

# The cyclin-dependent kinase PITSLRE/CDK11 is required for successful autophagy

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**Key words:** PITSLRE, CDK11, cyclin-dependent kinase, autophagy, human, *Drosophila*

**Abbreviations:** RNAi, RNA interference; siRNA, small interfering RNA; dsRNA, double-stranded RNA; CDK, cyclin-dependent kinase; GFP, green fluorescent protein; LC3, microtubule-associated protein 1 light chain 3

(Macro)autophagy is a membrane-trafficking process that serves to sequester cellular constituents in organelles termed autophagosomes, which target their degradation in the lysosome. Autophagy operates at basal levels in all cells where it serves as a homeostatic mechanism to maintain cellular integrity. The levels and cargoes of autophagy can, however, change in response to a variety of stimuli, and perturbations in autophagy are known to be involved in the etiology of various human diseases. Autophagy must therefore be tightly controlled. We report here that the *Drosophila* cyclin-dependent kinase *PITSLRE* is a modulator of autophagy. Loss of the human *PITSLRE* ortholog, *CDK11*, initially appears to induce autophagy, but at later time points *CDK11* is critically required for autophagic flux and cargo digestion. Since *PITSLRE/CDK11* regulates autophagy in both *Drosophila* and human cells, this kinase represents a novel phylogenetically conserved component of the autophagy machinery.

## Introduction

Autophagy literally means 'self-eating' and is a major catabolic process within the cell.<sup>1</sup> Three types of autophagy have been described: macroautophagy, microautophagy and chaperone-mediated autophagy.<sup>2</sup> Macroautophagy, which we will simply refer to here as autophagy, is the best characterized and is considered to operate in all cells. The process begins with the formation of a membranous structure called a phagophore or isolation membrane.<sup>3-5</sup> The membrane grows to form a spherical organelle termed an autophagosome which encapsulates cytoplasmic constituents.<sup>3-5</sup> Autophagosomes can then undergo fusion events with other organelles such as endosomes and multivesicular bodies, but ultimately fusion occurs with lysosomes forming a new organelle termed an autolysosome.<sup>3-5</sup> The hydrolases provided by the lysosome degrade the cargo of the autophagosome and these are then delivered back into the cytoplasm where they are either further catabolized or recycled in biosynthetic pathways.

At basal levels autophagy serves as a mechanism to degrade misfolded and damaged proteins and it is the only mechanism within the cell for the degradation of whole organelles.<sup>2</sup> In response to various stimuli, the levels and cargoes of autophagy can change to bring about desired effects within the cell. In this regard, autophagy is known to act as a protective mechanism within cells that acts to prevent various forms of human disease.<sup>2</sup>

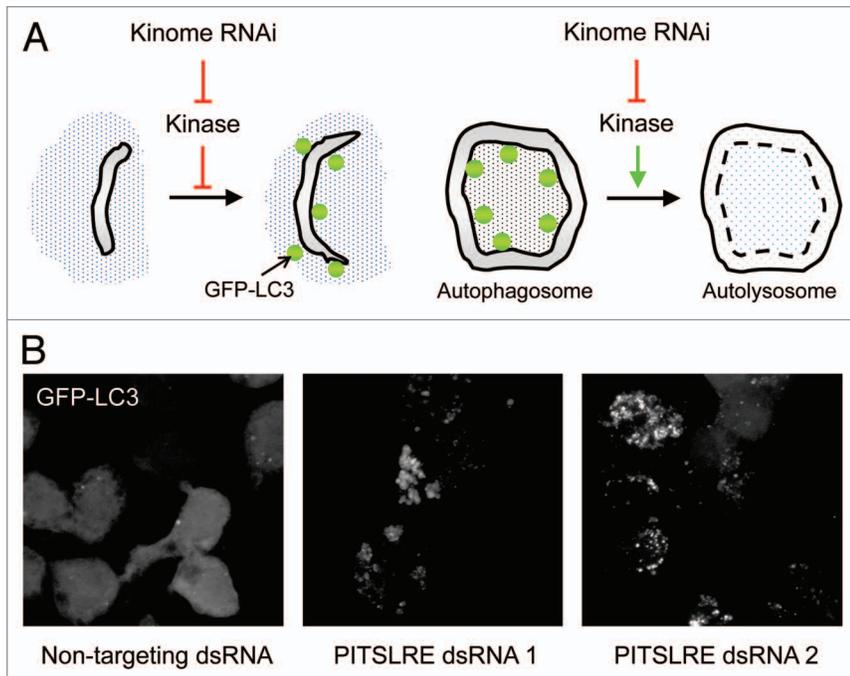
Perturbations in autophagy control are known to contribute to cancer and inflammatory conditions such as Crohn disease.<sup>4,6-9</sup> Autophagy is also known to be critically required for the removal of protein aggregates that form in neurodegenerative disease, and autophagy is central for the correct functioning of both the innate and acquired immune responses.<sup>10,11</sup> In light of these important and yet disparate roles of autophagy, it is critical that we understand the cellular factors required for autophagy and how the autophagic process is controlled.

A major mechanism involved in the control of protein function is phosphorylation. There are 518 kinases in human cells, which mediate phosphorylation events that control a multitude of processes within the cell by regulating protein function, protein folding, protein interaction and protein stability. Using a kinome-wide screen in *Drosophila* cells, we identify the cyclin-dependent kinase, *PITSLRE* and its human ortholog *CDK11* as critical regulators of basal autophagy. *CDK11* was first identified as a cell division control (CDC)-related kinase belonging to the p34<sup>cdc2</sup> family.<sup>12</sup> *CDK11* is the product of two duplicated genes, *CDK11A* and *CDK11B* (formerly named as *CDC2L1* and *CDC2L2*).<sup>13</sup> Subsequent studies on *PITSLRE/CDK11* have revealed diverse roles for the kinase in controlling RNA splicing, apoptosis, sister chromatid adhesion and cytokinesis.<sup>14-19</sup> We add to these roles by showing that *CDK11* is a fundamental regulator of autophagy. We show that while *CDK11* appears to act as a

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**Figure 1.** The *Drosophila* kinase PITSLRE is a modulator of autophagy. (A) Schematic diagram showing the screen for kinase regulators of autophagy. *Drosophila* S2R<sup>+</sup> GFP-LC3 cells growing in nutrient-rich conditions were transfected with the *Drosophila* kinome RNAi library. This screen theoretically allows for the identification of kinases that positively and negatively regulate GFP-LC3 puncta. (B) *Drosophila* S2R<sup>+</sup> GFP-LC3 cells were transfected with indicated dsRNA sequences, as described in Materials and Methods, and images of GFP-LC3 distribution obtained by confocal microscopy after a 72 h incubation period.

repressor of autophagy in cells grown under nutrient-rich conditions, prolonged loss of CDK11 results in the accumulation of immature autophagic intermediates and inhibits autophagic flux.

## Results

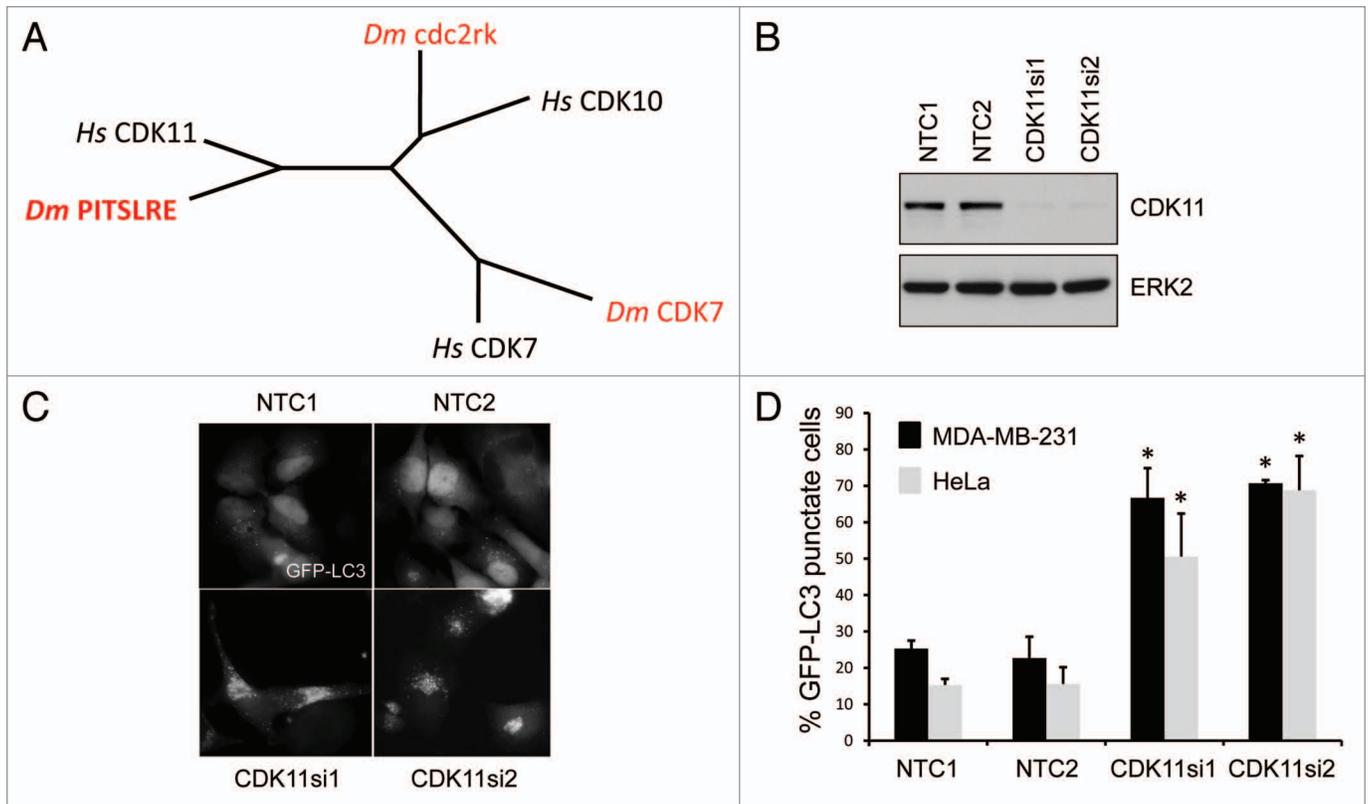
**PITSLRE is a modulator of autophagy.** In order to identify novel regulators of autophagy, we embarked on an RNA interference (RNAi) kinome screen in *Drosophila* S2R<sup>+</sup> cells. *Drosophila* cells were chosen for the screen due to the lower levels of functional redundancy compared with mammalian systems, and also for the ease with which human orthologs of *Drosophila* kinases can be identified. An S2R<sup>+</sup> cell line was therefore generated which expresses a GFP-LC3 transgene (GFP fused to *Drosophila* Atg8a).<sup>20</sup> LC3 is an integral component of the autophagosome membrane. In cells with basal autophagy, the majority of LC3 is in a form called LC3-I which is diffuse within the cytoplasm.<sup>21,22</sup> Upon induction of autophagy, LC3 becomes cleaved and lipidated and integrates into autophagosome membranes—as a form of LC3 termed LC3-II.<sup>21,22</sup> With the GFP-LC3 transgene, this event can easily be detected by fluorescence microscopy as the appearance of distinct GFP puncta within the cell. To validate whether the system can detect changes in autophagosome number, we incubated *Drosophila* S2R<sup>+</sup> GFP-LC3 cells with the lysosomotropic agent Bafilomycin A1. This agent is a potent inhibitor of lysosomal acidification and therefore blocks autophagosome

turnover. This revealed, as would be expected, that autophagosomes accumulate over time in the presence of Bafilomycin A1 because they cannot be turned over in the absence of lysosome function (Fig. S1).

Having validated the system, the S2R<sup>+</sup> GFP-LC3 cells were then challenged under normal growth conditions with a previously described *Drosophila* kinome RNAi library and cells were visualized by fluorescence microscopy for the accumulation of GFP-LC3 puncta.<sup>20</sup> This approach could potentially reveal kinases which block induction of autophagy or identify kinases which are required for turnover of autophagosomes (Fig. 1A). One kinase which when depleted by RNAi caused a particularly marked increase in GFP-LC3 puncta, was the cyclin-dependent kinase PITSLRE (Fig. 1B). In order to confirm the results of the screen, and to rule out the possibility of an off-target effect of the RNAi, a second double-stranded RNA (dsRNA) duplex targeting a different region of PITSLRE was used. Knockdown of PITSLRE with this dsRNA also caused a marked accumulation of autophagosomes, confirming the initial results from the screen (Fig. 1B). No effect on autophagosome accumulation was observed when cells were treated with a nontargeting dsRNA as control (Fig. 1B).

**The human PITSLRE ortholog CDK11 is a modulator of autophagy.** Phylogenetic analyses revealed that PITSLRE has one ortholog in human cells termed CDK11 which lies within the CMGC (cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases) group of kinases (Fig. 2A). CDK11 is encoded by two duplicated genes, *CDK11A* and *CDK11B*. Therefore we wanted to determine if human CDK11 was also involved in the modulation of autophagy. To this end, HeLa and MDA-MB-231 cells expressing GFP-LC3 were challenged with two siRNAs to knockdown CDK11 and two nontargeting siRNAs as controls. Both CDK11 siRNAs were able to significantly reduce CDK11 protein levels (Fig. 2B) and, similar to the result observed in the S2R<sup>+</sup> cells, this caused a considerable increase in GFP-LC3 puncta indicating accumulation of autophagosomes (Fig. 2C). No increase in autophagosome accumulation was observed in cells treated with nontargeting siRNAs as controls (Fig. 2C). Quantification of the number of cells exhibiting an increase in autophagosomes revealed that autophagy was clearly affected in 60–70% of the MDA-MB-231 and HeLa cell populations following CDK11 knockdown (Fig. 2D).

**CDK11 regulates autophagic flux.** Since autophagosomes are normally a transient mid-point in the complete autophagy process, their appearance can represent either an increase in autophagosome formation or a block to their turnover—as is seen following treatment with bafilomycin A1 (Fig. S1). In order

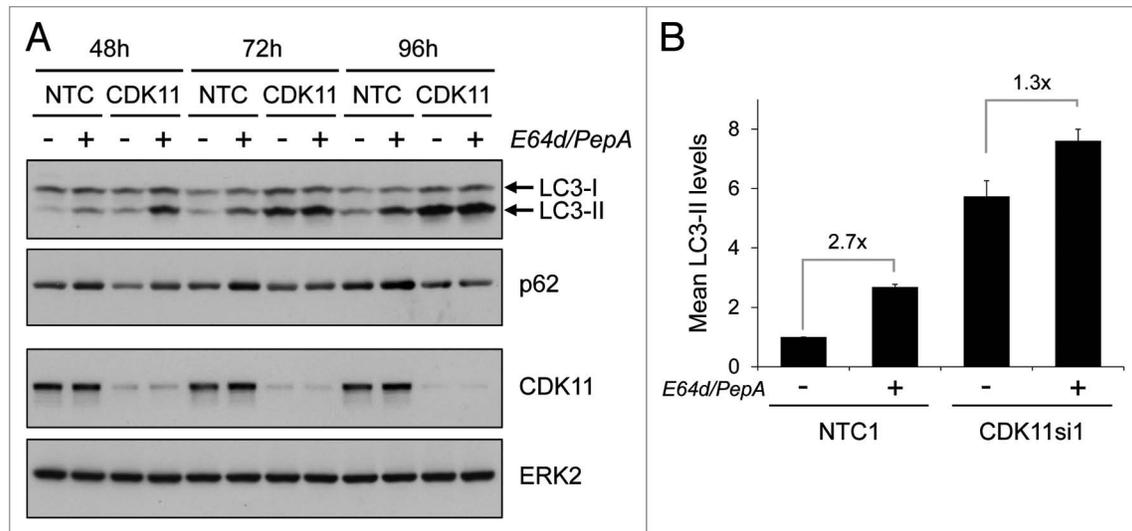


**Figure 2.** The human ortholog of PITSLRE, CDK11, is a regulator of autophagy. (A) Sequence alignment of Drosophila kinases with human kinases demonstrates that the nearest ortholog of *PITSLRE* is *CDK11*. (B and C) MDA-MB-231 GFP-LC3 cells were transfected with nontargeting control (NTC) or CDK11 siRNAs for 72 h and (B) protein samples immunoblotted with antibodies shown or (C) images captured by fluorescence microscopy. (D) MDA-MB-231 and HeLa GFP-LC3 cells were treated as described in (B and C) and images quantified for cells with overt GFP-LC3 puncta (\**p* < 0.05 for both CDK11 knockdowns in each cell line).

to determine which of these possibilities was causing autophagosome accumulation following CDK11 knockdown, assays were undertaken to measure autophagic flux. In the first assay, we monitored the accumulation of LC3-II levels by protein gel blotting following CDK11 knockdown either in the absence or presence of the lysosomal protease inhibitors, E64d and Pepstatin A. In the presence of these inhibitors, knockdown of an inhibitor of autophagy induction will cause a greater accumulation of LC3-II than occurs following treatment with E64d and Pepstatin A alone (more induction caused greater accumulation).<sup>22</sup> Conversely, knockdown of a factor required for autophagosome turnover will, by contrast, have a limited effect on LC3-II accumulation if autophagosome turnover is already impeded by E64d and Pepstatin A.<sup>22</sup> Our initial results at early time points revealed that knockdown of CDK11 in the presence of E64d/Pepstatin A caused enhanced LC3-II protein levels when compared with cells treated with E64d/Pepstatin A and a nontargeting siRNA control, indicating that CDK11 knockdown was inducing autophagy (Fig. 3A). At later time points, however, the combination of E64d/Pepstatin A with CDK11 knockdown only had a limited effect on LC3-II levels when compared with cells treated with E64d/Pepstatin A and a nontargeting siRNA control (Fig. 3A and B). We considered that the initial loss of CDK11 results in a cellular response to induce autophagy, but after longer periods it becomes

clear that the cell cannot cope with this increased induction of autophagosome accumulation in the absence of CDK11 and as a result autophagosomal turnover and therefore completion of autophagy is significantly retarded although not completely blocked (Fig. 3A and B).

In order to further assess whether successful autophagy was indeed blocked following prolonged CDK11 knockdown, we assayed autophagic flux by analyzing cargo entrapment. p62/SQSTM1 is an adaptor protein for autophagic cargo recruitment and is degraded by autophagy in multiple settings. Consistent with our analysis of LC3-I to LC3-II conversion, p62 protein levels initially decrease following CDK11 knockdown indicating that loss of CDK11 at early time points causes an increase in autophagic flux (Fig. 3A). The protease inhibitors E64d/Pepstatin A, through inhibition of autophagosome turnover, are known to cause an accumulation of p62 as a result of impeded digestion of p62 within autolysosomes. If CDK11 is required for autophagosome turnover at later time points, treatment of cells with E64d/Pepstatin A should have no effect on p62 levels in cells where CDK11 has been knocked down by RNAi. Indeed, after 72 and 96 h we observed that E64d/Pepstatin A markedly increased p62 protein levels in cells treated with a nontargeting siRNA control, whereas no induction of p62 was seen as a result of E64d/Pepstatin A treatment in cells where CDK11 was



**Figure 3.** CDK11 regulates autophagic flux. (A) MDA-MB-231 cells were transfected with nontargeting control (NTC1) or CDK11si1 siRNA for either 48, 72 or 96 h and then treated with vehicle control or 10  $\mu$ g/ml E64d/Pepstatin A for 16 h. Lysates were immunoblotted for the indicated proteins. (B) Quantification of LC3-II levels following 96 h of CDK11 knockdown. Blots were probed using an anti-LC3B antibody and the relevant Alexa-Fluor 680 secondary antibody. LC3-II bands were detected by Li-Cor Odyssey infrared scanning and normalized to ERK2. Data are shown as mean LC3-II levels  $\pm$  SEM (n = 5). A two-tailed, paired Student's t-test was used to determine the statistical significance of LC3-II upregulation following E64d/Pepstatin A treatment between NTC1 and CDK11si1 transfected cells ( $p = 0.001$ ).

knocked down by siRNA (Fig. 3A). In addition, it was observed that p62 protein levels were lower in CDK11 knockdown cells compared with nontargeting controls. This can be explained by our observation that p62 mRNA levels are reduced following CDK11 knockdown (Fig. S2).

A block in the turnover of LC3-II and p62 levels as assessed by protein gel blotting does not discern whether the effects of CDK11 knockdown are through inhibition of fusion of autophagosomes with lysosomes, or due to inhibition of turnover subsequent to autophagosome/lysosome fusion. To address this issue and to assess for fusion between autophagosomes and lysosomes, we infected cells with an adenovirus expressing mCherry-LC3 and stained for the lysosomal membrane protein LAMP2. This revealed that fusion between autophagosomes and lysosomes could easily be detected in cells following CDK11 knockdown indicating that while prolonged CDK11 knockdown impedes the turnover stage of autophagy, it does not do this by blocking autophagosome/lysosome fusion (Fig. 4). When taken together our data show that, while CDK11 may repress basal autophagy, prolonged CDK11 inhibition results in inhibition of successful autophagy.

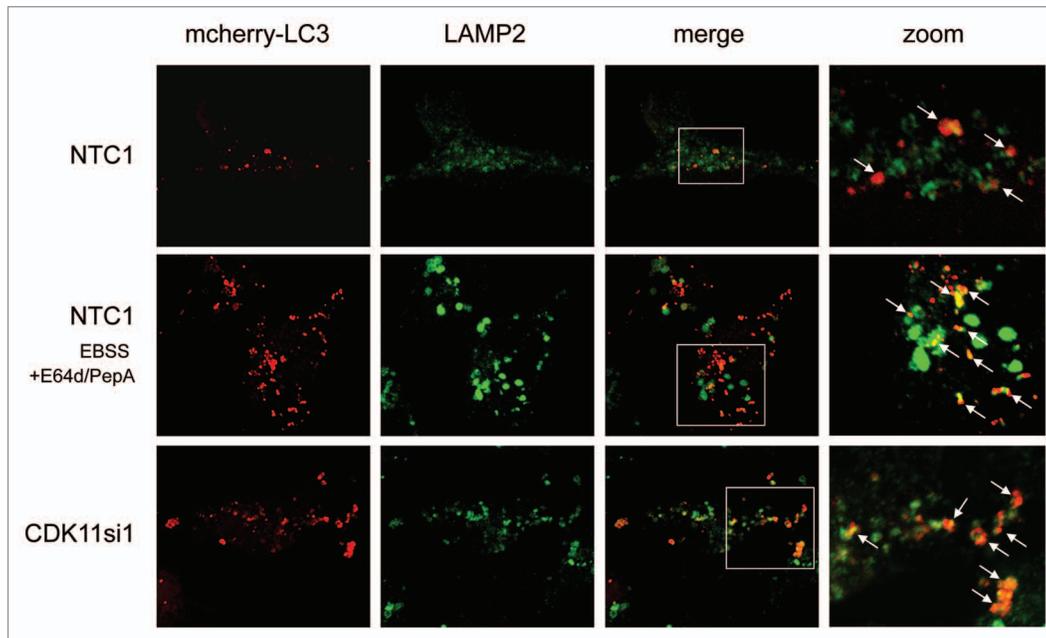
## Discussion

We report here for the first time that the cyclin-dependent kinase PITSLRE/CDK11 is a new factor regulating autophagy. We show that depletion of PITSLRE in *Drosophila* cells and CDK11, the one ortholog of PITSLRE in human cells, both result in accumulation of autophagosomes indicating that the modulation of autophagy by the kinase is evolutionarily conserved. We also show in human cells that the initial depletion of CDK11 causes cells to undergo an initial induction of autophagic flux whereas at

later time points it is clear that autophagosome turnover is significantly retarded, although not completely blocked. How then can we explain these different effects on autophagy at different times? As no substrates of PITSLRE are currently known, it is only possible to speculate as to the role played by PITSLRE/CDK11 in autophagy and how it can have different effects at different times. One possibility is that at early time points the depletion of CDK11 results in induction of autophagy, but at later time points loss of CDK11 also results in a decrease in lysosomal function. This potential explanation is consistent with the data we provide showing that while at later time points autolysosomes are clearly formed, their turnover is markedly reduced (Fig. 4).

One interesting observation from our studies is the downregulation of p62 mRNA following protracted CDK11 knockdown (Fig. S2). We can only assume that p62 mRNA levels decrease following CDK11 knockdown through a mechanism that decreases either p62 transcription or p62 mRNA stability. Previous studies have shown that p62 protein levels feed-forward to regulate p62 transcription via the transcription factor NRF2.<sup>23</sup> Following prolonged CDK11 knockdown, p62 may become sequestered in autophagosomes/autolysosomes which are retarded in their turnover. As a result, this may well break the feed-forward loop regulating p62 transcription leading to decreased levels of p62 mRNA. Further studies are therefore required to ascertain if this mechanism is the cause of p62 mRNA downregulation following long-term loss of CDK11.

Although no clear mechanistic functions for CDK11 are known, in terms of targets for phosphorylation, the kinase has been reported to have effects on multiple biological functions including apoptosis, RNA splicing and mitosis.<sup>14-19</sup> It is important therefore not to simply focus on how CDK11 regulates components of the autophagy machinery to bring about the effects we report



**Figure 4.** CDK11 does not inhibit autophagosome and lysosome fusion. MDA-MB-231 cells expressing mCherry-LC3 were transfected with nontargeting control (NTC1) or CDK11si1 siRNA for 72 h. As a control, NTC1 transfected cells were also treated with vehicle control or 10  $\mu$ g/ml E64d/Pepstatin A for 16 h, and starved by incubation in EBSS for the remaining 6 h. Following fixation and permeabilization, cells were stained with a LAMP2 antibody and imaged by confocal microscopy. Arrows indicate co-localization of mCherry-LC3 with LAMP2 in autolysosomes.

here, but also how the effects of CDK11 on autophagy impinge on these other CDK11 functions and vice versa. In this regard, we examined the effects of CDK11 knockdown on cell cycle progression and cell death in MDA-MB-231 cells. This revealed that in the time frame studied, CDK11 knockdown had no effect on apoptosis as assessed by analysis for sub- $G_1$  DNA content—a reliable marker of apoptotic death (Fig. S3).<sup>24</sup> Moreover, accumulation of autophagosomes following CDK11 knockdown was not inhibited with a concentration of the pan-caspase inhibitor, zVAD-fmk that was sufficient to inhibit TNF $\alpha$ -driven apoptotic cell death (Fig. S4A and B). A small increase (15%) in cells in  $G_2/M$  phase was observed following 72 h of CDK11 knockdown (Fig. S3), but we do not consider this to be the cause of autophagy inhibition, since 70–80% of the cells exhibited marked autophagosome accumulation at this time point (Fig. 2D).

CDK11 has been reported to be perturbed in various human cancers including neuroblastoma, non-Hodgkin's lymphoma and melanoma.<sup>25–27</sup> In light of the findings we report here, it is natural to consider whether the regulation of autophagy is the reason for selection for alterations in CDK11 in these forms of cancer or again whether the involvement of CDK11 in other cellular functions is more important. Ultimately, extensive further studies are required to not only determine the role of CDK11 in the regulation of autophagy, but also to ascertain if CDK11 represents another control point at which autophagy can be deregulated in human disease.

## Materials and Methods

**Cell culture and reagents.** GFP-LC3 Drosophila cells are described in Wilkinson et al. 2009<sup>20</sup> and were cultured in

Schneider's medium. MDA-MB-231 and HeLa cells were cultured in DMEM (Gibco, 21969) + 10% FCS (Hyclone, SH30080.03), supplemented with antibiotics. Mammalian cell lines were authenticated by microsatellite genotyping. Stable expression of GFP-LC3 was achieved with pBabe-puro GFP-LC3 retroviral vector.<sup>20</sup> E64d (330005) and Pepstatin A (516481) were purchased from Calbiochem.

**RNA interference.** Drosophila cells were transfected with double-stranded RNA as previously described in reference 20. dsRNA sequences used were derived with T7-binding sequence tagged primers, followed by in vitro transcription with T7 polymerase. Gene-specific sequences of primers were: nontargeting control: 5' GCC AAA AAC ATA AAG AAA GGG CCC G and 3' CCC TGG TAA TCC GTT TTA GAA TCC, PITSLRE 1: ATG ACG ATG AGG AAA GCG AGG AGA G and CGG GAT AAT AGT TGG GCA GGG GAA T, PITSLRE 2: CTC CGA ACG AGA AGA TCT GG and CCC AAT TGC TTT GTA TGG CT. For mammalian cells, siRNAs used were NTC1 (Dharmacon, D-001210-01), NTC2 (Dharmacon, D-001210-02), CDK11si1 (Targeting sequence: CAA GAT CTA CAT CGT GAT GAA; Qiagen, S100287700) and CDK11si2 (Targeting sequence: ATG GAG TGG TCT ACA GAG CAA; Qiagen, S103050978). Cells plated in 6 well plates were transfected with Oligofectamine (Invitrogen, 12252-011) according to the manufacturer's instructions, using 10 nM oligonucleotide concentration.

**Protein gel blotting.** Samples for analysis were obtained by direct lysis in lysis buffer (50 mM Hepes, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM sodium pyrophosphate, protease inhibitors, 1% (v/v) Triton X-100, 0.1% (v/v) SDS). Protein

gel blotting was performed as previously described in reference 28. Antibodies used for protein gel blotting were CDK11 (Rabbit polyclonal, Abcam ab19393), ERK2 (Ab122, CJ Marshall, Institute of Cancer Research, London), LC3B (Rabbit polyclonal, Abcam, ab48394) and p62/SQSTM1 (Mouse monoclonal, BD Transduction Labs; 610833). For quantification of LC3-II levels, blots were visualized with fluorophore-conjugated secondary antibodies and detected using the Odyssey system (LI-COR Biosciences).

**Fluorescence microscopy.** Images of GFP-LC3 expressing cells were captured with an Olympus BX51 microscope and blind scored for cells that contained prominent GFP-LC3 puncta above background. For the screen in *Drosophila* cells, due to the low level of autophagosomes under basal conditions, a positive hit was when 10 autophagosomes or more were observed per cell (on average) following treatment with kinase RNAi.

**Immunofluorescence.** Cells grown on glass coverslips were fixed for 15 min in 4% (w/v) para-formaldehyde (PFA)/PBS and then permeabilized for 15 min in 0.3% (v/v) Triton X-100/PBS. After fixation and permeabilization, cells were washed three times in PBS and then blocked with 0.3% (w/v) Avidin BSA (Sigma), 10% (v/v) FCS, 5% (w/v) nonfat milk in 0.3% (v/v) Triton X-100/PBS for 1 h. Cells were incubated with mouse anti-LAMP2 (BD Pharmingen, 555803) for 60 min, followed by three washes with PBS and a 30 min incubation with goat anti-mouse AlexaFluor 488 secondary antibody (Invitrogen, A11001). Coverslips were

mounted in Dako fluorescent mounting medium (S3023) and visualized using a Zeiss LSM710 confocal microscope.

**Cell cycle analysis.** Total populations of cells were harvested, and then fixed and permeabilized in 100% ice-cold methanol. PI staining was performed by incubation with propidium iodide (50  $\mu$ g/ml) plus RNase A (125  $\mu$ g/ml) for 45 min at room temperature. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest software.

**qPCR analysis.** RNA was prepared using an RNeasy Mini kit (Qiagen, 74104). cDNA was synthesized and qPCR performed using the DyNAmo SYBR Green 2-step qRT-PCR kit (Finnzymes, F430L) and validated QuantiTect human primer sets for p62/SQSTM1 (Qiagen, QT00095676). Data collection was performed using a Chromo4 real-time PCR detector (BioRad) and MJ Opticon Monitor software. Expression was normalized relative to 18S rRNA levels (Fwd, 5'-GTA ACC CGT TGA ACC CCA TT-3'; Rev, 5'-CCA TCC AAT CGG TAG TAG CG-3').

#### Acknowledgements

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#### Note

Supplementary material can be found at: [www.landesbioscience.com/journals/autophagy/article/16646](http://www.landesbioscience.com/journals/autophagy/article/16646)

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