:609–622.
- Debdad M, Carreaux F, Renault S, Soundararajan M, Fedorov O, Filipakopoulos P, Lozach O, Babault L, Tahtouh T, and Baratte B et al. (2011) Leucettines, a class of potent inhibitors of cdc2-like kinases and dual specificity, tyrosine phosphorylation regulated kinases derived from the marine sponge leucettamine B: modulation of alternative pre-mRNA splicing. *J Med Chem* **54**:4172–4186.
- Deegan S, Saveljeva S, Gorman AM, and Samali A (2013) Stress-induced self-cannibalism: on the regulation of autophagy by endoplasmic reticulum stress. *Cell Mol Life Sci* **70**:2425–2441.
- de Graaf K, Hekerman P, Spelten O, Herrmann A, Packman LC, Büsow K, Müller-Newen G, and Becker W (2004) Characterization of cyclin L2, a novel cyclin with an arginine/serine-rich domain: phosphorylation by DYRK1A and colocalization with splicing factors. *J Biol Chem* **279**:4612–4624.
- Dehay B, Bové J, Rodríguez-Muela N, Perier C, Recasens A, Boya P, and Vila M (2010) Pathogenic lysosomal depletion in Parkinson's disease. *J Neurosci* **30**:12535–12544.
- de Lartigue J, Polson H, Feldman M, Shokat K, Tooze SA, Urbé S, and Clague MJ (2009) PIKfyve regulation of endosome-linked pathways. *Traffic* **10**:883–893.
- Duncan PI, Stojdl DF, Marius RM, and Bell JC (1997) In vivo regulation of alternative pre-mRNA splicing by the Clk1 protein kinase. *Mol Cell Biol* **17**:5996–6001.
- Ertmer A, Huber V, Gilch S, Yoshimori T, Erfle V, Duyster J, Elsässer HP, and Schätzl HM (2007) The anticancer drug imatinib induces cellular autophagy. *Leukemia* **21**:936–942.
- Faustino NA and Cooper TA (2003) Pre-mRNA splicing and human disease. *Genes Dev* **17**:419–437.
- Gui JF, Tronchère H, Chandler SD, and Fu XD (1994) Purification and characterization of a kinase specific for the serine- and arginine-rich pre-mRNA splicing factors. *Proc Natl Acad Sci USA* **91**:10824–10828.
- Hagiwara M (2005) Alternative splicing: a new drug target of the post-genome era. *Biochim Biophys Acta* **1754**:324–331.
- He C and Klionsky DJ (2009) Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet* **43**:67–93.
- Hochfeld WE, Lee S, and Rubinstein DC (2013) Therapeutic induction of autophagy to modulate neurodegenerative disease progression. *Acta Pharmacol Sin* **34**:600–604.
- Høyer-Hansen M and Jäättelä M (2007) AMP-activated protein kinase: a universal regulator of autophagy? *Autophagy* **3**:381–383.
- Ikononov OC, Sbrissa D, and Shisheva A (2009) YM201636, an inhibitor of retroviral budding and PIKfyve-catalyzed PtdIns(3,5)P₂ synthesis, halts glucose entry by insulin in adipocytes. *Biochem Biophys Res Commun* **382**:566–570.
- Jefferies HB, Cooke FT, Jat P, Boucheron C, Koizumi T, Hayakawa M, Kaizawa H, Ohishi T, Workman P, and Waterfield MD et al. (2008) A selective PIKfyve inhibitor blocks PtdIns(3,5)P₂ production and disrupts endomembrane transport and retroviral budding. *EMBO Rep* **9**:164–170.
- Kaida D, Schneider-Poetsch T, and Yoshida M (2012) Splicing in oncogenesis and tumor suppression. *Cancer Sci* **103**:1611–1616.
- Kimura S, Noda T, and Yoshimori T (2007) Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* **3**:452–460.
- Kuma A, Matsui M, and Mizushima N (2007) LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: caution in the interpretation of LC3 localization. *Autophagy* **3**:323–328.
- Leost M, Schultz C, Link A, Wu YZ, Biernat J, Mandelkow EM, Bibb JA, Snyder GL, Greengard P, and Zaharevitz DW et al. (2000) Paullones are potent inhibitors of glycogen synthase kinase-3beta and cyclin-dependent kinase 5/p25. *Eur J Biochem* **267**:5983–5994.
- Long JC and Caceres JF (2009) The SR protein family of splicing factors: master regulators of gene expression. *Biochem J* **417**:15–27.
- Maiuri MC, Galluzzi L, Morselli E, Kepp O, Malik SA, and Kroemer G (2010) Autophagy regulation by p53. *Curr Opin Cell Biol* **22**:181–185.
- Majumder S, Richardson A, Strong R, and Oddo S (2011) Inducing autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits. *PLoS ONE* **6**:e25416.
- Marcel V, Khoury MP, Fernandes K, Diot A, Lane DP, and Bourdon JC (2013) Detecting p53 isoforms at protein level. *Methods Mol Biol* **962**:15–29.
- Margariti A, Li H, Chen T, Martin D, Vizcay-Barrena G, Alam S, Karamariti E, Xiao Q, Zampetaki A, and Zhang Z et al. (2013) XBP1 mRNA splicing triggers an autophagic response in endothelial cells through BECLIN-1 transcriptional activation. *J Biol Chem* **288**:859–872.
- Mariño G, Madeo F, and Kroemer G (2011) Autophagy for tissue homeostasis and neuroprotection. *Curr Opin Cell Biol* **23**:198–206.
- Martin S, Harper CB, May LM, Coulson EJ, Meunier FA, and Osborne SL (2013) Inhibition of PIKfyve by YM-201636 dysregulates autophagy and leads to apoptosis-independent neuronal cell death. *PLoS ONE* **8**:e60152.
- Metzger Y, Gompel M, Thomas V, Garnier M, Leost M, Ceballos-Picot I, Noble M, Endicott J, Vierfond JM, and Meijer L (2003) Aloisines, a new family of CDK/GSK-3 inhibitors. SAR study, crystal structure in complex with CDK2, enzyme selectivity, and cellular effects. *J Med Chem* **46**:222–236.
- Mills JD and Janitz M (2012) Alternative splicing of mRNA in the molecular pathology of neurodegenerative diseases. *Neurobiol Aging* **33**:1012–1024.
- Mizushima N and Levine B (2010) Autophagy in mammalian development and differentiation. *Nat Cell Biol* **12**:823–830.
- Muraki M, Ohkawara B, Hosoya T, Onogi H, Koizumi J, Koizumi T, Sumi K, Yomoda J, Murray MV, and Kimura H et al. (2004) Manipulation of alternative splicing by a newly developed inhibitor of Clks. *J Biol Chem* **279**:24246–24254.
- Quidville V, Alsafadi S, Goubar A, Commo F, Scott V, Pioche-Durieu C, Girault I, Baconnais S, Le Cam E, and Lazar V et al. (2013) Targeting the deregulated spliceosome core machinery in cancer cells triggers mTOR blockade and autophagy. *Cancer Res* **73**:2247–2258.
- Romeo V (2012) Myotonic Dystrophy Type 1 or Steinert's disease. *Adv Exp Med Biol* **724**:239–257.
- Sarkar S, Floto RA, Berger Z, Imarisio S, Cordenier A, Pasco M, Cook LJ, and Rubinstein DC (2005) Lithium induces autophagy by inhibiting inositol monophosphatase. *J Cell Biol* **170**:1101–1111.
- Sbrissa D, Ikononov OC, and Shisheva A (1999) PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. *J Biol Chem* **274**:21589–21597.
- Sbrissa D, Ikononov OC, Deeb R, and Shisheva A (2002) Phosphatidylinositol 5-phosphate biosynthesis is linked to PIKfyve and is involved in osmotic response pathway in mammalian cells. *J Biol Chem* **277**:47276–47284.
- Sbrissa D, Ikononov OC, Filios C, Delvecchio K, and Shisheva A (2012) Functional dissociation between PIKfyve-synthesized PtdIns5P and PtdIns(3,5)P₂ by means of the PIKfyve inhibitor YM201636. *Am J Physiol Cell Physiol* **303**:C436–C446.
- Schaeffer V and Goedert M (2012) Stimulation of autophagy is neuroprotective in a mouse model of human tauopathy. *Autophagy* **8**:1686–1687.
- Shisheva A (2012) PIKfyve and its lipid products in health and in sickness. *Curr Top Microbiol Immunol* **362**:127–162.
- Shisheva A (2013) PtdIns5P: news and views of its appearance, disappearance and deeds. *Arch Biochem Biophys* **538**:171–180.
- Singh RK and Cooper TA (2012) Pre-mRNA splicing in disease and therapeutics. *Trends Mol Med* **18**:472–482.
- Smith B, Medda F, Gokhale V, Dunckley T, and Hulme C (2012) Recent advances in the design, synthesis, and biological evaluation of selective DYRK1A inhibitors: a new avenue for a disease modifying treatment of Alzheimer's? *ACS Chem Neurosci* **3**:857–872.
- Spilman P, Podlutska N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, Richardson A, Strong R, and Galvan V (2010) Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PLoS ONE* **5**:e9979.
- Steele JW and Gandy S (2013) Latrepirdine (Dimebon®), a potential Alzheimer therapeutic, regulates autophagy and neuropathology in an Alzheimer mouse model. *Autophagy* **9**:617–618.
- Tahtouh T, Elkins JM, Filipakopoulos P, Soundararajan M, Burgi G, Durieu E, Cochet C, Schmid RS, Lo DC, and Delhomel F et al. (2012) Selectivity, cocrystal structures, and neuroprotective properties of leucettines, a family of protein kinase inhibitors derived from the marine sponge alkaloid leucettamine B. *J Med Chem* **55**:9312–9330.

- Tchevkina E and Komelkov A (2012) Protein phosphorylation as a key mechanism of mTORC1/2 signaling pathways, in *Protein Phosphorylation in Human Health* (Huang C ed) InTech, Rijeka, Croatia.
- Tian Y, Bustos V, Flajolet M, and Greengard P (2011) A small-molecule enhancer of autophagy decreases levels of Abeta and APP-CTF via Atg5-dependent autophagy pathway. *FASEB J* **25**:1934–1942.
- Vila M, Bove J, Dehay B, Rodriguez-Muela N, and Boya P (2011) Lysosomal membrane permeabilization in Parkinson disease. *Autophagy* **7**:98–100.
- Wullschlegel S, Loewith R, and Hall MN (2006) TOR signaling in growth and metabolism. *Cell* **124**:471–484.
- Yang Z and Klionsky DJ (2010) Eaten alive: a history of macroautophagy. *Nat Cell Biol* **12**:814–822.
- Yomoda J, Muraki M, Kataoka N, Hosoya T, Suzuki M, Hagiwara M, and Kimura H (2008) Combination of Clk family kinase and SRp75 modulates alternative splicing of Adenovirus E1A. *Genes Cells* **13**:233–244.

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