The LCLAT1/LYCAT acyltransferase is required for EGF-mediated phosphatidylinositol-3,4,5-trisphosphate and Akt signalling

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Abbreviations: BSA: bovine serum albumin; EGF: epidermal growth factor: EGFR: epidermal growth factor receptor; IP_3: inositol-1,4,5-trisphosphate; ER: endoplasmic reticulum; PtdIns: phosphatidylinositol; PtdIns(3)P: phosphatidylinositol-3-phosphate; PtdIns(4,5)P_2:

phosphatidylinositol-4,5-bisphosphate; PtdIns(3,4,5)P_3: phosphatidylinositol-3,4,5-trisphosphate; PtdInsP: phosphoinositide; TIRF: total internal reflection fluorescence; TNBC: triple negative breast cancer

Abstract

Receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) stimulate phosphoinositide 3-kinases (PI3Ks) to convert phosphatidylinositol-4,5-bisphosophate [PtdIns(4,5)P_2] into phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3]. PtdIns(3,4,5)P_3 then remodels actin and gene expression patterns, and boosts cell survival and proliferation. PtdIns(3,4,5)P_3 partly achieves these functions by stimulating the kinase Akt, which phosphorylates targets like Tsc2 and GSK3β. Consequently, unchecked upregulation of PtdIns(3,4,5)P_3-Akt signalling promotes tumourgenesis. Interestingly, 50-70% of PtdIns and PtdInsPs have stearate and arachidonate at *sn*-1 and *sn*-2 positions of glycerol, respectively, forming a species known as 38:4-PtdIns/PtdInsPs. LCLAT1 and MBOAT7 acyltransferases are respectively responsible for enriching PtdIns in this acyl format. We previously showed that disruption of LCLAT1 lowered PtdIns(4,5)P_2 levels and perturbed endocytosis and endocytic trafficking. However, the role of LCLAT1 in receptor tyrosine kinase and PtdIns(3,4,5)P_3 signaling was not explored. Here, we show that LCLAT1 silencing in MDA-MB-231 and ARPE-19 cells abated the levels of PtdIns(3,4,5)P_3 in response to EGF signalling. Importantly, LCLAT1-silenced cells were also impaired for EGF-driven Akt activation and downstream signalling, and consequently, were depressed for cell proliferation and survival. Thus, our work provides first evidence that the LCLAT1 acyltransferase is required for receptor tyrosine kinase signalling and may represent a novel target for therapeutic development against cancers.

Introduction

Phosphoinositide (PtdInsP) lipid signaling orchestrates a variety of cellular functions such as organelle identity and membrane trafficking, ion channel activity, cytoskeletal organization, regulation of gene expression, modulation of metabolic activity, and cell proliferation and survival (Balla, 2013; Choy et al., 2017; Dickson and Hille, 2019; Doumane et al., 2022; Idevall-Hagren and De Camilli, 2015; Posor et al., 2022). PtdInsPs are generated by the reversible phosphorylation of the phosphatidylinositol (PtdIns) headgroup by several types of lipid kinases and phosphatases. Collectively, and based on the headgroup phosphorylation, these enzymes can generate up to seven species of PtdInsPs (Balla, 2013; Choy et al., 2017; Dickson and Hille, 2019; Posor et al., 2022). Nonetheless, there is another facet of PtdInsP biology that is poorly defined at the regulatory and functional levels – the control and function of the acyl composition of PtdInsPs (Barneda et al., 2019; Bozelli and Epand, 2019; Choy et al., 2017; D'Souza and Epand, 2014; Traynor-Kaplan et al., 2017).

In many mammalian tissues and cells, 50-70% of PtdIns and PtdInsPs are enriched for stearate and arachidonate at the *sn*-1 and *sn*-2 positions, respectively – this acyl combination is referred to as 38:4-PtdIns or 38:4-PtdInsPs (Anderson et al., 2013; Anderson et al., 2016; Barneda et al., 2019; D'Souza and Epand, 2014; Haag et al., 2012; Imae et al., 2012; Lee et al., 2012; Milne et al., 2005; Traynor-Kaplan et al., 2017). Additionally, this acyl composition is unique to PtdIns and PtdInsPs since other phospholipids have distinct acyl profiles (Barneda et al., 2019; Bozelli and Epand, 2019; Hicks et al., 2006; Traynor-Kaplan et al., 2017). This suggests that the acyl groups of PtdIns and PtdInsPs do more than simply embedding the lipids into the membrane bilayer (Barneda et al., 2019; Bozelli and Epand, 2019; Choy et al., 2017). However, the exact acyl profile of PtdIns and PtdInsPs can vary between cell types, environmental conditions, and

patho-physiological conditions such as cancer (Anderson et al., 2016, 10; Barneda et al., 2019; Bozelli and Epand, 2019; Hicks et al., 2006; Imae et al., 2012; Mujalli et al., 2018; Traynor-Kaplan et al., 2017). For example, $p53^{-/-}$ cancer cells, prostate cancer cells and triple negative breast cancer tissue all have distinct acyl profiles of PtdIns relative to normal tissue (Freyr Eiriksson et al., 2020; Koizumi et al., 2019; Naguib et al., 2015; Rueda-Rincon et al., 2015). Yet, there is much to be understood about the regulatory mechanisms that establish and remodel the acyl profile of PtdIns and PtdInsPs, and their functional implications.

The LCLAT1 acyltransferase has been identified as one of the enzymes that remodels and enriches PtdIns and/or PtdInsPs in stearate at the *sn*-1 position (Bone et al., 2017; D'Souza and Epand, 2014; Imae et al., 2012). LCLAT1 is an ER-localized protein and is thought to act during the Lands' Cycle or PtdIns Cycle to enrich PtdIns in stearate at *sn*-1 (Barneda et al., 2019; Blunsom and Cockcroft, 2020; Bone et al., 2017; Imae et al., 2012). Murine tissues deleted for LCLAT1 had reduced levels of 38:4-PtdIns, mono-PtdInsP, and bis-phosphorylated PtdInsPs (Imae et al., 2012). More recently, we observed that LCLAT1 silencing reduced the relative levels of endosomal phosphatidylinositol-3-phosphate [PtdIns(3)P] and phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] on the plasma membrane, but not phosphatidylinositol-4-phosphate [PtdIns(4)P] (Bone et al., 2017). Importantly, PtdIns and bis-phosphorylated PtdInsPs (mostly PtdIns(4,5)P_2), but not mono-phosphorylated PtdInsPs (mostly PtdIns(4)P] were altered in their acyl profile upon LCLAT1 silencing. This effect on specific pools of PtdInsPs has also been observed in cells disrupted for LPIAT1/MBOAT7, an acyltransferase thought to enrich PtdIns/PtdInsPs in arachidonic acid (Anderson et al., 2013).

PtdIns(4,5)P_2 regulates a number of functions including endocytosis, ion transport, and the cytoskeleton organization (Balla, 2013; Katan and Cockcroft, 2020; Sun et al., 2013).

PtdIns(4,5)P_2 is also a precursor for other signalling intermediates regulated by growth factor receptors like the Epidermal Growth Factor (EGF) and its receptor, the EGF receptor (EGFR), a major receptor tyrosine kinase (Katan and Cockcroft, 2020; Orofiamma et al., 2022). EGF binds and dimerizes EGFR, leading to receptor trans-autophosphorylation on various tyrosine residues (Böni-Schnetzler and Pilch, 1987; Gullick et al., 1985; Honegger et al., 1987; Koland and Cerione, 1988; Linggi and Carpenter, 2006; Yarden and Schlessinger, 1987). These phospho-tyrosines serve as docking sites for adaptor proteins like Gab1 and Grb2, which assemble a signaling complex composed of other protein kinases and phosphatases, and lipid-metabolizing enzymes (Holgado-Madruga et al., 1996, 2; Margolis et al., 1990a; Orofiamma et al., 2022; Rodrigues et al., 2000). For example, active EGFR recruits and activates Phospholipase Cγ (PLCγ), which hydrolyses PtdIns(4,5)P_2 into diacylglycerol and inositol-1,4,5-trisphosphate (IP_3), and which releases Ca^{2+} from endoplasmic reticulum stores (Delos Santos et al., 2017; Margolis et al., 1990a; Margolis et al., 1990b). In addition, Gab1 recruits the Class I PI3Ks to convert PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 (Kiyatkin et al., 2006; Rodrigues et al., 2000). This burst of PtdIns(3,4,5)P_3 then recruits and activates PDK1 and Akt protein kinases (Alessi et al., 1997; Bellacosa et al., 1998; Manning and Toker, 2017; Stokoe et al., 1997). Akt is a major driver of cell metabolism and growth by phosphorylating numerous targets like GSK3β and TSC2 (Cross et al., 1995; Inoki et al., 2002; Manning and Toker, 2017; Rodgers et al., 2017; Sugiyama et al., 2019). For example, Akt inactivates TSC2, a GTPase activating protein (GAP) for the Rheb GTPase, thus promoting the mTORC1 pathway (Inoki et al., 2002; Inoki et al., 2003). The PtdIns(3,4,5)P_3-Akt-mTORC1 pathway is a major driver of cell growth and proliferation (Dey et al., 2017; Dibble and Manning, 2013; Manning and Toker, 2017; Sugiyama et al., 2019). As a result, mutations that hyperactivate

this pathway are often associated with human cancers like triple-negative breast cancer (TNBC) (Dey et al., 2017; Li et al., 2017).

Overall, given that LCLAT1 is a PI acyltransferase and the connection between EGF and PI3K-Akt pathway, we postulated that LCLAT1 disruption would impair EGF-mediated PtdIns(3,4,5)P_3-Akt signalling. In fact, we found that LCLAT1 silencing in at least two cell lines impeded generation of PtdIns(3,4,5)P_3 and Akt activation upon addition of EGF. Moreover, LCLAT1 disruption significantly hindered cell proliferation and survival, positioning LCLAT1 as a target for drug development.

Methods and Materials

Cell Culture

The male human-derived ARPE-19 retinal pigment epithelial cell line was obtained from ATCC (CRL-2302, Manassas, VA) and was cultured in DMEM/F12 medium (ThermoFisher Scientific, Mississauga, ON) supplemented with 10% fetal bovine serum (Wisent, St. Bruno, QB), 100 U/mL penicillin and 100 µg/mL streptomycin (ThermoFisher Scientific). The female human-derived MDA-MB-231 triple negative breast cancer cell line was obtained from ATCC (CRM-HTB-26). Wild-type MDA-MB-231 cells and its derivatives (see below) were cultured in DMEM medium (Wisent) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The female human-derived HEK293T cell line was cultured in DMEM with 10% fetal calf serum and 1% penicillin/streptomycin. All cells were cultured at 37°C and 5% CO_2. *Mycoplasma* screening is performed at least annually.

Transfection and siRNA-mediated gene silencing

To silence gene expression of LCLAT1 in both ARPE-19 and MDA-MB-231 cells, custom-synthesized siRNA oligonucleotides against LCLAT1 were designed using Horizon Discovery siDESIGN Centre. We designed and tested siLCLAT1-1, siLCLAT1-7, and siLCLAT1-8 with the respective sequences 5'-GGAAAUGGAAGGAUGACAAUU-3', 5'CAGCAAGUCUCGAAGUAAUU-3', and 5'-UCGAAGACAUGAUUGAUUAUU-3'. Synthesis was by Sigma-Aldrich (Oakville, ON). In addition, we used siLCLAT1-5, a siGenome-validated oligonucleotide from Horizon (cat. D-010307-01-0002). Moreover, a non-targeting control siRNA (NT siRNA, or siCON) with the sequence 5'-CGUACUGCUUGCGAUACGGUU-3' was used (Sigma-Aldrich). Cells were transfected with 22 pmol of siRNA oligonucleotides/well using Lipofectamine RNAiMAX (ThermoFisher Scientifc) in Opti-MEM reduced serum media (ThermoFisher Scientific) for 3 h at 37°C and 5% CO_2 as per manufacturer's instructions. After transfection, cells were washed and incubated with fresh growth medium. Two rounds of transfection were performed, 72 and 48 h before each experiment.

Plasmids and transfections

Plasmid encoding PH-Akt-GFP was a kind gift from the Balla lab, NIH (Addgene: #51465) and was previously described in (Várnai and Balla, 1998). PH-Akt-GFP plasmid was transfected into ARPE-19 and MDA-MB-231 cells using Lipofectamine 3000 as instructed by the manufacturer. The plasmid encoding PLCδ-PH-GFP was previously described in (Stauffer et al., 1998) and used to generate MDA-MB-231 cells stably expressing the PtdIns(4,5)P_2 biosensor.

Other plasmids used in this work were used to generate MDA-MB-231 cells equipped for inducible deletion of LCLAT1 by CRISPR-Cas9. All plasmids were obtained from Addgene (Watertown, MA): pMD2.G (Addgene: #12259) and psPAX2(Addgene: #12260) were kind gifts

from Dr. Didier Trono; we also used lenti Cas9-blast (Addgene: #52962; (Sanjana et al., 2014)), lentiguide-Puro (Addgene: #52963; (Sanjana et al., 2014)), and PXPR_011 (Addgene: #59702; (Doench et al., 2014)). The plasmid pEZ-Tet-pLKO-hygro (Addgene plasmid 85972; (Frank et al., 2017)) was adapted to express sgRNAs, and is hereafter referred to as "pEZ-TET-pLKO-guide-hygro".

Lentivirus Production

9 x10^6 HEK293T cells were seeded on 15 cm plates 24 hours prior to transfection with 50 µg of lentiviral vector, 37.5 µg of psPAX2, and 15 µg of pMD2.G per plate using the Calcium Phosphate method. Viral media was harvested 48 hours post-transfection, cleared via centrifugation at 500 *g* for 10 min and filtered prior to concentration by ultracentrifugation at 50,000 g for 2.5 hours.

MDA-MB-231 Cas9 Generation

MDA-MB-231 cells were infected with lentiCas9-blast virus in the presence of 8 µg/mL protamine sulfate in a 6-well plate. Forty-eight hours post-transduction, media were changed and transduced cells were selected with 13 µg/mL blasticidin for seven days. CRISPR-Cas9 genome editing capability of MDA-MB-231 cells was tested as follows using an EGFP ablation assay. MDA-MB-231 and MDA-MB-231 Cas9 cells were infected with pXPR_011 virus in the presence of 8 µg/mL protamine sulfate in a 6-well plate. Forty-eight hours post-transfection, media were changed and transduced cells were selected with 4 µg/mL puromycin for 2 days. GFP fluorescence was measured by flow cytometry 5 days post selection. The pXPR_011 plasmid contains both EGFP and a gRNA sequence that targets *EGFP*, and the percentage of GFP loss compared to control was used as a measurement of Cas9 activity.

Inducible gRNA Construction

gRNAs (Scaffold and Spacer) were PCR amplified using AccuPrime Pfx SuperMix (template and primers listed below) and digested with EcoRI and NheI overnight at 37°C. The pEZ-tet-pLKO-gRNA-hygR vector was digested with EcoRI and NheI overnight at 37°C, treated with Antarctic Phosphatase at 37°C for 1 hour, and gel purified using the QIAquick Gel Extraction Kit. The digested PCR products were purified and ligated into the linearized vector using T4 DNA ligase following the manufacturer's instructions. Ligation products were transformed into DH5α, prepped with the QIAGEN DNA Maxi Kit, and verified by Sanger Sequencing.

PCR Template: lentiGuide-puro

gLacZ forward: gctagcCCCGAATCTCTATCGTGCGGgttttagagctagaaatagcaagt

gLCLAT1-1 forward: GGCCgctagcACAACCGCCTTGTGGCAACAgttttagagctagaaatagcaagt

gLCLAT1-2 forward: GGCCgctagcTCTGATTGAGGAATGTTGTGgttttagagctagaaatagcaagt

gLCLAT1-3 forward: GGCCgctagcTGTAAAAGTGATTATAACTGgttttagagctagaaatagcaagt

Universal Scaffold reverse: CCGAATTCtcaagatctagttacgccaagctt

Inducible Cas9-mediated LCLAT1 Knockout

MDA-MB-231 Cas9 cells were infected with pEZ-tet-pLKO-gLacZ-hygR or pEZ-tet-pLKO-gLCLAT1-hygR virus in the presence of 8 µg/mL protamine sulfate in a 6-well plate. Forty-eight hours post-transduction, media was changed and transduced cells were selected with 500 µg/mL hygromycin for 10 days. Thereafter, expression of LCLAT1-sgRNAs were induced by treatment of cells with 1 µg/ml of doxycycline for at least 48 hours.

EGF signaling and Western blotting

Before lysate preparation, cells were incubated with 2 mL of serum free growth medium for 1 h. After serum starvation, cells were stimulated with 5 ng/mL EGF for 5 or 10 min or left unstimulated (basal). Following serum starvation and subsequent EGF stimulation, whole cell lysates were prepared in 200 µL 2x Laemmli Sample Buffer (0.5M Tris, pH 6.8, glycerol and 10% sodium dodecyl sulfate (SDS)) supplemented with a protease and phosphatase inhibitor cocktail (Complete 1x protease inhibitor (Sigma-Aldrich), 1 mM sodium orthovanadate, and 10 nM okadaic acid). Lysates were heated at 65°C for 15 min and passed through a 27-gauge needle 10 times. Finally, 10% ß-mercaptoethanol and 5% bromophenol blue were added to cell lysates.

Proteins were resolved by Tris-glycine SDS-PAGE and transferred on to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked for 1 h at room temperature in blocking buffer composed of 3% bovine serum albumin (BSA) in 1X final Tris-buffered saline-Tween (TBS-T; 20 nM Tris, 150 mM NaCl and 0.1% Tween-20). After blocking, membranes were washed 3 times with wash buffer and incubated with 1:1000 primary antibody overnight at 4°C. The next day, membranes were washed 3 times, for 5 min each, and then subjected to 1:1000 secondary antibody for 1 h at room temperature. After incubation with secondary antibody, membranes were washed 3 times, 5 min each, and imaged using the ChemiDoc Imaging System (BioRad). Membranes were exposed to Immobilon Crescendo Western HRP substrate (Millipore Sigma) for 30-60 s and chemiluminescent images were acquired by the ChemiDoc System. Western blot signals were analyzed and quantified using the ImageLab 6.1 (BioRad). Band intensity was obtained by signal integration in an area corresponding to the appropriate band. This value was then normalized to the loading control signal. Phosphorylated protein levels were normalized to total protein levels, and if necessary, the loading control.

Primary antibodies raised in rabbit were anti-LCLAT1 (cat. 106759, GeneTex), anti-phospho-Akt (S473, cat. 9271), anti-phospho-Akt1 (S473, cat. 9018), anti-phospho-Akt2 (S474, cat. 8599), anti-Akt1 (cat. 2938), anti-phospho-EGFR (Y1068. Cat. 2234), anti-phospho-tuberin/TSC2 (T1462, cat. 3611), anti-tuberin/TSC2 (cat. 3612), anti- phospho-MDM2 (S166, cat. 3521), anti-MDM2 (cat. 86934), anti-phospho-GSK3ß (S9, cat. 9323), anti-clathrin (cat. 4796), and anti-GAPDH (cat. 2118) were all from Cell Signaling Technologies. Antibodies raised in mouse were anti-Akt (cat. 2920), anti-Akt2 (cat. 5239), anti-GSK3ß (cat. 9832), and anti-puromycin (cat. MABE343) and were all from Cell Signaling Technology. Goat anti-EGFR antibodies were from Santa Cruz Biotechnology (sc-03-G). Horseradish peroxidase (HRP)-linked secondary anti-rabbit, anti-mouse, and anti-goat IgG antibodies were from Cell Signaling Technology.

Immunofluorescence

To detect surface levels of EGFR in MDA-MB-231 and ARPE-19 cells, cells were blocked with 3% BSA in PBS supplemented with 1 mM $CaCl_2$ and $MgCl_2$ for 30 min on ice, followed by 1 h incubation with a 1:200 dilution of mouse anti-EGFR antibody collected in-house from the mAb108 hybridoma obtained from ATCC (Cabral-Dias et al., 2022). After washing with PBS, cells were fixed with 4% paraformaldehyde in PBS for 15 min and then quenched with 100 mM glycine in PBS for 10 min, followed by washing with PBS, and then fluorescent secondary mouse antibodies (Jackson ImmunoResearch Labs Inc., West Grove, PA) at a 1:500 dilution in 1% BSA in PBS for 1 h at room temperature. The coverslips were mounted using Dako fluorescence mounting medium (Agilent Technologies, Inc. Mississauga, ON, Canada).

Microscopy

Confocal and TIRF micrographs were obtained using a Quorum Diskovery spinning disc confocal system coupled to a TIRF module (Quorum Technologies, Inc., Guelph, ON). The microscope itself consisted of an inverted fluorescence microscope (DMi8; Leica) equipped with an Andor Zyla 4.2 Megapixel sCMOS camera (Oxford Instruments, Belfast, UK), a 63x oil immersion objective (1.4 NA), and standard excitation and emission filter sets and lasers were used for all fluorophores. The TIRF module used a 63x/NA1.49 objective with a 1.8x camera relay (total magnification 108x). The microscope system was controlled by the MetaMorph acquisition software (Molecular Devices, LLC, San Jose, CA, USA).

For fixed cells, z-stacks of 10-30 images were acquired with an inter-planal distance of 0.6 µm distance. For live-cell imaging of Akt-PH-GFP dynamics in ARPE-19 cells or of PLCδ-PH-GFP in MDA-MB-231 cells using spinning disc confocal or TIRF microscopy, cells were maintained in DMEM free of phenol red or serum in a Chamlide microscope-mounted chamber at 37°C and 5% CO_2. For timelapse of Akt-PH-GFP in MDA-MB-231 cells, a baseline was obtained by acquiring images for 1 min at 15 s, then EGF added and images acquired every 15 s for 10 min.

Image Analysis and Processing

Image processing and quantitative analysis were performed using ImageJ or FIJI v. 2.3 (Schindelin et al., 2012) or Volocity v. 7 (Quorum Technologies), where image enhancements were completed without altering the quantitative relationship between image elements. For quantification of EGFR cell surface, regions of interest were generated by freehand to define the cell outline, the mean fluorescence intensity over the whole cell area was calculated, and then background corrected (Cabral-Dias et al., 2022). Mean fluorescence from at least 30 cells per condition per experiment

was then normalized against control condition. To obtain the relative cell surface localization index for the Akt-PH-GFP probe in ARPE-19 cells, we used ImageJ to determine the ratio of TIRF/epifluorescence fluorescence for >100 cells per condition per experiment. To measure the plasma membrane to cytosolic ratio of PLCδ-PH-GFP or Akt-PH-GFP in MDA-MB-231 cells (Cabral-Dias et al., 2021), we used randomly selected regions of the plasma membrane and cytosol for each cell over timelapse movies using ImageJ and then calculated the fluorescence ratio. We examined at least 23 cells over three experiments.

Cell Proliferation

Cellular viability and cytotoxicity were measured using an Incucyte SX5 Live-Cell Analysis System (Sartorius). Cell death was assessed using CellTox Green Cytotoxicity Assay reagent (Promega G8741) at 1x final dilution in 12 mL of media and added to cells prior to imaging. Images were acquired at 30-min intervals for 72 h under 10x magnification using brightfield phase contrast and 300 ms exposure of 460 nm excitation, 524 nm emission to detect green fluorescence. Time-lapse image series were analyzed using the Incucyte Basic Analysis Software. Cellular confluence was normalized to initial phase contrast confluence for each sample.

Lipid extraction

Cells were grown to ~0.5×10^6 per well in a 6-well plate. Cells were then placed on ice and the media removed. Cells were scraped in 500 μL of ice cold 1M HCl and transferred to a pre-cooled safe lock 2 mL microcentrifuge tube. Cells were then collected by centrifugation at 13,000 xg for 10 min at 4°C. The supernatant was aspirated, and the pellet was then snap frozen in liquid nitrogen.

Mass Spectrometry Lipid Analysis

Mass spectrometry was used to measure PtdInsPs from lipid extracts prepared from 0.5-1x10^6 MDA-MB-231 cells or 0.6x10^6 ARPE-19 cells as previously described (Clark et al., 2011). Briefly, we used a QTRAP 4000 mass spectrometer (AB Sciex, Macclesfield, UK) and employing the lipid extraction and derivatization method described for cultured cells (Clark et al., 2011). The response ratios of a specific PtdInsP acyl species were calculated by normalizing the targeted lipid integrated response area to that of a known amount of added relevant internal standard. A ratio of ratios was then calculated by taking the response ratios of 38:4-PtdIns, 38-4-mono-PtdInsP, 38-4-bis-PtdInsP, and 38:4-PtdIns(3,4,5)P_3 against the sum of response ratios for 36:1 and 36:2 (36:x) of PtdIns or the corresponding 36:x-mono-PtdInsP or 36:x-bis-PtdInsP. Data are presented as mean ± STD from four separate experiments.

Statistical analysis

All experiments were repeated a minimum of three independent times - specific repeat number for each experiment is indicated in the respective figure legend and/or graph as individual data points. Data were collected as mean ± standard deviation (STD) or ± standard error of the mean (SEM) as appropriate. Statistical comparisons of means were then performed with GraphPad Prism v. 10. Statistical tests were selected based on data conditions such as number of parameters, sample size, and normality or assumed normality. Figure legends specify the tests employed for a given data set such as Mann-Whitney, one-way or two-way ANOVA tests, and associated post-hoc tests. $p < 0.05$ was used to determine statistical significance.

Results

LCLAT1 acyltransferase regulation of EGFR trafficking

In our previous work, LCLAT1 silencing altered the endocytosis and endosomal trafficking of the transferrin receptor in ARPE-19 cells (Bone et al., 2017). We thus queried if LCLAT1 disruption would alter the total and surface levels of EGFR and/or affect EGFR signalling in at least two human-derived cells lines: the non-cancerous, male-derived ARPE-19 and the female-derived triple negative breast cancer cell line, MDA-MB-231. To do this, we used previously designed and validated siRNA oligonucleotides against LCLAT1 (Bone et al., 2017) and a non-targeting oligonucleotide (see Methods), transiently transfected cells twice, and after 48 h, we lysed the cells and probed for LCLAT1 levels by Western blotting. As shown in Figure 1A and Figure 2A respectively, control ARPE-19 and MDA-MB-231 cells displayed a major band at 35 kDa. After transfection with siLCLAT1-1, this band was reduced by >70% intensity in both cell types, manifesting efficient LCLAT1 silencing (Fig. 1A, B and Fig. 2A, B). We also observed reduced LCLAT1 expression in ARPE-19 and MDA-MB-231 cells transfected with independent siRNA oligonucleotides against LCLAT1 (Supplemental Figure S1A, B and S2A, B). We note that silencing of LCLAT1 often reduced protein levels in our samples despite starting with the same cell number, likely due to cell death and abated growth (see below). We thus normalize our Western blot signals to proteins such as GADPH and clathrin heavy chain to account for this apparent cell loss.

We next examined the effects of LCLAT1 silencing on total and surface levels of EGFR in these cells. We observed that LCLAT1 silencing in ARPE-19 and MDA-MB-231 cells did not alter total EGFR levels as measured by Western blotting (Fig. 1C, 1D and 2C, 2D) nor the EGFR surface levels as measured by immunofluorescence of unpermeabilized cells under basal conditions (Fig. 1E and 2E). Moreover, EGFR was internalized at similar rates in non-targeted and

LCLAT1-silenced ARPE-19 (Fig. 1E) and MDA-MB-231 cells (Fig. 2E) after 1 h of 100 ng/mL EGF stimulation. Importantly, LCLAT1-silencing did not impair EGF-mediated phosphorylation of Y1068 of EGFR in ARPE-19 cells (Fig. 1C, F) and MDA-MB-231 cells (Fig. 2C, F). Overall, these data suggest that the steady-state levels and trafficking of EGFR, and immediate response to EGF in ARPE-19 and MDA-MB-231 cells were not affected by LCLAT1 silencing.

LCLAT1 acyltransferase silencing reduces PtdIns(3,4,5)P_3 synthesis in response to EGF

We previously observed that ARPE-19 cells silenced for LCLAT1 had ~30% reduction in PtdIns(4,5)P_2 levels (Bone et al., 2017). To determine if this was recapitulated in MDA-MB-231 cells, we generated cells stably expressing PLCδ-PH-GFP, a reporter for PtdIns(4,5)P_2 (Stauffer et al., 1998). Upon silencing of LCLAT1 in these cells, we observed a significant decrease in the PLCδ-PH-GFP fluorescence ratio of plasma membrane to cytosol relative to non-silenced cells (Fig. 3A,B) suggesting that LCLAT1-silenced MDA-MB-231 cells also had less PtdIns(4,5)P_2. Next, a key outcome of EGFR stimulation is the activation of PI3Ks to convert PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 (Hu et al., 1992; Orofiamma et al., 2022; Rodrigues et al., 2000). To determine if PtdIns(3,4,5)P_3 synthesis was affected in LCLAT1-disturbed cells, we transfected ARPE-19 and MDA-MB-231 cells with plasmids encoding Akt-PH-GFP, a biosensor for 3-phosphorylated PtdInsPs (Várnai and Balla, 1998). The recruitment of Akt-PH-GFP to the plasma membrane in response to EGF was then quantified using two different methods. Given that ARPE-19 cells are exceptionally flat, we quantified the ratio of TIRF to epifluorescence fields (TIRF/Epi fluorescence ratio) as an indicator for PtdIns(3,4,5)P_3 levels at the plasma membrane. By comparing EGF-stimulated non-targeted and LCLAT1-silenced cells, we observed a lower Akt-PH-GFP TIRF/Epi fluorescence ratio in latter cells, nearly comparable to cells incubated with the PI3K inhibitor,

LY294002 (Fig. 3C, D). Thus, despite normal EGFR levels and early response, ARPE-19 cells were defective in EGF-mediated PI3K signalling. We then assessed if MDA-MB-231 cells were also impaired for PI3K signalling. Since these cells are rounder, we measured Akt-PH-GFP on the plasma membrane more readily by sampling Akt-PH-GFP at the cell periphery against cytosolic signal. We tracked the Akt-PH-GFP recruitment over 10 min after adding EGF. We observed an increase in Akt-PH-GFP to the cell periphery after EGF stimulation of non-silenced MDA-MB-231 cells (Fig. 3E, F). Importantly, this increase in Akt-PH-GFP at the cell periphery was suppressed in the LCLAT1-silenced cell group (Fig. 3E, F). Hence, we reveal that LCLAT1 expression is required to support EGF-EGFR lipid signalling in at least two cell types.

The impact of LCLAT1 acyltransferase expression on the acyl profile of PtdInsPs

We next examined the PtdInsP acyl profile and their relative levels by mass spectrometry in control, serum starved, and EGF-stimulated non-silenced and LCLAT1-silenced ARPE-19 and MDA-MB-231 cells. For this analysis, we normalized lipid spectral counts against synthetic standards added to the samples to generate a response ratio (see methods). In addition, to correct for variation in cell input between experiments, we further normalized against an internal benchmark by comparing changes in 38:4 PtdIns, *mono*-PtdInsPs, *bis*-PtdInsPs, and PtdIns(3,4,5)P_3 relative to standardized 36:x-PtdIns and the corresponding 36:x-mono-PtdInsP and 36:xbis-PtdInsP since these species have previously been shown to be less affected by LCLAT1 expression relative to 38:4 species (Bone et al., 2017; Imae et al., 2012). We note that our previous normalization benchmark used the 38:x PtdInsP (not just 38:4) to its respective 36:x PtdInsP and an internal benchmark was not used (Bone et al., 2017).

For ARPE-19 cells, the major acyl species of PtdIns, *mono*-, *bis*-, and *tris*-PtdInsP was 38:4, as expected. In fact, we only detected 38:4 acyl species for PtdIns(3,4,5)P_3. We then compared each group of 38:4 PtdInsPs to the 36:x-PtdIns and the corresponding 36:x-PtdInsP. Relative to 36:x-PtdIns, we saw that the relative levels of 38:4-PtdIns was reduced in LCLAT1-silenced cells (Fig. 4A). In comparison, there was no significant difference in 38:4-*mono*-PtdInsPs relative to 36:x-PtdIns or 36:x-mono-PtdInsP in any treatment (Fig. 4B, Sup. Fig. S3A). In comparison, 38:4-*bis*-PtdInsPs was lower in EGF-stimulated LCLAT1-silenced cells relative to 36:x-PtdIns (Fig. 4C). In addition, while statistical significance was not reached as defined by $p<0.05$, all p values were low in resting and serum-starved cells (Fig. 4C) and 36:x-bis-PtdInsPs (Sup. Fig. S3B). Lastly, EGF-stimulated LCLAT1-silenced cells had lower 38:4-PtdIns(3,4,5)P_3 levels relative to 36:x-PtdIns than non-targeted ARPE-19 cells (Fig. 4D). We could not quantitatively compare 38:4-PtdIns(3,4,5)P_3 to 36:x-PtdIns(3,4,5)P_3 since we did not detect the latter; but nonetheless, this suggests that 38:4-PtdIns(3,4,5)P_3 is the predominant acyl species of this phosphoinositide upon EGF stimulation and that it depends on LCLAT1 for efficient synthesis. Overall, we reveal that ARPE-19 cells shift their acyl profile of PtdIns, *bis*-PtdInsPs, and PtdIns(3,4,5)P_3 upon LCLAT1-disruption, but not for *mono*-PtdInsPs.

We similarly investigated the lipidomic profile of MDA-MB-231 cells. The major acyl species for all PtdInsPs and PtdIns was again 38:4 and we once again only detected 38:4-PtdIns(3,4,5)P_3 acyl species in our samples. Interestingly, and unlike ARPE-19 cells, LCLAT1 suppression did not alter the relative levels of 38:4-PtdIns, 38:4-*mono*-PtdInsPs or 38:4-*bis*-PtdInsPs relative to 36:x-PtdIns or the respective 36:x-*mono*-PtdInsP or 36:x-*bis*-PtdInsP whether cells were resting, serum-starved or EGF-stimulated (Figure 4E, F, G, Sup. Fig. S3C, D). Despite these observations, the relative levels of 38:4-PtdIns(3,4,5)P_3 were once again abated after EGF

stimulation in LCLAT1-perturbed cells relative to 36:x-PtdIns (Fig. 4H). Overall, we propose that LCLAT1 expression is essential to support EGFR-dependent activation of PI3K signalling in at least two distinct cell lines, but the impact on acyl profile can vary between cell type, lipid benchmarks, and treatments.

Akt activation by EGF is defective in LCLAT1-silenced cells

Since LCLAT1-silenced cells had lower EGF-induced PtdInsP(3,4,5)P_3 levels relative to non-silenced counterparts, we next examined if Akt activation was also impaired in ARPE-19 and MDA-MB-231 cells after EGF exposure. To do this, we probed for phosphorylation at S473 using an antibody that recognizes all isoforms of Akt when phosphorylated (pan-phospho-Akt antibody). Relative to serum-starved ARPE-19 and MDA-MB-231 cells, EGF caused a large increase in phospho-Akt in non-silenced control cells (Fig. 5A, B and Fig. 6A, B). Importantly, both ARPE-19 and MDA-MB-231 cells silenced for LCLAT1 displayed substantially reduced phospho-Akt levels after EGF stimulation (Fig. 5A, B and Fig. 6A, B). We also observed impaired p-Akt levels in ARPE-19 cells (Supplemental Figure S1A, C) and MDA-MB-231 cells (Supplemental Figure S2A, C) treated with independent oligonucleotides against LCLAT1. We then probed with anti-p-Akt1 (S473) and p-Akt2 (S474) antibodies to test if there was an isoform specific effect. We reveal that ARPE-19 and MDA-MB-231 silenced for LCLAT1 after EGF stimulation have lower levels of both p-Akt1 and p-Akt2 relative to their respective total Akt1 and Akt2 (Fig. 5C-F and Fig. 6C-F). Thus, LCLAT1 silencing negatively and broadly impacts Akt activation.

LCLAT1 acyltransferase silencing impairs Akt-mediated regulation of downstream targets

Since Akt activation is defective in LCLAT1-silenced cells after addition of EGF, we next examined if this effect percolated to several known Akt targets. To test for specific targets, we measured the phosphorylation state of Tsc2, GSK3β, and Mdm2 by Western blotting. In ARPE-19 and MDA-MB-231 cells transfected with non-targeting oligonucleotides, EGF promoted phosphorylation of these specific Akt substrates (Fig. 7). By contrast, LCLAT1-silenced cells suffered a considerable reduction in this EGF-induced phosphorylation of Tsc2 and GSK3β normalized to their respective totals (Fig. 7A-D, 7G-J). Interestingly, while there was an apparent decrease in p-MDM2 after EGF stimulation in LCLAT1-silenced cells, when normalized to total MDM2, there was little difference (Fig. 7E, F, K, L). Instead, total MDM2 protein expression was significantly reduced in LCLAT1-sienced cells indicating an additional regulatory level imposed on MDM2 that depends on LCLAT1 expression (Fig. 7E, F, K, L).

LCLAT1 acyltransferase disruption impairs cell growth

Our observations hereto suggest that LCLAT1 is required to activate the PtdIns(3,4,5)P_3-Akt pathway in response to EGF signaling. This pathway is essential to promote cell proliferation and survival. We thus predicted that loss of LCLAT1 expression would impede cell proliferation and/or survival. For these experiments, we first focused on MDA-MB-231 cells transiently silenced for LCLAT1. We tracked growth of LCLAT1-silenced and control MDA-MB-231 cells in an Incucyte system and quantified change in confluency as a measure of growth (Supplemental Videos 1 and 2). Concurrently, we tracked cell death by staining with CellTox Green and quantifying number of stained dead cells (Supplemental Videos 1 and 2). As shown in Fig. 8A-C and Supplemental Videos, we found that MDA-MB-231 cells stagnated in their growth and died at a greater rate than cells exposed to non-targeting siRNA oligonucleotides. However, since these

observations require prolonged observation, we complemented transient gene silencing with a model that allows for inducible, yet stable disruption of LCLAT1 expression. We generated MDA-MB-231 cells that constitutively expressed the Cas9 recombinase (Fig. 8D). These cells were then further modified by the stable insertion of doxycycline-inducible expression of gRNAs targeting LCLAT1. We generated three cell lines using three different gRNAs and tested the efficiency of induced CRISPR-based knockout of LCLAT1 in these MDA-MB-231-Cas9 sublines by treating cells with 0.1 or 1 µg/mL doxycycline over 3-5 days and then probed for LCLAT1 expression. We found that gRNA2 was most efficient at disrupting LCLAT1 expression (Fig. 8E, F).

Having established this new model system to investigate the role of LCLAT1 in MDA-MB-231 cells, we next assessed the ability of these cells to proliferate and survive after LCLAT1 deletion. To do this, we used the Incucyte SX5 imaging incubator system over 72 h and tracked change in confluency as an indicator or proliferation. As shown in Fig. 8G, H and Supplemental Videos 3 and 4, MDA-MB-231-Cas9 cells became confluent over 72 h with or without doxycycline. In comparison, MDA-MB-231-Cas9 cells engineered with the LCLAT1 gRNA2 and exposed to doxycycline to delete LCLAT1 grew at a slower rate relative to its counterparts without doxycycline and parent MDA-MB-231-Cas9 cells (Fig. 8D, E, Sup. Videos 5 and 6). Overall, LCLAT1 expression is needed to support EGF signalling and its PtdIns(3,4,5)P_3-Akt pathway in at least two distinct cell types and to promote cell proliferation and survival.

Discussion

Here, we reveal that the LCLAT1 acyltransferase is needed to promote EGFR-mediated PtdIns(3,4,5)P_3-Akt signaling in at least two cell lines. While EGFR levels and its early activation by EGF were unaffected, cells perturbed for LCLAT1 had reduced PtdIns(3,4,5)P_3 levels,

diminished Akt activation and downstream factors, and consequently these cells were impaired for proliferation. We thus identified an important role for the poorly studied LCLAT1 acyltransferase and reveal that this enzyme is a target for drug targeting to treat cancer.

The role of LCLAT1 in generating PtdIns(3,4,5)P_3

We provide at least three pieces of evidence that LCLAT1 is important to generate PtdIns(3,4,5)P_3 during EGF-mediated signalling. First, we revealed that LCLAT1-silenced MDA-MB-231 and ARPE-19 cells were impaired for the recruitment of the Akt-PH-GFP, a biosensor for PtdIns(3,4,5)P_3 levels (Haugh et al., 2000; Marshall et al., 2001), to the plasma membrane after EGF stimulation. Second, we observed that LCLAT1-silenced cells possessed lower relative levels of 38:4-PtdIns(3,4,5)P_3. Incidentally, 38:4-PtdIns(3,4,5)P_3 was the only observable acyl-based species for PtdIns(3,4,5)P_3 in both cell types, likely because the other acyl isoforms of PtdIns(3,4,5)P_3 were below the detection limit of our current analysis. Regardless, this is consistent with other studies examining the acyl profile of PtdIns(3,4,5)P_3, identifying 38:4 as the major species, though this can vary with tissue, cell type, and genetics (Clark et al., 2011; Koizumi et al., 2019; Morioka et al., 2022; Mujalli et al., 2018). Third, Akt activation was impaired in LCLAT1-silenced cells after EGF stimulation. Hence, collectively these varied approaches indicate that LCLAT1-silenced cells are defective in promoting PtdIns(3,4,5)P_3 levels, and possibly PtdIns(3,4)P_2.

A key question then is how does LCLAT1 contribute to PtdIns(3,4,5)P_3 synthesis. We propose at least three possible, non-mutually exclusive mechanisms that will need to be defined in future studies. First, enzymes involved in PtdIns(3,4,5)P_3 metabolism such as Class I PI3Ks, the PTEN 3-phosphatase, and SHIP1/2 5-phosphatase may display acyl sensitivity towards their

substrates as previously suggested (Anderson et al., 2016), thus affecting PtdIns(3,4,5)P_3 generation or its turnover. This was observed for Type I PIPKs, Vps34 Class III PI3Ks, and Type II phosphatases (Ohashi et al., 2020; Schmid et al., 2004; Shulga et al., 2012). Second, PtdIns(3,4,5)P_3 may be reduced in LCLAT1-disturbed cells due to lower PtdIns(4,5)P_2 substrate levels or availability, which we observed here for MDA-MB-231 cells and previously in ARPE-19 (Bone et al., 2017). Third, and perhaps linked to the second model above, PtdIns(3,4,5)P_3 synthesis may depend on specific substrate pools. These pools may be highly localized or even channeled through scaffolds (Choi et al., 2016) or transferred at membrane contact sites (Zaman et al., 2020). For example, depletion of bulk PtdIns(4)P from the plasma membrane did not impact PtdIns(4,5)P_2 levels (Hammond et al., 2012), possibly because PtdIns(4,5)P_2 depends on specific pools of PtdIns(4)P. Moreover, contact sites between endoplasmic reticulum and the plasma membrane are important to generate PtdIns and PtdIns(4,5)P_2 (Chang and Liou, 2015; Cockcroft et al., 2016; Kim et al., 2015; Lees et al., 2017; Saheki et al., 2016; Zaman et al., 2020). Thus, LCLAT1 may play a role in generating specific substrate pools to support PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 at the plasma membrane.

The notion that LCLAT1 may act on specific pools of PtdInsPs is consistent with observations that LCLAT1 and LPIAT/MBOAT7 do not affect the levels of and the acyl profile of all PtdInsPs. For example, their disruption preferentially affects the acyl profile of PtdIns and/or *bis*-phosphorylated PtdInsPs, but not mono-PtdInsPs (Anderson et al., 2013; Bone et al., 2017) and in this work. Moreover, other than PtdIns(3,4,5)P_3, we were surprised to see that LCLAT1 expression did not impact the acyl profile of PtdInsPs in MDA-MB-231 cells – this may be because LCLAT1 acts on highly specialized pools of lipids in these cells and/or that other enzymes are at

play. Overall, while we know that LCLAT1 is needed to boost PtdIns(3,4,5)P_3 levels in response to EGF, the exact mechanism of action remains to be defined.

LCLAT1 and PtdIns(3,4,5)P_3 functions

We witnessed that LCLAT1 supports PtdIns(3,4,5)P_3-mediated activation of Akt isoforms after EGF signalling. Consequently, targets of Akt such as TSC2 and GSK3β, exhibited reduced phosphorylation in LCLAT1-silenced cells, while Mdm2 stability was abated. Hence, we anticipate that LCLAT1 affects other effector functions of PtdIns(3,4,5)P_3 including activation of other kinases such as Btk (Bolland et al., 1998; Lien et al., 2017) and GEFs for the Rho-family of GTPases such as Vav1 and Tiam1 (Salamon and Backer, 2013; Wang et al., 2006, 2; Zhu et al., 2015, 1). Additionally, while our work focused on EGFR-mediated signalling, we postulate that LCLAT1 also supports signalling by other receptor tyrosine kinases such as the Met receptor, FGF, and insulin receptor, and may also support signalling by GPCRs and peripherally-associated kinase receptors such as immune receptors (Bresnick and Backer, 2019; Dowling and Mansell, 2016; Getahun and Cambier, 2015; Takeuchi and Ito, 2011). Conceivably, the putative role of LCLAT1 in promoting PI3K signalling among these receptor classes may depend on which isoforms of Class I and/or Class II PI3K are engaged (Bilanges et al., 2019; Duncan et al., 2020). Overall, our observations establish a key relationship between LCLAT1 and EGFR-PtdIns(3,4,5)P_3-Akt axis and sets a course to determine the universality of LCLAT1 acyltransferase in PtdIns(3,4,5)P_3 signalling.

LCLAT1 in cellular function and therapeutic potential

While the LCLAT1 acyltransferase remains relatively under-investigated, LCLAT1 is associated with hematopoiesis and cell differentiation (Huang et al., 2014; Huang et al., 2017; Wang et al., 2007; Xiong et al., 2008), metabolic regulation (Cao et al., 2009, 1; Liu et al., 2012, 1), mitochondrial stability, dynamics and function (Huang et al., 2020; Li et al., 2010, 1; Li et al., 2012, 1), sensitivity to oxidative stress (Li et al., 2010; Liu et al., 2012, 1), endocytosis and endosomal trafficking (Bone et al., 2017), and now EGFR signalling. LCLAT1 is also proposed to remodel the acyl profile of both cardiolipin and PtdIns/PtdInsPs (Bone et al., 2017; Cao et al., 2004, 1; Imae et al., 2012; Li et al., 2010, 1). It is generally thought that mitochondrial and oxidative stress occurs through cardiolipin remodelling, while endocytosis and receptor signalling is connected to PtdIns acyl function, as proposed here. However, the specific roles of LCLAT1 in cardiolipin and PtdInsP acyl remodelling have not been reconciled. It may be that LCLAT1 has independent roles in acylating these two distinct lipids, or alternatively, one may depend on the other. For example, mitochondria contain PtdIns on their outer membrane, which is important for mitochondria dynamics and function (Pemberton et al., 2020; Zewe et al., 2020). Conceivably, then LCLAT1 acylation of PtdIns may impact lipidomic properties of cardiolipin in mitochondria. Regardless, LCLAT1 and its putative partner, MBOAT7/LPIAT1, remain relatively understudied and without well-established inhibitors. Given the role of LCLAT1 in PtdInsP biology and receptor signalling, we propose that these acyltransferases represent new targets for therapeutic development to complement existing PI3K and Akt inhibitors.

Overall, our work shows that LCLAT1 acyltransferase supports receptor tyrosine kinase signalling by enabling PtdIns(3,4,5)P_3 synthesis and Akt signalling, supporting cell survival and proliferation. While the exact mechanism of action is not known, our work positions LCLAT1 and

acyltransferases such as MBOAT7/LPIAT1 for further study and as potential drug targets against conditions like cancer that depend on PtdIns(3,4,5)P_3 signalling.

Figure legends

Figure 1: LCLAT1 silencing has no negative impact on EGFR activation, EGFR total levels, and EGFR surface levels in ARPE-19 cells. A. Western blots showing repressed LCLAT1 expression in ARPE-19 cells transfected with siLCLAT1-1 oligonucleotides relative to cells exposed to a non-targeting control siRNA (NT siRNA). B = Basal standard media; SS = Serum-starved; EGF = EGF for 5 min. Clathrin heavy chain (CHC) was used as a loading control. **B.** Quantification of LCLAT1 silencing by normalizing LCLAT1 signal to CHC in ARPE-19 cells. **C.** ARPE-19 cells silenced for LCLAT1 or treated with non-targeting oligonucleotide were serum-starved and then stimulated with EGF for 5 min. Lysates were then probed for total EGFR or phospho-EGFR. GAPDH was employed as the loading control. **D.** Quantification of total EGFR relative to the respective GAPDH signal. **E.** Normalized cell surface EGFR detected by immunofluorescence before and after 1 h EGF stimulation in non-targeted and LCLAT1-silenced ARPE-19 cells. **F.** Quantification of phosphor-EGFR (p-Y1068) relative to the respective total EGFR signal. All experiments were repeated a minimum of three times. For B, D, and F shown is mean ±STD. Data in E is shown as mean ± SEM where at least 40-80 cells were scored per condition per experiment. Data in B and D were analysed by an unpaired Mann-Whitney test. For data in E and F, a two-way ANOVA followed by Sidak's post-hoc test was used. * represents $p<0.05$.

Figure 2: LCLAT1 silencing has no negative impact on EGFR activation, total EGFR levels, and surface EGFR levels in MDA-MB-231 cells. A. Western blot showing LCLAT1 silencing in MDA-MB-231 cells transfected with siLCLAT1-1 (siLC-1) relative to cells exposed to non-targeting control siRNA (NT siRNA). SS = Serum-starved; EGF = EGF for 5 min. Clathrin heavy chain (CHC) was used as a loading control. **B.** Quantification of LCLAT1 silencing by normalizing LCLAT1 to CHC signal. **C.** MDA-MB-231 cells silenced for LCLAT1 or treated with non-targeting oligonucleotides were serum-starved and then stimulated with EGF for 5 min. Lysates were then probed for total EGFR or phospho-EGFR (pY-1068). GAPDH was employed as the loading control. **D.** Quantification of total EGFR relative to respective GAPDH. **E.** Normalized cell surface EGFR detected by immunofluorescence before and after 1 h EGF stimulation in non-targeted and LCLAT1-silenced MDA-MB-231 cells. **F.** Quantification of phospho-Y1068-EGFR relative to EGFR. All experiments were repeated at least three independent times. For B, D, and F, shown are the mean ± STD. For E, the mean ± SEM is shown where 50-100 cells were scored per condition per experiment. Data in B and D was tested by an unpaired Mann Whitney test. For data in E and F, a two-way ANOVA and Sidak's post-hoc test were used. * represents $p<0.05$.

Figure 3. Defective PI(4,5)P_2 and EGF-stimulated PtdIns(3,4,5)P_3 synthesis in LCLAT1-silenced cells. MDA-MB-231 cells (A, E), and ARPE-19 (C) were mock-silenced or LCLAT1-silenced. **A.** Confocal images of MDA-MB-231 cells expressing PLCδ-PH-GFP. **B.** Quantification of PLCδ-PH-GFP fluorescence at the cell rim relative to its cytosolic signal. **C.** TIRF and epifluorescence microscopy of ARPE-19 cells mock-silenced or silenced for LCLAT1 and expressing Akt-PH-GFP after 5 min of EGF stimulation. As control, cells were treated with

LY294002 to inhibit PI 3-kinase. **D.** Quantification of TIRF/epifluorescence ratio of Akt-PH-GFP. Total Akt-PH-GFP fluorescence in TIRF field is expressed as a ratio against the corresponding total fluorescence in the epifluorescence field. **E.** Spinning disc confocal images of MDA-MB-231 cells mock-silenced or LCLAT1-silenced before and EGF stimulation. **F.** Quantification of Akt-PH-GFP fluorescence at the plasma membrane relative to its cytosolic signal from time-lapse imaging over 10 min of EGF stimulation. Scale bar = 20 µm. All experiments were repeated three independent times. Shown is the mean ± SEM, where data in D were binned every 45 sec (three images). For B and D, data are based on 30-50 transfected cells per condition per experiment. For F, a total of 23 transfected cells were traced over time over three independent experiments. Data in B was analysed by paired Student's t-test. A one-way ANOVA and Sidak's post-hoc test was used to test data in D. Repeated measures ANOVA was used for data in F. * and *** respectively represent $p<0.05$ and $p<0.001$.

Figure 4. Relative levels of 38:4 PtdIns and PtdInPs in ARPE-19 and MDA-MB-231 cells silenced for LCLAT1. ARPE-19 cells (**A-D**) and MBA-MB-231 cells (**E-I**) were mock silenced (siNT) or LCLAT1-silenced. Cells were then grown in regular medium (control), serum-starved (ss), and stimulated with EGF for 5 min (EGF). Reactions were quenched and lipid extracted after addition of internal standards to primary cell extracts. PtdInsPs were measured by mass spectrometry (HPLC-MS). Shown is the ratio of standardized 38:4-PtdIns (A, E), 38:4-mono-PtdInsP (B, G), 38:4-bis-PtdInsP (C, H), and 38:4-PtdIns(3,4,5)P_3 (D, I) to the standardized sum of 36:1 and 36:2-PtdIns (referred to as 36:x-PtdIns). Lipid analysis was repeated four independent times. Shown are the mean ±STD. A two-way ANOVA and Sidak's post-hoc test was used to test data. In this figure, we disclose the p values to assess statistical confidence.

Figure 5: LCLAT1 is required for EGF-stimulated Akt activation in ARPE-19 cells. **A, C, E:** Mock-silenced (siNT) and LCLAT1-silenced ARPE-19 cells were serum-starved (0 min) or stimulated with EGF for 5 min. Lysates were then prepared, separated by SDS-PAGE and probed by Western blotting for pan-phospho-Akt and total pan-Akt (A), phospho-Akt1 and total Akt1 (C), and phospho-Akt2 and total Akt2 (E). Clathrin heavy chain (CHC) or GAPDH were used as loading controls. # indicates that the GAPDH blot was also used as loading control for p-TSC2 in Fig. 7G since they originated from the same membrane cut across to probe for different sized proteins. **B, D, F**: Quantification of pan-pAkt (B), pAkt1 (D), and pAkt2 (F) normalized to respective total pan-Akt, Akt1, and Akt2. Mean ±STD are shown from n=4 independent experiments are shown. A two-way ANOVA and Sidak's post-hoc test was used to statistically test the data, where ** or *** represents $p<0.01$ or $p<0.001$.

Figure 6: LCLAT1 is required for EGF-stimulated Akt activation in MDA-MB-231 cells. **A, C, E:** Mock-silenced and LCLAT1-silenced MDA-MB-231 cells were serum-starved (0 min) or stimulated with EGF for 5 min or 10 min. Lysates were then prepared, separated by SDS-PAGE and probed by Western blotting for pan-phospho-Akt and total pan-Akt (A), phospho-Akt1 and total Akt1 (C), and phospho-Akt2 and total Akt2 (E). Clathrin heavy chain (CHC) or GAPDH were used as loading controls. **B, D, F**: Quantification of pan-pAkt (B), pAkt1 (D), and pAkt2 (F) normalized to respective total pan-Akt, Akt1, and Akt2. Mean ± STD are shown from n=3 independent experiments are shown. A two-way ANOVA and Sidak's post-hoc test was used to statistically test the data, where *, **, and *** respectively represent $p<0.05$, $p<0.01$, and $p<0.001$.

Figure 7: LCLAT1 is required for activation of Akt substrates after EGF stimulation. **A, C, E:** Mock-silenced and LCLAT1-silenced ARPE-19 cells were serum-starved (0 min) or stimulated with EGF for 5 min. Lysates were then separated by SDS-PAGE and probed by Western blotting for phospho-Tsc2 and total Tsc2 (A), phospho-GSK3β and total GSK3β (C), and phospho-Mdm2 and total Mdm2 (E). Clathrin heavy chain (CHC) or GAPDH were used as loading controls. # indicates that the GAPDH blot was also used as loading control for total pan-Akt in Fig. 5A since they originated from the same membrane cut across to probe for different sized proteins. **B, D, F**: Quantification of pTsc2 (B), pGSK3β (D), and pMdm2 (F) normalized to respective total Tsc2, GSK3β, and Mdm2. **G, I, K**: Mock-silenced and LCLAT1-silenced MDA-MB-231 cells were serum-starved (0 min) or stimulated with EGF for 5 min. Lysates were then separated by SDS-PAGE and probed by Western blotting for phospho-Tsc2 and total Tsc2 (G), phospho-GSK3β and total GSK3β (I), and phospho-Mdm2 and total Mdm2 (K). Clathrin heavy chain (CHC) or GAPDH were used as loading controls. ## indicates that the GAPDH blot was used as loading control for both total TSC2 (G) and total GSK3β (I) since they originated from the same membrane cut across to probe for different sized proteins. **H, J, L**: Quantification of pTsc2 (H), pGSK3β (I), and pMdm2 (K) normalized to respective total Tsc2, GSK3β, and Mdm2. For G, I, K, H, J, and L, mean ± STD are shown from n=3 independent experiments. A two-way ANOVA and Sidak's post-hoc test was used to statistically test the data, where * and ** represents $p<0.05$ and $p<0.01$, respectively.

Figure 8: LCLAT1 is required for cell growth and/or survival. A. Frames at indicated time points of mock and LCLAT1-silenced MDA-MB-231 cells over 72 h using Incucyte imaging incubator. Cells were stained with CytoTox Green to track cell death. Images were acquired from

Supplemental Videos X and Y. **B.** Quantification of cell confluency over 72 h. **C**. Quantification of cell death (CytoTox Green objects) over 72 h. **D.** Western blotting testing for Cas9 expression in parental MDA-MB-231 versus MDA-MB-231-Cas9 cells. **E.** Western blotting testing LCLAT1 expression in MDA-MB-231-Cas9 and MDA-MB-231-Cas9 cells stably transfected with gRNA1, gRNA2, and gRNA3 against LCLAT1. Cells were treated with 0.1 or 1 µg/mL doxycycline for 3 (shown) or 5 days. Clathrin was used as loading control **F.** Quantification of LCLAT1 expression relative to clathrin in MDA-MB-231-Cas9 cells stably transfected with gRNA2 after 5 days of doxycycline at indicated concentrations. **G.** Frames at indicated time points of cells expressing Cas9 and non-targeted gRNA (top panels) or Cas9-gRNA against LCLAT1 (bottom panels). Non-targeted and gRNA-LCLAT1 expression were either not expressed (no doxycycline) or induced by adding doxycycline. Cells were exposed to CytoTox Green to track cell death. **H.** Quantification of cell confluency of CRISPR-engineered MDA-MB-231 cells as shown in D. Data shown are the mean ± STD of three independent experiments. Individual time points in B, C, and H were analysed by two-way ANOVA and Tukey's post-hoc test. Data in F was analysed by one-way ANOVA and Tukey's post-hoc test. * represents $p<0.05$.

Supplemental Information

Supplemental Figure S1: LCLAT1 silencing in ARPE-19 cells with an independent siRNA oligonucleotide disrupts Akt signalling. ARPE-19 cells were transfected with oligonucleotide siLCLAT1-5 or non-targeting control. Cells were then serum-starved (SS), followed by EGF stimulation for 5 min. **A.** Lysates were probed for LCLAT1 expression, p-Akt, Akt, and clathrin

heavy chain (CHC), which was used as a loading control. **B.** Quantification of LCLAT1 silencing by normalizing LCLAT1 to CHC signal in ARPE-19 cells. **C.** Quantification of p-Akt levels relative to total Akt. Data is shown as mean ±STD are shown from n=3 independent experiments. Data was analysed by a one-way ANOVA and Sidak's post-hoc test, where ** and *** represent $p<0.01$ and $p<0.001$, respectively.

Supplemental Figure S2: LCLAT1 silencing in MDA-MB-231 cells with independent siRNA oligonucleotides disrupts Akt signalling. **A.** Western blotting showing LCLAT1 silencing in MDA-MB-231 cells transfected with non-targeting siRNA, siLCLAT1-1, siLCLAT1-7, or siLCLAT1-8 oligonucleotides. Cells were then serum-starved (SS) or stimulated with EGF for 5 min. Lysates were probed for LCLAT1 expression, p-Akt, and clathrin heavy chain (CHC), which was used as a loading control. **B.** Quantification of LCLAT1 silencing by normalizing LCLAT1 to CHC signal in MDA-MB-231 cells transfected as in D. **C.** Quantification of p-Akt levels relative to clathrin in MDA-MB-231 cells mock-silenced or LCLAT1-silenced with one of three siRNA oligonucleotides and either serum-starved or stimulated with EGF for 5 min. Show is the mean ±STD from n=3 independent experiments. Data in B was analysed with a one-way ANOVA and Dunnett's post-hoc test. Data in C was analysed with a two-way ANOVA and Sidak's post-hoc test, where *, **, ***, and **** respectively represent $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively.

Supplemental Figure S3. Relative levels of 38:4-PtdInsPs to 36:x-PtdInsPs in ARPE-19 and MDA-MB-231 cells silenced for LCLAT1. ARPE-19 cells (**A-B**) and MBA-MB-231 cells (**C-D**) were mock silenced (siCon) or LCLAT1-silenced. Cells were then grown in regular medium

(control), serum-starved (ss), and stimulated with EGF for 5 min (EGF). Reactions were quenched and lipid extracted after addition of internal standards to primary cell extracts. PtdInsPs were measured by mass spectrometry (HPLC-MS). Shown is the ratio of standardized 38:4-mono-PtdIns (A, C) and 38:4-bis-PtdInsP2 (B, D) to the respective standardized sum of 36:1 and 36:2- (referred to as 36:x-PtdIns) mono-PtdInsP and bis-PtdInsP2. Lipid analysis was repeated four independent times. Shown are the mean ±STD. A two-way ANOVA and Sidak's post-hoc test was used to test data. Here, we disclose the p value as statistical confidence.

Supplemental Video 1: Growth and cell death of MDA-MB-231 cells transiently mock silenced. MDA-MB-231 cells were mock transfected with non-targeting siRNA and imaged in an Incucyte system in the presence of CellTox Green over 3 days at 2 frames per hour. Scale bar = 400 µm.

Supplemental Video 2: Growth and cell death of MDA-MB-231 cells transiently silenced for LCLAT1. MDA-MB-231 cells were silenced for LCLAT1 expression by transfected with siLCLAT1 oligonucleotides. Cells were imaged in an Incucyte system in the presence of CellTox Green over 3 days at 2 frames per hour. Scale bar = 400 µm.

Supplemental Video 3: Growth and cell death of MDA-MB-231-Cas9 cells with no doxycycline. MDA-MB-231-Cas9 cells in normal growth medium were imaged in an Incucyte system in the presence of CellTox Green over 3 days at 2 frames per hour. Scale bar = 400 µm.

Supplemental Video 4: Growth and cell death of MDA-MB-231-Cas9 cells in the presence of doxycycline. MDA-MB-231-Cas9 cells in growth medium supplemented with 1 µg/mL doxycycline were imaged in an Incucyte system in the presence of CellTox Green over 3 days at 2 frames per hour. Scale bar = 400 µm.

Supplemental Video 5: Growth and cell death of MDA-MB-231-Cas9::gRNA2 cells without doxycycline. MDA-MB-231-Cas9 cells stably-transfected with gRNA2 against LCLAT1 were maintained in normal growth medium and imaged in an Incucyte system in the presence of CellTox Green over 3 days at 2 frames per hour. Scale bar = 400 µm.

Supplemental Video 6: Growth and cell death of MDA-MB-231-Cas9::gRNA2 cells in the presence of doxycycline. MDA-MB-231-Cas9 cells stably-transfected with gRNA2 against LCLAT1 and exposed to growth medium supplemented with 1 µg/mL doxycycline were imaged in an Incucyte system in the presence of CellTox Green over 3 days at 2 frames per hour. Scale bar = 400 µm.

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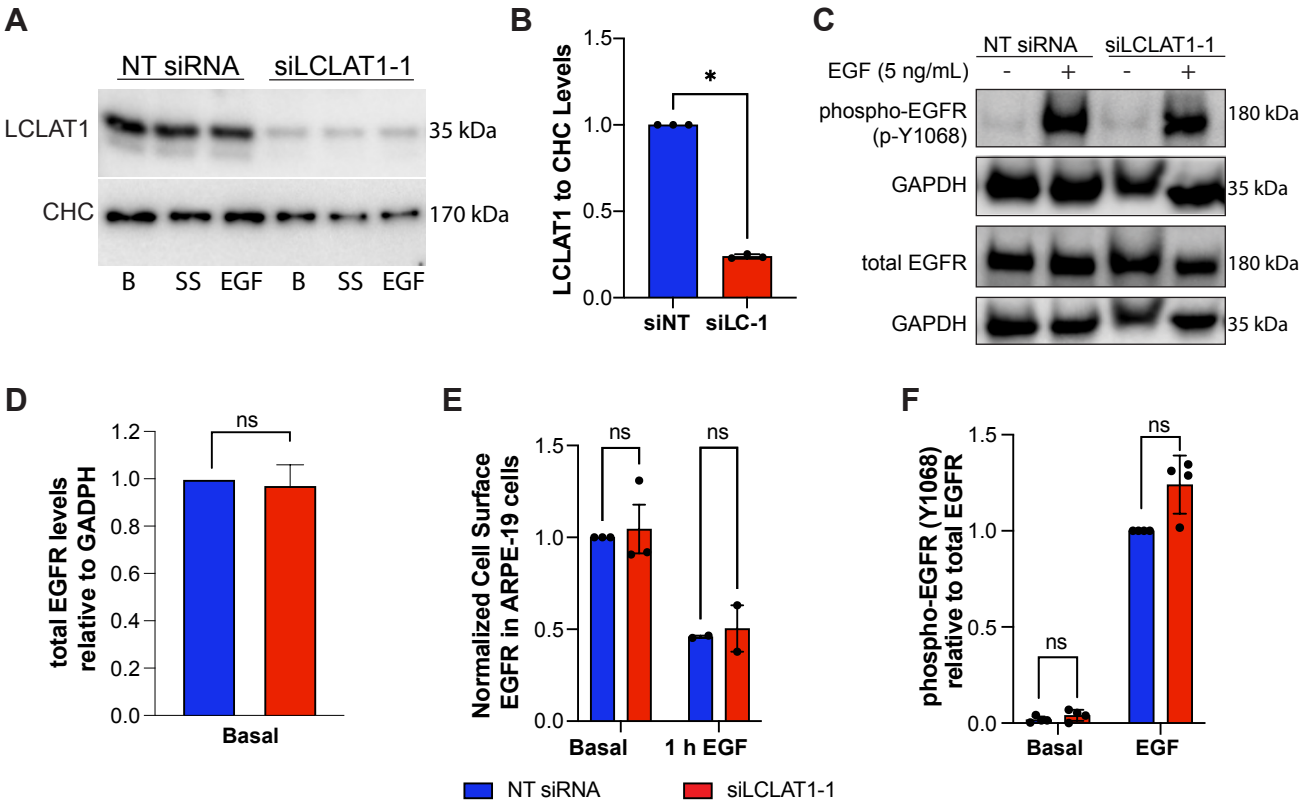
A
NT siRNA
siLCLAT1-1
LCLAT1
35 kDa
CHC
170 kDa
B
SS
EGF
B
SS
EGF
B
LCLAT1 to CHC Levels
1.5
1.0
0.5
0.0
*
siNT
siLC-1
C
NT siRNA
siLCLAT1-1
EGF (5 ng/mL)
-
+
-
+
phospho-EGFR
(p-Y1068)
180 kDa
GAPDH
35 kDa
total EGFR
180 kDa
GAPDH
35 kDa
D
total EGFR levels
relative to GADPH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ns
Basal
E
Normalized Cell Surface
EGFR in ARPE-19 cells
1.5
1.0
0.5
0.0
ns
ns
Basal
1 h EGF
NT siRNA
siLCLAT1-1
F
phospho-EGFR (Y1068)
relative to total EGFR
1.5
1.0
0.5
0.0
ns
ns
Basal
EGF

Figure 1

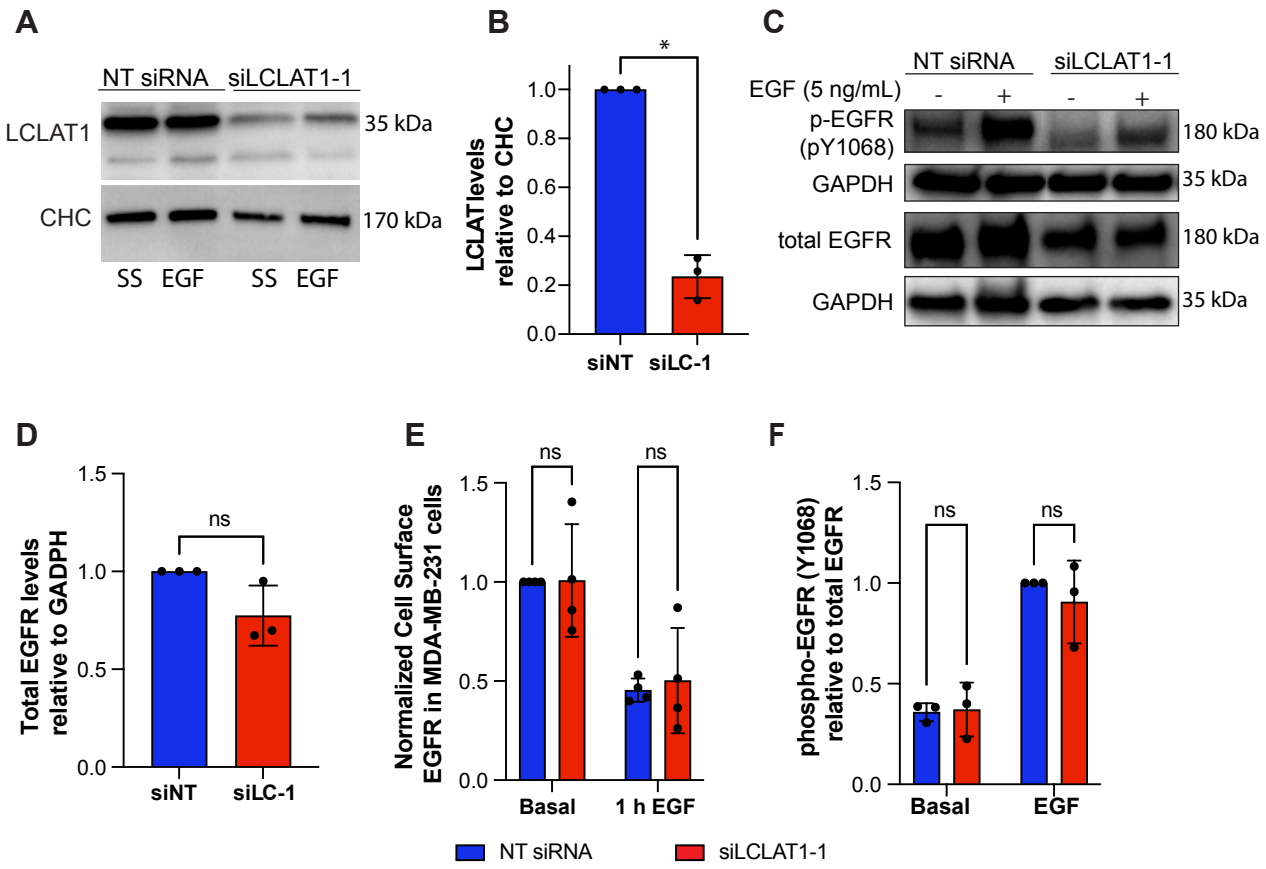
A
NT siRNA
siLCLAT1-1
LCLAT1
35 kDa
CHC
170 kDa
SS
EGF
SS
EGF
B
*
LCLAT levels relative to CHC
1.0
0.8
0.6
0.4
0.2
0.0
siNT
siLC-1
C
NT siRNA
siLCLAT1-1
EGF (5 ng/mL)
-
+
-
+
p-EGFR (pY1068)
180 kDa
GAPDH
35 kDa
total EGFR
180 kDa
GAPDH
35 kDa
D
Total EGFR levels relative to GADPH
1.5
1.0
0.5
0.0
ns
siNT
siLC-1
E
Normalized Cell Surface EGFR in MDA-MB-231 cells
ns
ns
1.5
1.0
0.5
0.0
Basal
1 h EGF
F
phospho-EGFR (Y1068) relative to total EGFR
ns
ns
1.5
1.0
0.5
0.0
Basal
EGF
NT siRNA
siLCLAT1-1

Figure 2

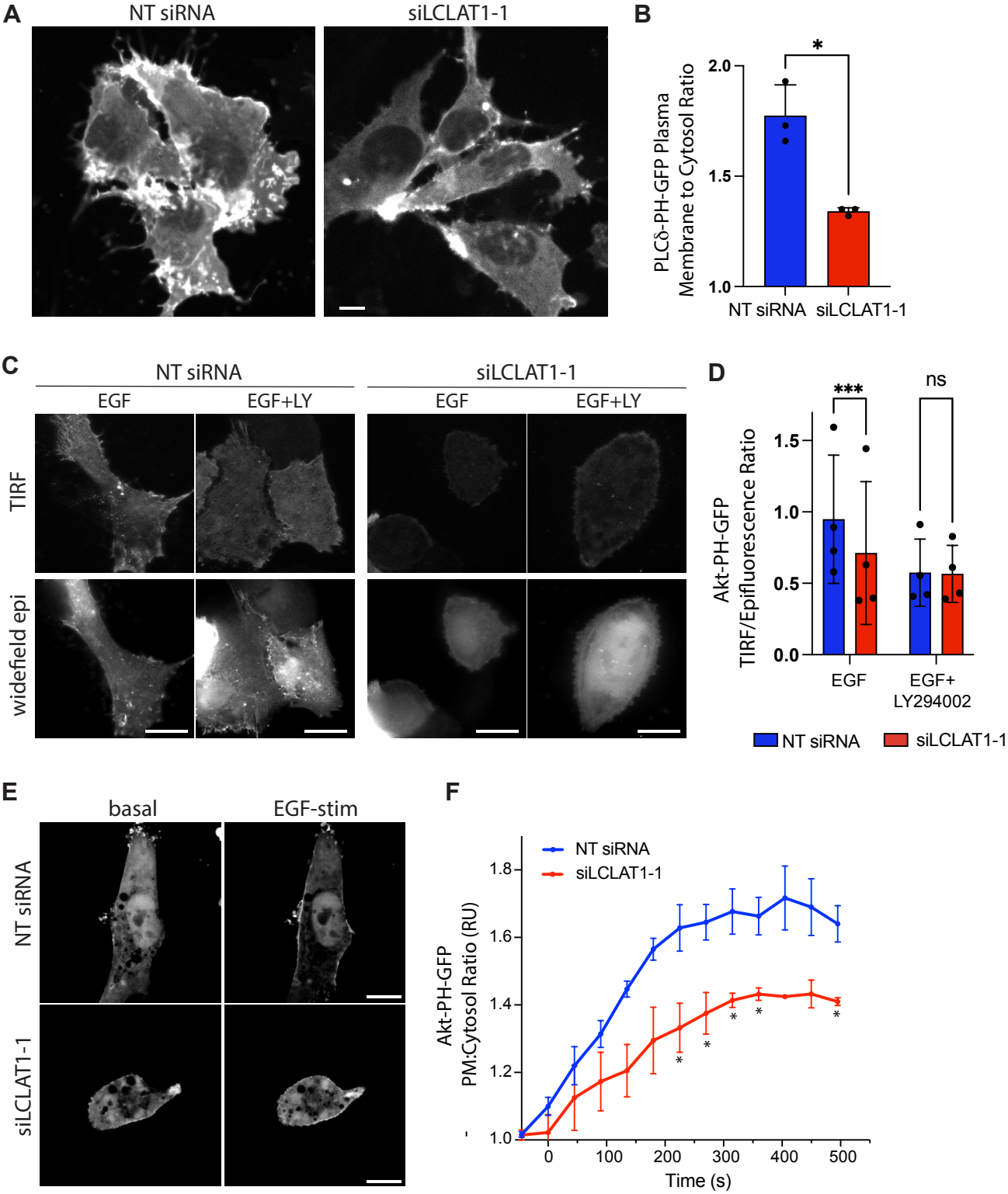
A
NT siRNA
siLCLAT1-1
B
PLCδ-PH-GFP Plasma Membrane to Cytosol Ratio
2.0
1.5
1.0
*
NT siRNA
siLCLAT1-1
C
NT siRNA
siLCLAT1-1
EGF
EGF+LY
EGF
EGF+LY
TIRF
widefield epi
D
Akt-PH-GFP TIRF/Epifluorescence Ratio
1.5
1.0
0.5
0.0
***
ns
EGF
EGF+ LY294002
NT siRNA
siLCLAT1-1
E
basal
EGF-stim
NT siRNA
siLCLAT1-1
F
NT siRNA
siLCLAT1-1
Akt-PH-GFP PM:Cytosol Ratio (RU)
1.8
1.6
1.4
1.2
1.0
0
100
200
300
400
500
Time (s)

Figure 3

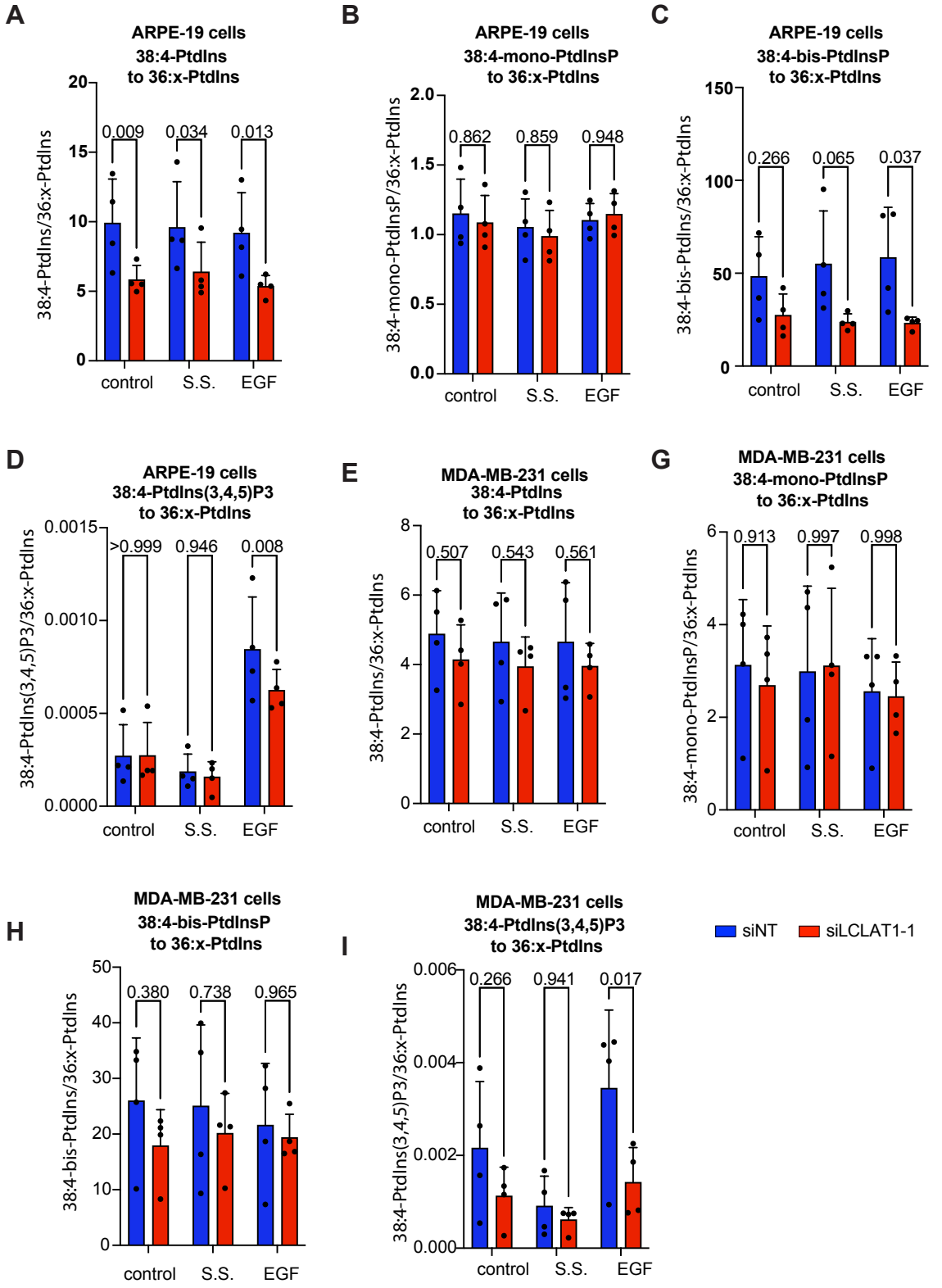
A
ARPE-19 cells
38:4-PtdIns
to 36:x-PtdIns
38:4-PtdIns/36:x-PtdIns
0.009
0.034
0.013
control
S.S.
EGF
B
ARPE-19 cells
38:4-mono-PtdInsP
to 36:x-PtdIns
38:4-mono-PtdInsP/36:x-PtdIns
0.862
0.859
0.948
C
ARPE-19 cells
38:4-bis-PtdInsP
to 36:x-PtdIns
38:4-bis-PtdIns/36:x-PtdIns
0.266
0.065
0.037
D
ARPE-19 cells
38:4-PtdIns(3,4,5)P3
to 36:x-PtdIns
38:4-PtdIns(3,4,5)P3/36:x-PtdIns
>0.999
0.946
0.008
E
MDA-MB-231 cells
38:4-PtdIns
to 36:x-PtdIns
38:4-PtdIns/36:x-PtdIns
0.507
0.543
0.561
G
MDA-MB-231 cells
38:4-mono-PtdInsP
to 36:x-PtdIns
38:4-mono-PtdInsP/36:x-PtdIns
0.913
0.997
0.998
H
MDA-MB-231 cells
38:4-bis-PtdInsP
to 36:x-PtdIns
38:4-bis-PtdIns/36:x-PtdIns
0.380
0.738
0.965
I
MDA-MB-231 cells
38:4-PtdIns(3,4,5)P3
to 36:x-PtdIns
38:4-PtdIns(3,4,5)P3/36:x-PtdIns
0.266
0.941
0.017
siNT
siLCLAT1-1

Figure 4

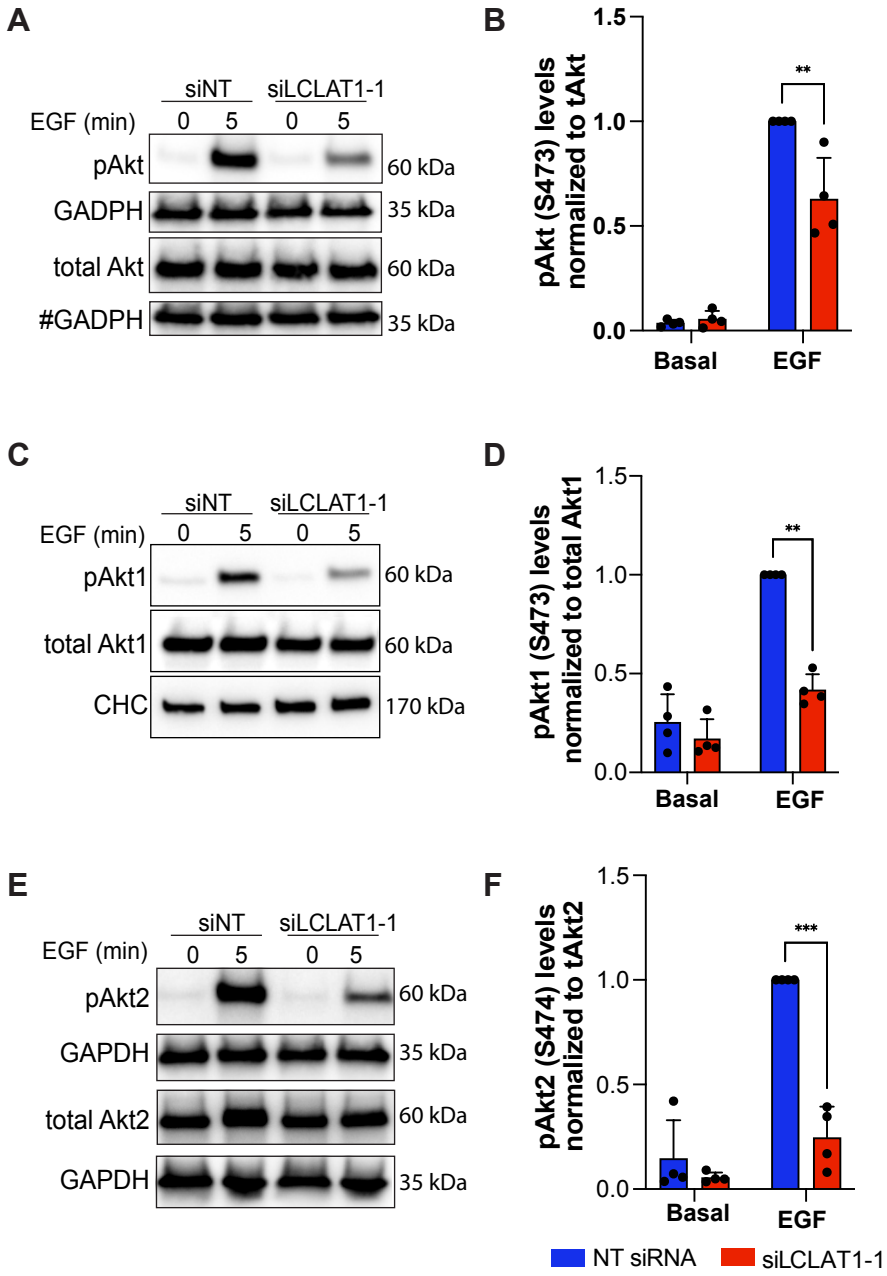
A
siNT
siLCLAT1-1
EGF (min)
0
5
0
5
pAkt
60 kDa
GADPH
35 kDa
total Akt
60 kDa
#GADPH
35 kDa
B
pAkt (S473) levels normalized to tAkt
1.5
1.0
0.5
0.0
**
Basal
EGF
C
siNT
siLCLAT1-1
EGF (min)
0
5
0
5
pAkt1
60 kDa
total Akt1
60 kDa
CHC
170 kDa
D
pAkt1 (S473) levels normalized to total Akt1
1.5
1.0
0.5
0.0
**
Basal
EGF
E
siNT
siLCLAT1-1
EGF (min)
0
5
0
5
pAkt2
60 kDa
GAPDH
35 kDa
total Akt2
60 kDa
GAPDH
35 kDa
F
pAkt2 (S474) levels normalized to tAkt2
1.5
1.0
0.5
0.0
***
Basal
EGF
NT siRNA
siLCLAT1-1

Figure 5

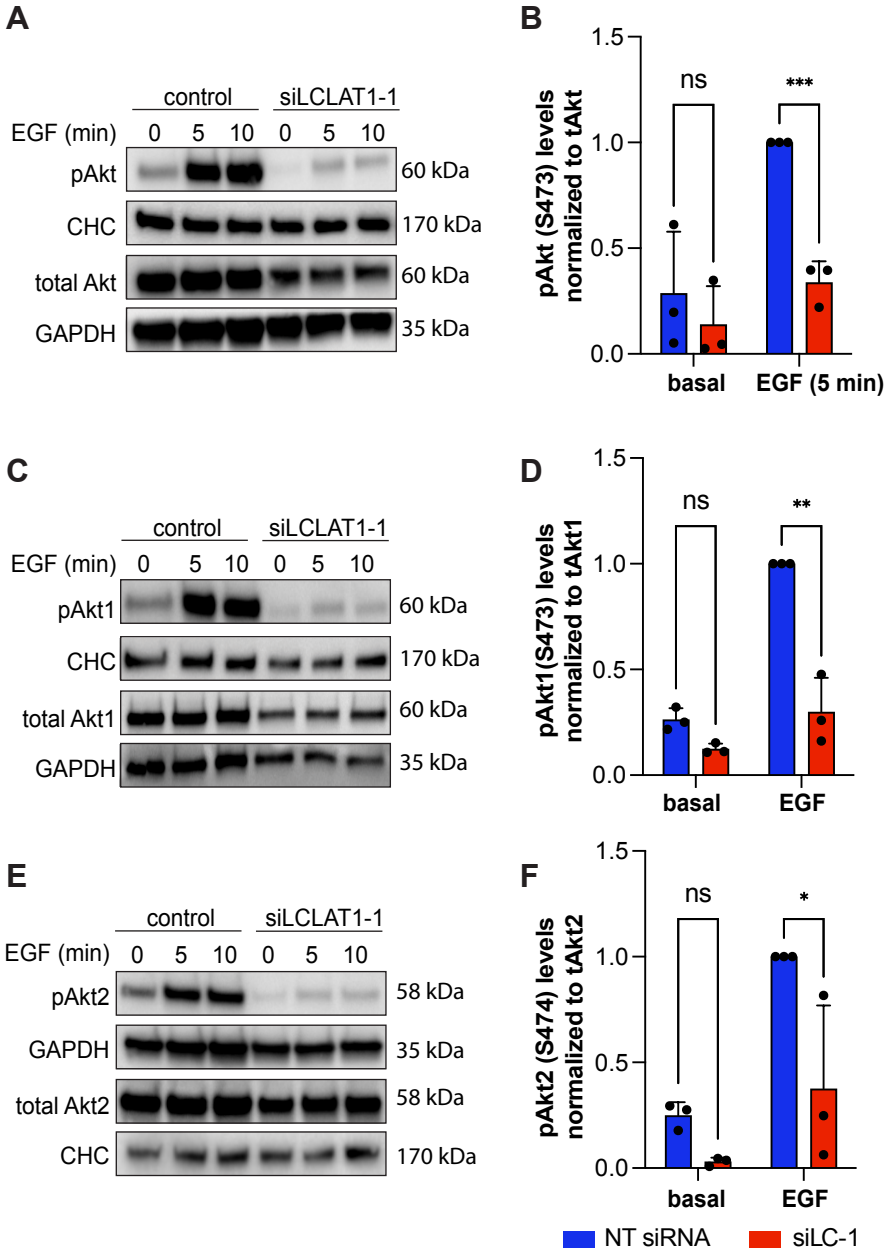
A
control
siLCLAT1-1
EGF (min)
0 5 10 0 5 10
pAkt
CHC
total Akt
GAPDH
60 kDa
170 kDa
60 kDa
35 kDa
B
pAkt (S473) levels normalized to tAkt
1.5
1.0
0.5
0.0
ns
***
basal
EGF (5 min)
C
control
siLCLAT1-1
EGF (min)
0 5 10 0 5 10
pAkt1
CHC
total Akt1
GAPDH
60 kDa
170 kDa
60 kDa
35 kDa
D
pAkt1(S473) levels normalized to tAkt1
1.5
1.0
0.5
0.0
ns
**
basal
EGF
E
control
siLCLAT1-1
EGF (min)
0 5 10 0 5 10
pAkt2
GAPDH
total Akt2
CHC
58 kDa
35 kDa
58 kDa
170 kDa
F
pAkt2 (S474) levels normalized to tAkt2
1.5
1.0
0.5
0.0
ns
*
basal
EGF
NT siRNA
siLC-1

Figure 6

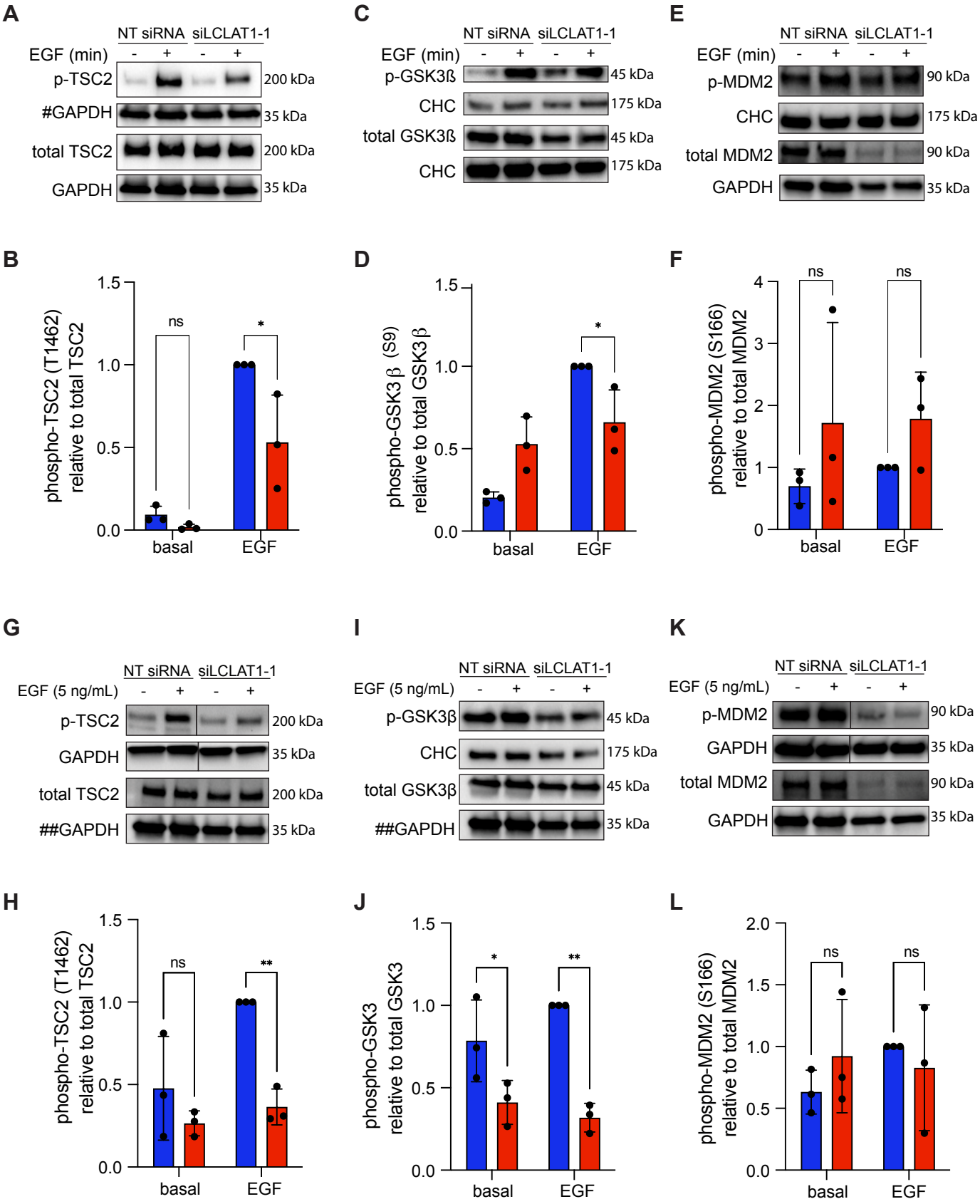
A
NT siRNA
siLCLAT1-1
EGF (min)
- + - +
p-TSC2
200 kDa
#GAPDH
35 kDa
total TSC2
200 kDa
GAPDH
35 kDa
B
phospho-TSC2 (T1462) relative to total TSC2
1.5
1.0
0.5
0.0
ns
*
basal
EGF
C
NT siRNA
siLCLAT1-1
EGF (min)
- + - +
p-GSK3ß
45 kDa
CHC
175 kDa
total GSK3ß
45 kDa
CHC
175 kDa
D
phospho-GSK3 β (S9) relative to total GSK3 β
1.5
1.0
0.5
0.0
*
basal
EGF
E
NT siRNA
siLCLAT1-1
EGF (min)
- + - +
p-MDM2
90 kDa
CHC
175 kDa
total MDM2
90 kDa
GAPDH
35 kDa
F
phospho-MDM2 (S166) relative to total MDM2
4
3
2
1
0
ns
ns
basal
EGF
G
NT siRNA
siLCLAT1-1
EGF (5 ng/mL)
- + - +
p-TSC2
200 kDa
GAPDH
35 kDa
total TSC2
200 kDa
##GAPDH
35 kDa
H
phospho-TSC2 (T1462) relative to total TSC2
1.5
1.0
0.5
0.0
ns
**
basal
EGF
I
NT siRNA
siLCLAT1-1
EGF (5 ng/mL)
- + - +
p-GSK3β
45 kDa
CHC
175 kDa
total GSK3β
45 kDa
##GAPDH
35 kDa
J
phospho-GSK3 relative to total GSK3
1.5
1.0
0.5
0.0
*
**
basal
EGF
K
NT siRNA
siLCLAT1-1
EGF (5 ng/mL)
- + - +
p-MDM2
90 kDa
GAPDH
35 kDa
total MDM2
90 kDa
GAPDH
35 kDa
L
phospho-MDM2 (S166) relative to total MDM2
2.0
1.5
1.0
0.5
0.0
ns
ns
basal
EGF

Figure 7

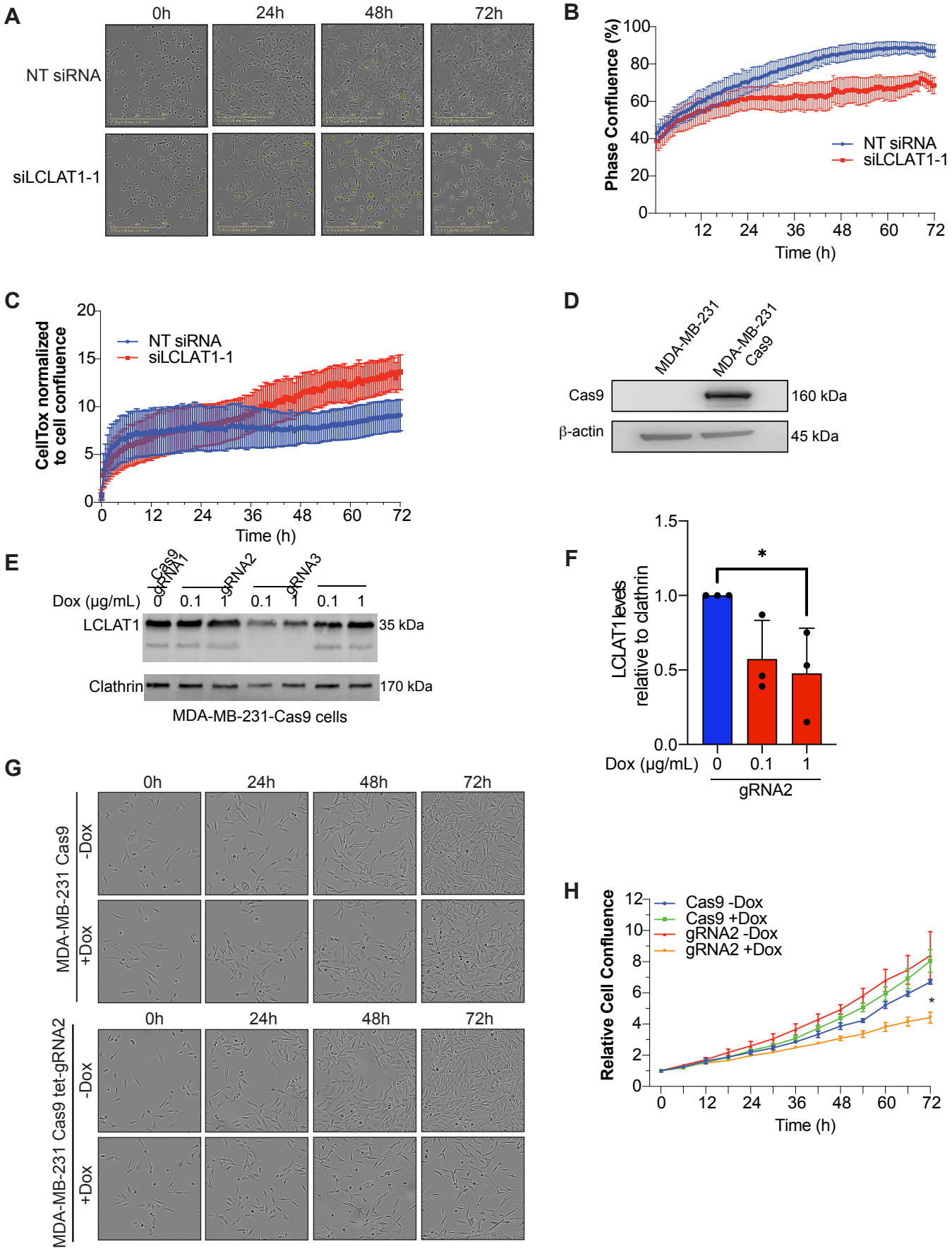
A
0h
24h
48h
72h
NT siRNA
siLCLAT1-1
B
Phase Confluence (%)
100
80
60
40
20
0
12
24
36
48
60
72
Time (h)
NT siRNA
siLCLAT1-1
C
CellTox normalized to cell confluence
20
15
10
5
0
NT siRNA
siLCLAT1-1
0
12
24
36
48
60
72
Time (h)
D
MDA-MB-231
MDA-MB-231 Cas9
Cas9
160 kDa
β-actin
45 kDa
E
Cas9
gRNA1
gRNA2
gRNA3
Dox (μg/mL)
0
0.1
1
0.1
1
0.1
1
LCLAT1
35 kDa
Clathrin
170 kDa
MDA-MB-231-Cas9 cells
F
LCLAT1 levels relative to clathrin
1.5
1.0
0.5
0.0
*
Dox (μg/mL)
0
0.1
1
gRNA2
G
0h
24h
48h
72h
MDA-MB-231 Cas9
-Dox
+Dox
MDA-MB-231 Cas9 tet-gRNA2
-Dox
+Dox
H
Relative Cell Confluence
12
10
8
6
4
2
0
Cas9 -Dox
Cas9 +Dox
gRNA2 -Dox
gRNA2 +Dox
*
0
12
24
36
48
60
72
Time (h)

Figure 8