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The serine/threonine kinase ULK1 is a target of multiple phosphorylation events

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Autophagy is a cellular degradation process that is up-regulated upon starvation. Nutrition-dependent regulation of mTOR (mammalian target of rapamycin) is a major determinant of autophagy. RTK (receptor tyrosine kinase) signalling and AMPK (AMP-activated protein kinase) converge upon mTOR to suppress or activate autophagy. Nutrition-dependent regulation of autophagy is mediated via mTOR phosphorylation of the serine/threonine kinase ULK1 (unc51-like kinase 1). In the present study, we also describe ULK1 as an mTOR-independent convergence point for AMPK and RTK signalling. We initially identified ULK1 as a 14-3-3-binding protein and this interaction was enhanced by treatment with AMPK agonists. AMPK interacted with ULK1 and phosphorylated ULK1 at Ser^{555} *in vitro*. Mutation of this residue to alanine abrogated 14-3-3 binding to ULK1, and *in vivo* phosphorylation of ULK1 was blocked by a dominant-negative AMPK mutant. We next identified a high-stringency Akt site in ULK1 at Ser^{774} and showed that phosphorylation at this site was increased by insulin. Finally, we found that the kinase-activation loop of ULK1 contains a consensus phosphorylation site at Thr^{180} that is required for ULK1 autophosphorylation activity. Collectively, our results suggest that ULK1 may act as a major node for regulation by multiple kinases including AMPK and Akt that play both stimulatory and inhibitory roles in regulating autophagy.

Key words: AMP-activated protein kinase (AMPK), autophagy, 14-3-3 protein, mammalian target of rapamycin (mTOR), receptor tyrosine kinase (RTK), unc51-like kinase 1 (ULK1).

INTRODUCTION

Autophagy is a highly regulated cellular degradation pathway [1]. It forms an important cellular response to starvation and plays a vital role in the removal of damaged organelles during stress responses. Autophagy has been implicated in a large number of diseases, including neurological disorders, diabetes and cancer [2]. During autophagy, a double membrane surrounds cytoplasmic content or organelles, such as mitochondria, endoplasmic reticulum or peroxisomes. Closure of the double membrane isolates these structures and leads to the formation of the autophagosome. Subsequent fusion of the autophagosome with the lysosome results in degradation of the content of the newly formed autolysosome and membrane transporters in the limiting membrane facilitate the release of amino acids and other degradation products into the cytoplasm.

Autophagy is precisely regulated both by stimulatory and inhibitory inputs. In the fed state, autophagy is repressed due to activation of the inhibitory pathway. This can be mediated either directly by nutrients or via nutrient-mediated activation of RTKs (receptor tyrosine kinases) such as the insulin signalling pathway. These inputs converge on the mTOR (mammalian target of rapamycin) complex, a major node in the nutrient-sensing machinery of eukaryotic cells. mTOR is found in two separate complexes: mTORC1 and mTORC2 (mTOR complex 1 and 2). mTORC1, a complex of mTOR, raptor (regulatory associated protein of mTOR) and GβL (G-protein β-like), is the major regulator of autophagy. RTKs feed into mTOR via Akt-dependent phosphorylation of TSC2 (tuberous sclerosis complex 2), a GTPase-activating protein for the small molecular GTPase Rheb. Akt phosphorylation at Ser^{939}, Ser^{981}, Ser^{1130}, Ser^{1132} and Thr^{1462} causes inhibition of TSC2 [3], which, in conjunction with Akt phosphorylation of the upstream regulator of raptor PRAS40 (proline-rich Akt substrate of 40 kDa) at Thr^{246}, leads to activation of mTOR [4,5]. Nutrients, such as amino acids and glucose, activate mTOR via at least two different pathways. One involves a complex of Rag GTPases [6,7], whereas the other involves repression of AMPK (AMP-activated protein kinase) via alterations in the intracellular AMP/ATP ratio. AMPK phosphorylation of TSC2 at Thr^{1271} and Ser^{1387} activates TSC2 and, together with AMPK phosphorylation of raptor at Ser^{722} and Ser^{792} [8], this leads to inhibition of mTOR. Treatment of mammalian or yeast cells with rapamycin inhibits mTOR function and induces autophagy, even in the presence of amino acids [9,10]. In *Drosophila* fat bodies, autophagy is highly induced in TOR (target of rapamycin)-null animals even in the fed state and disruption of inhibitory inputs, such as deletion of the insulin receptor or Rheb, enhances autophagy whereas interruption of stimulatory pathways such as TSC1/2 inhibits autophagy [11].

Genetic screens in yeast identified a number of autophagy-related genes (ATG) including the serine/threonine protein kinase Atg1 [12], which forms an active complex with Atg13 and Atg17

Abbreviations used: AMPK, AMP-activated protein kinase; AS160, Akt substrate of 160 kDa; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; 4E-BP, eukaryotic initiation factor 4E-binding protein; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; FIP200, focal adhesion kinase family-interacting protein of 200 kDa; GFP, green fluorescent protein; GST, glutathione transferase; HA, haemagglutinin; HBSS, Hanks balanced salt solution; HEK, human embryonic kidney; KRP, Krebs–Ringer phosphate; mAtg13, mammalian Atg13; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; NA, numerical aperture; PAS, phospho-Akt substrate; PKA, protein kinase A; PRAS40, proline-rich Akt substrate of 40 kDa; raptor, regulatory associated protein of mTOR; RTK, receptor tyrosine kinase; TOR, target of rapamycin; TSC, tuberous sclerosis complex; ULK, Unc51-like kinase; WT, wild-type.

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[13,14]. Increased nutrient supply leads to phosphorylation of Atg13 by TOR, triggering dissociation of the Atg1–Atg13–Atg17 complex, deactivation of Atg1 and inhibition of autophagy [15]. In mammals, the Atg1 kinase homologues ULK1 (Unc51-like kinase 1) and ULK2 [16] have been implicated in starvation and rapamycin-induced autophagy [17–19]. ULK1-knockout mice have impaired autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. However, autophagy in other cells is largely unimpaired, suggesting a potential redundancy with ULK2 [20]. ULK1 and ULK2 each form complexes with the mammalian homologue of Atg13 and FIP200 (Rb1CC1) [18,21–23]. In contrast with yeast cells, in mammalian cells the complex of ULK, mAtg13 (mammalian Atg13) and FIP200 (focal adhesion kinase family-interacting protein of 200 kDa) is stable during nutrient abundance. ULK1, ULK2 and mAtg13 are directly phosphorylated by mTOR *in vitro* [18,21,23] and this is associated with inhibition of ULK1. Together, these findings point to mTOR as the major convergence point for regulation of autophagy. While the present paper was in review, phosphorylation of ULK1 by mTOR and AMPK on distinct sites has been reported [24–26]. Our data support the notion that Ser^{555} is a key AMPK phosphorylation site and we also show that Ser^{555} is the major site for AMPK-dependent 14-3-3 binding. In addition, we provide evidence for a phosphorylation site at Thr^{180} in the ULK kinase-activation loop and a potential Akt phosphorylation site at Ser^{774}. The latter was shown to be insulin-responsive. Collectively, these data point to an intriguing mechanism for the regulation of ULK1 function through the integration of multiple signal transduction pathways.

MATERIALS AND METHODS

Materials

DMEM (Dulbecco's modified Eagle's medium), antibiotics and Lipofectamine™ 2000 were purchased from Invitrogen. FBS (fetal bovine serum) was obtained from Trace Scientific, insulin was from Calbiochem, BSA was from Bovogen, bicinchoninic acid reagent, Supersignal West Pico chemiluminescent substrate and Protein G–Sepharose beads were from Pierce, PVDF membrane was from Millipore and Complete™ protease inhibitor cocktail tablets were from Roche. All other materials were obtained from Sigma–Aldrich. [γ-^{32}P]ATP was sourced from MP Biochemicals.

Antibodies against the FLAG epitope (M2), LC3 and β-actin were from Sigma–Aldrich, those against the HA (haemagglutinin) epitope (16B12) were from Covance, those against the Myc epitope were from Abcam, and those against LC3 were from MBL International. Anti-ULK1 and anti-14-3-3 antibodies were from Santa Cruz Biotechnology, and anti-AMPK, anti-PAS (phospho-Akt substrate) and anti-GST (glutathione transferase) antibodies were from Cell Signaling Technology. The bacterially produced AMPK trimer and LKB1 as well as A769662 were a gift from Dr Bronwyn Hegarty (Garvan Institute, Sydney, Australia) and Dr David Carling (Imperial College London, London, U.K.). Active Akt was purchased from Abcam.

DNA constructs

The HA-tagged ULK1 and $ULK1^{K46R}$ constructs (GenBank® accession number NM_009469) were a gift from Dr Toshifumi Tomoda (City of Hope, Duarte, CA, U.S.A.). Myc-tagged FIP200 was kindly provided by Dr Tkuhiro Chano (Shiga University, Otsu, Japan). The cDNA for Atg13 (GenBank® accession number NM_014741.2) was obtained from Origene. Atg13 was cloned into the HindIII and EcoRI restriction sites of the p3xFlag-CMV-10 vector using PCR amplification. Human WT (wild-type) AMPK α1 and AMPK $\alpha 1^{D159A}$ constructs were obtained from Dr David Stapleton (Bio21, Melbourne, Australia) and subcloned into pDEST40.

The phosphorylation site point mutations T180A, S467A, S555A, T659A and S774A in both WT ULK1 and $ULK1^{K46R}$ were obtained by site-directed mutagenesis using Phusion High-Fidelity DNA polymerase from Finnzyme. For expression in 3T3-L1 fibroblasts, the ULK1 and GFP (green fluorescent protein)–LC3 constructs were cloned into pBABE-puro.

Cell culture

HEK (human embryonic kidney)-293FT cells were obtained from Invitrogen, HEK-293 c18 cells (HEK-293E) were from the A.T.C.C. (Manassas, VA, U.S.A.) (CRL-10852), and 3T3-L1 cells were from Dr Howard Green (Harvard University, Boston MA, U.S.A.). Cells were cultured according to standard tissue culture procedures. HEK-293 cells were transfected with Lipofectamine™ 2000 1 day after seeding following the manufacturer's protocol and were used 24 h post-transfection. 3T3-L1 cells were infected with retrovirus containing pBABE-puro ULK1 or GFP–LC3 constructs and selected by incubation in 2 μg/ml puromycin [27]. For starvation, HEK-293 cells were incubated for 2 h in KRP (Krebs–Ringer phosphate) supplemented with either 2.5 mM glucose or with the amino acids leucine (408 μM), tyrosine (196 μM), proline (874 μM), methionine (120 μM), histidine (184 μM) and tryptophan (186 μM) [28], as indicated. Cells were treated with 500 nM rapamycin and 100 nM bafilomycin for 2 h, 50 μM A769662, 2 mM phenformin and 10 μM berberine for 30 min, 100 nM wortmannin for 20 min or for the indicated times with 100 or 350 nM insulin.

Immunoprecipitation and Western blotting

Immunoprecipitation of HA-tagged ULK1 from HEK-293 cells was performed using Nonidet P40 buffer (1 % Nonidet P40, 137 mM NaCl, 10 % glycerol, 25 mM Tris/HCl, pH 7.4, 2 mM sodium orthovanadate, 1 mM PP_i, 10 mM NaF and Complete™ protease inhibitor mixture) as described in [27]. Alternatively, cells were lysed directly in 2% SDS in HES buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 250 mM sucrose, 2 mM sodium orthovanadate, 1 mM PP_i, 10 mM NaF and Complete™ protease inhibitor cocktail). Samples were subjected to standard SDS/PAGE (8, 10 or 12 % gels) and Western blotting. The GST–14-3-3 overlay was performed by incubating membranes in 1–5 μg/ml bacterially produced GST–14-3-3β in TBS (Tris-buffered saline: 7.7 mM Tris/HCl, pH 7.5, and 150 mM NaCl)/Tween 20/5 % BSA followed by probing with anti-GST antibody. For quantification purposes, membranes were stripped of primary antibody and blotted with anti-HA antibody against total protein levels or separate Western blotting was performed with either anti-HA or anti-β-actin antibodies.

Fluorescence microscopy

GFP–LC3-expressing HEK-293E cells were generated by transfection with pBABE-puro GFP–LC3, selection for puromycin resistance and cell sorting for GFP expression. Cells were grown on coverslips, washed in PBS and incubated in DMEM without FBS for 2 h as indicated with or without 500 nM rapamycin, 350 nM insulin and/or 100 nM bafilomycin. 3T3-L1 cells expressing GFP–LC3 were incubated overnight in

DMEM and rapamycin and/or insulin was added as indicated. For ULK1 localization, 3T3-L1 fibroblasts were starved in the presence of HBSS (Hanks balanced salt solution) (no glucose). Cells were fixed and imaged for GFP fluorescence on a Nikon C1 inverted microscope using a Plan Apo 60 × lens [NA (numerical aperture) = 1.2] or a Plan Apo 100 × lens (NA = 1.4). Quantification of GFP–LC3 puncta was performed manually.

In vitro phosphorylation

Phosphorylation of ULK1 by AMPK was performed *in vitro* as described by Sanders et al. [29] with ULK1 instead of the SAMS peptide as a substrate in a reaction buffer [25 mM Hepes (pH 7.4), 7.5 mM $MgCl_2$, 0.3 mM ATP, 0.3 mM AMP, 0.5 mM DTT (dithiothreitol) and 0.25 % Triton X-100] with 1 μCi of [γ-^{32}P]ATP per reaction. ULK1 was expressed in HEK-293 cells and used for *in vitro* phosphorylation by AMPK after prior *in vitro* activation of AMPK by LKB1 (reaction buffer: 50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM AMP and 2 mM DTT).

Autophosphorylation of ULK1 was carried out in a reaction buffer (25 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM 2-glycerophosphate, 0.1 mM sodium orthovanadate, 2 mM DTT and 200 μM ATP) with 1 μCi of [γ-^{32}P]ATP per reaction. Similar studies were performed using recombinant Akt to determine Akt-dependent phosphorylation (reation buffer: 25 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM 2-glycerophosphate, 0.1 mM sodium orthovanadate, 2 mM DTT, 200 μM ATP and 220 ng of Akt/reaction).

RESULTS

ULK1 binds directly to 14-3-3 via phosphorylation of Ser^{555}

In an earlier screen for 14-3-3-binding proteins, we identified ULK1 [30]. It was shown recently that 14-3-3 interacts with the ULK1–raptor complex via an interaction with raptor [31]. To show that 14-3-3 binds to ULK1 directly, we performed a far-Western blot using 14-3-3 fused to GST as a probe. HEK-293FT cells expressing HA-tagged ULK1 were incubated in the absence or presence of glucose and amino acids, or in full medium with or without the addition of rapamycin. ULK1 was immunoprecipitated, subjected to SDS/PAGE and probed using recombinant GST–14-3-3. A direct interaction between GST–14-3-3 and ULK1 was observed, and this interaction was enhanced by starvation (Figure 1A). The interaction was dependent on the nutritional state and was markedly decreased in the presence of glucose, with the addition of amino acids having no further effect. Inhibition of mTOR with rapamycin in the presence of nutrients did not affect 14-3-3 binding to ULK1. Incubation of cells with the drug A769662, a stimulator of AMPK activity, further increased binding of 14-3-3 to ULK1, hinting at a possible role for AMPK in ULK1 phosphorylation. Endogenous 14-3-3 immunoprecipitated with HA–ULK1 showed the same pattern as the GST–14-3-3 overlay. Intriguingly, AMPK was also present in the ULK1 immunoprecipitates, consistent with recent studies [31]. Interestingly, the level of Thr^{172} phosphorylation, an indicator of AMPK activity, correlated with the level of 14-3-3 binding. In addition, treatment of HEK-293T cells with phenformin, another activator of AMPK, increased binding of 14-3-3 to ULK1 (see Supplementary Figure S1A at http://www.BiochemJ.org/bj/440/bj4400283add.htm).

Using Scansite [32], two putative 14-3-3-binding motifs were identified in ULK1 at Ser^{555} and Thr^{659}. Whereas the Ser^{555} site shows conservation in both ULK1 and ULK2, the Thr^{659} site was less conserved and not present in ULK2 (Figure 1B). Each of these motifs were mutated to alanine and retested for 14-3-3 binding. Mutation of Ser^{555} to alanine resulted in a significant reduction in 14-3-3 binding, whereas no change in binding was observed for the T659A mutant (Figure 1C). Furthermore, in the presence of A769662, the binding of GST–14-3-3 to ULK1 was greatly reduced (Figure 1D and see Supplementary Figure S1B). This suggests that Ser^{555}, which is conserved in ULK2 as well, is part of the major 14-3-3-binding site in ULK1.

AMPK phosphorylates ULK1 both *in vivo* and *in vitro*

The ability of AMPK agonists, but not rapamycin, to regulate 14-3-3 binding to ULK1 suggests a direct and mTOR-independent role for AMPK in ULK1 function (Figure 1A). To investigate further the role of AMPK, we performed a phosphorylation experiment *in vitro* using activated recombinant AMPK. The bacterially expressed AMPK complex consisting of the α-, β- and γ-subunits was activated *in vitro* by treatment with recombinant LKB1. WT ULK1 or the kinase-inactive ULK1^{K46R} or ULK1^{T180A} (see below) was incubated with activated AMPK or LKB1 kinase alone (Figure 2A). WT ULK1 showed significant autophosphorylation in accordance with the described autophosphorylation domain adjacent to its kinase domain [33], thus masking the potential effect of AMPK. In contrast, the kinase-dead ULK1 mutants were *in vitro* phosphorylated by AMPK, but not by LKB1. To test further whether this phosphorylation was site-specific, we performed *in vitro* phosphorylation using kinase-inactive ULK1^{K46R}, which lacked potential phosphorylation sites. Compared with controls, phosphorylation of ULK1^{K46R,S555A} was reduced by approximately 25 % (Figures 2B and 2C). This suggests that AMPK phosphorylates ULK1 at Ser^{555} and other sites. Screening for AMPK phosphorylation sites using Scansite software revealed a potential second phosphorylation site at Ser^{467}. However, incubation of the ULK1^{K46R,S467A} mutant with recombinant AMPK did not result in further reduction in *in vitro* phosphorylation of ULK1 (Figure 2B). To determine whether AMPK phosphorylation stimulates 14-3-3 binding, we performed *in vitro* phosphorylation of various ULK1 mutants with AMPK followed by far-Western blotting with GST–14-3-3 (Figure 2D). Only WT ULK1 and mutants containing Ser^{555} showed increased GST–14-3-3 binding in response to AMPK phosphorylation. We next explored phosphorylation of ULK1 by AMPK *in vivo* (Figures 2E and 2F). Expression of the dominant-negative AMPK^{D159A} α-subunit inhibits AMPK function by competing with the endogenous AMPK α-subunit in the AMPK complex [34]. ULK1 was co-expressed with either WT AMPK or the AMPK^{D159A} mutant. When ULK1 was co-expressed with WT AMPK, ULK1 phosphorylation, as assessed by 14-3-3 binding, was unchanged relative to the empty vector control. In contrast, co-expression of the dominant-negative AMPK^{D159A} mutant resulted in significant inhibition of 14-3-3 binding. The same effect was seen with the kinase-inactive ULK1^{K46R} mutant, which had a higher electrophoretic mobility due to defective autophosphosphorylation. Collectively, these results suggest a direct regulation of ULK1 by AMPK both *in vivo* and *in vitro*.

Phosphorylation of ULK1 at Ser^{774}

Autophagy is controlled by a number of intracellular pathways. In contrast with AMPK, the growth factor/Akt pathway represses autophagy. Scansite revealed a high-stringency Akt phosphorylation site at Ser^{774} in ULK1 (0.008 percentile) that is highly conserved in ULK1 and ULK2 isoforms (Figure 3A). The

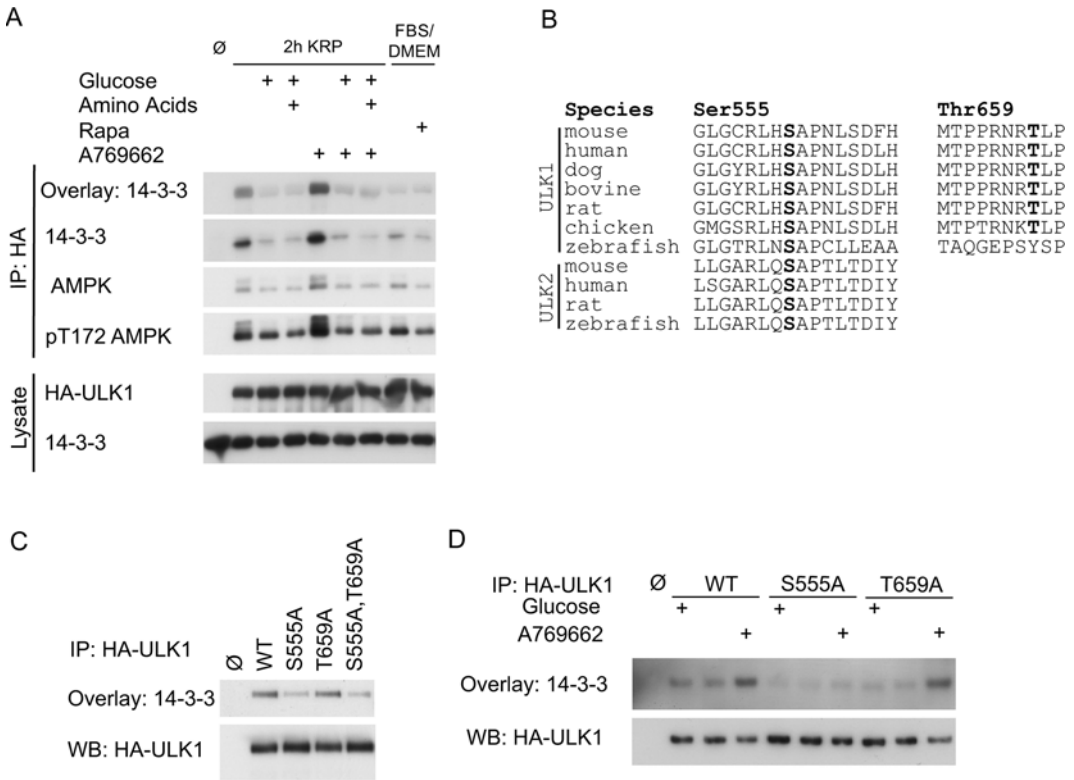

Figure 1 ULK1 binds directly to 14-3-3 via phosphorylation of Ser^{555}

(**A**) HA-tagged ULK1 was expressed in HEK-293FT cells. Cells were starved for 2 h in KRP supplemented with either glucose or glucose and regulatory amino acids in the absence or presence of 50 μM A769662 for 30 min. Alternatively, cells were incubated in FBS/DMEM with or without 500 nM rapamycin (Rapa) for 2 h. ULK1 was immunoprecipitated (IP) using an anti-HA antibody and subjected to SDS/PAGE and Western blotting using either an overlay with GST–14-3-3 followed by an anti-GST antibody or with anti-14-3-3, anti-(total AMPK), anti-(pThr^{172} AMPK) or anti-HA antibodies. (**B**) Conservation of the potential 14-3-3-binding sites Ser^{555} and Thr^{659}. (**C**) HEK-293 cells were transfected with vector alone, WT ULK1 or the ULK1 point mutants ULK1^{S555A} or ULK1^{T659A}, or the double mutant ULK1^{S555A,T659A}. Cells were incubated with KRP, HA–ULK1 was isolated by immunoprecipitation (IP) and detected by Western blotting (WB) using GST–14-3-3 overlay or an anti-HA antibody as in (**A**). (**D**) HEK-293 cells were transfected with WT ULK1, ULK1^{S555A} or ULK1^{T659A} and incubated for 2 h in KRP with or without glucose or in the presence of 50 μM A769662 for 30 min. HA–ULK1 was isolated by immunoprecipitation (IP) and detected by Western blotting using GST–14-3-3 overlay or an anti-HA antibody as in (**A**).

anti-PAS antibody specifically recognizes sites that have the Akt consensus phosphorylation motif R/K-X-R/K-X-X-pS [35,36] and the ULK1 sequence around Ser^{774} (**R**T**R**MF**S**V) fits this motif. The anti-PAS antibody recognized ULK1 immunoprecipitated from HEK-293 cells that had been incubated with or without berberine, an AMPK agonist that activates AMPK via the same mechanism as phenformin [37]. The binding of the anti-PAS antibody to ULK1 after berberine treatment of HEK-293 cells was, if anything, slightly reduced in contrast with the increase in GST–14-3-3 binding (Figure 3B). Mutation of Ser^{774} in ULK1 to alanine, on the other hand, resulted in reduced binding of the anti-PAS antibody. Interestingly, the kinase-inactive ULK1^{K46R} mutant did not show any labelling with the anti-PAS antibody, indicating that ULK1 kinase activity might be required in some way as a prerequisite for Ser^{774} phosphorylation. We next tested whether Ser^{774} ULK1 phosphorylation was insulin-responsive. To address this, we used HEK-293E cells, an insulin-responsive subline of HEK-293 cells (Figure 3C). These cells display robust insulin-dependent and wortmannin-sensitive phosphorylation of Akt at Ser^{473}. A similar pattern was observed for PAS binding to ULK1 (Figure 3C). The insulin-dependent binding of PAS was time-dependent, reaching a maximum after 15 min of incubation with insulin (see Supplementary Figure S2A at http://www.BiochemJ.org/bj/440/bj4400283add.htm). We next tested whether *in vitro* phosphorylation of ULK1 by Akt results in increased binding of anti-PAS antibody (Figure 3D). In WT ULK1 isolated from HEK-293 cells, a clear increase in anti-PAS antibody binding was observed in contrast with the S774A mutant. Interestingly, incubation of ULK1 in the presence of ATP alone already resulted in increased anti-PAS antibody binding, both to WT ULK1 and to the S774A mutant, indicating that the anti-PAS antibody may not only bind to Ser^{774}, but also recognize an autophosphorylation site. The same effect was observed when ULK1 was isolated from HBSS-starved cells before *in vitro* Akt phosphorylation (see Supplementary Figure S2B). However, the non-Akt-dependent PAS binding was much higher in the case of the S774A mutant, which could be consistent with a repressive role for Ser^{774} on autophosphorylation.

To test whether insulin repression of autophagy was dependent on mTOR, we treated HEK-293E cells with rapamycin in the absence and presence of insulin (Figure 4). In the HEK-293E cells, rapamycin slightly increased the level of membrane-associated LC3-II, as indicated by immunoblotting and by immunofluorescence localization of GFP–LC3 (Figures 4A–4D) consistent with increased autophagy. If the effects of insulin to repress autophagy are mediated entirely via activation of mTOR, then no repressive effect of insulin should be observed in the presence of rapamycin. However, this was not the case, as the rapamycin-dependent accumulation of LC3-II and the membrane localization of GFP–LC3 were completely blocked by insulin (Figures 4A–4D). In 3T3-L1 fibroblasts, autophagy was more robustly induced by rapamycin and, again, this effect was blocked in the presence of insulin (Figure 4E and see Supplementary Figure S3 at http://www.

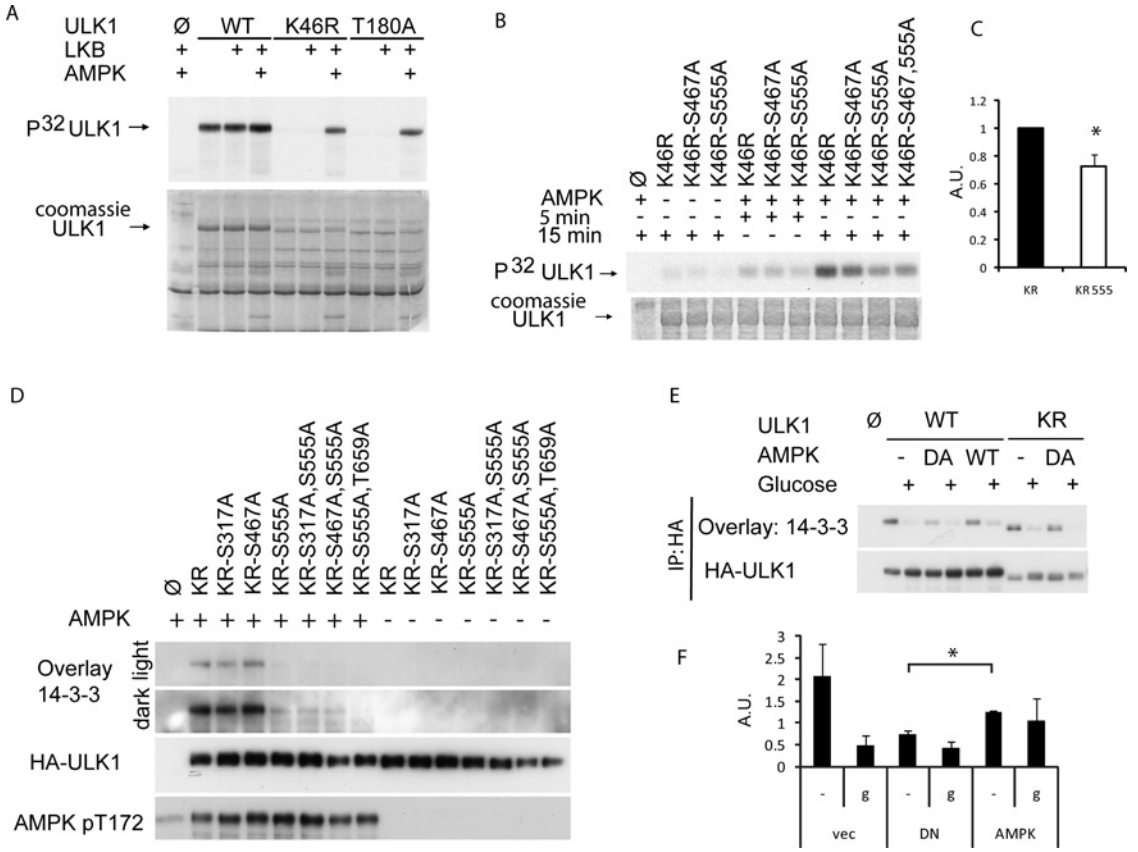

Figure 2 ULK1 is phosphorylated by AMPK *in vitro* and *in vivo*

(**A**) WT ULK1 or kinase-inactive mutant ULK1^{K46R} or ULK1^{T180A} was immunoprecipitated from transfected HEK-293FT cells and incubated without or with either LKB1 or with LKB1-activated AMPK in the presence of [γ-^{32}P]ATP. Immunoprecipitates were subjected to SDS/PAGE and phosphorylated material was visualized by autoradiography and Coomassie Blue staining. WT ULK1 shows evidence of autophosphorylation, whereas kinase-inactive ULK1 mutants are phosphorylated only by AMPK. (**B**) ULK1^{K46R}, ULK1^{K46R,S467A}, ULK1^{K46R,S555A} and ULK1^{K46R,S467A,S555A} were incubated with or without LKB1-activated AMPK in the presence of [γ-^{32}P]ATP for the indicated times at 37°C. ULK1 protein constructs were run on SDS/PAGE and visualized by autoradiography and Coomassie Blue staining. (**C**) Quantification of relative phosphorylation of ULK1^{K46R,S555A} compared with ULK1^{K46R} ($n = 5$, means $\pm$ S.E.M., $^*P < 0.05$). (**D**) ULK1^{K46R}, ULK1^{K46R,S317A}, ULK1^{K46R,S467A}, ULK1^{K46R,S555A}, ULK1^{K46R,S317A,S555A}, ULK1^{K46R,S467A,S555A} and ULK1^{K46R,S555A,T659A} were incubated with or without LKB1-activated AMPK in the presence of unlabelled ATP for 15 min as in (**B**). Samples were subjected to SDS/PAGE and Western blotting using either an overlay with GST–14-3-3 followed by an anti-GST antibody, or blotting with anti-HA or anti-(pThr^{172} AMPK) antibodies. (**E**) The dominant-negative AMPK^{D159A} inhibits ULK1 phosphorylation *in vivo*. WT ULK1 or ULK1^{K46R} (KR) was transfected with vector, WT AMPK or AMPK^{D159A} (DA) in HEK-293FT cells. Cells were starved in KRP for 2 h with or without glucose. Cells were lysed and ULK1 was immunoprecipitated (IP) and, after SDS/PAGE, detected by Western blotting using GST–14-3-3 overlay or an anti-HA antibody. (**F**) Quantification of GST–14-3-3 binding over total protein level (anti-HA) from (**E**). ($n = 3$, means $\pm$ S.E.M., $^*P < 0.005$). A.U., arbitrary units.

BiochemJ.org/bj/440/bj4400283add.htm). Taken together, these results are consistent with an mTOR-independent role of the insulin/Akt pathway in autophagy regulation.

ULK1–Atg13–FIP200 complex formation is independent of protein phosphorylation

Many kinases contain regulatory phosphorylation sites in their activation loop that are necessary for kinase activity. ULK1 contains a conserved threonine residue in its activation loop domain (Figure 5A) and a homologous site has been described in yeast [38]. To test whether this residue is required for ULK1 activation, the effect of mutating this residue to alanine in ULK1 autophosphorylation was measured. As shown in Figure 5(B), the ULK1^{T180A} mutant displayed no significant autophosphorylation activity. Atg13 has been described as a substrate for ULK1, and, to test its kinase activity towards an exogenous substrate, we co-expressed ULK1 with Atg13. An increase in Atg13 mobility indicating reduced phosphorylation was found for ULK1^{T180A} and, to a lesser extent, for ULK1^{K46R}, but not for other ULK1 mutants (Figure 5C). ULK1 inhibits mTOR-dependent phosphorylation of 4E-BP (eukaryotic initiation factor 4E-binding protein) via a negative-feedback loop [39]. This inhibitory effect was abrogated in the case of the kinase-inactive ULK1^{K46R} mutant and also for the ULK1^{T180A} mutant (Figure 5D). These results show that ULK1 activation requires phosphorylation at Thr^{180}, but not at Ser^{555} or Ser^{774}, for its activity. We next tested whether phosphorylation at Thr^{180}, Ser^{555} or Ser^{774} controls ULK1 localization. We retrovirally expressed WT ULK1 and phosphorylation mutants in 3T3-L1 fibroblasts and incubated the cells in full medium or in HBSS for 2 h (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/440/bj4400283add.htm). In response to starvation, WT ULK1, ULK1^{S555A} and ULK1^{S774A} were localized to small puncta in contrast with ULK1^{T180A}, consistent with ULK1's localization to phagophores.

To gain insight into the role of ULK1 phosphorylation on complex formation, we examined complex formation in cells expressing ULK1 mutants in the absence and presence of amino acids and glucose (Figure 5E). Neither starvation nor any of the ULK1 phosphorylation mutants affected complex formation, and, even in the case of the kinase-inactive T180A mutant, although there was a clear effect on Atg13 and FIP200 electrophoretic

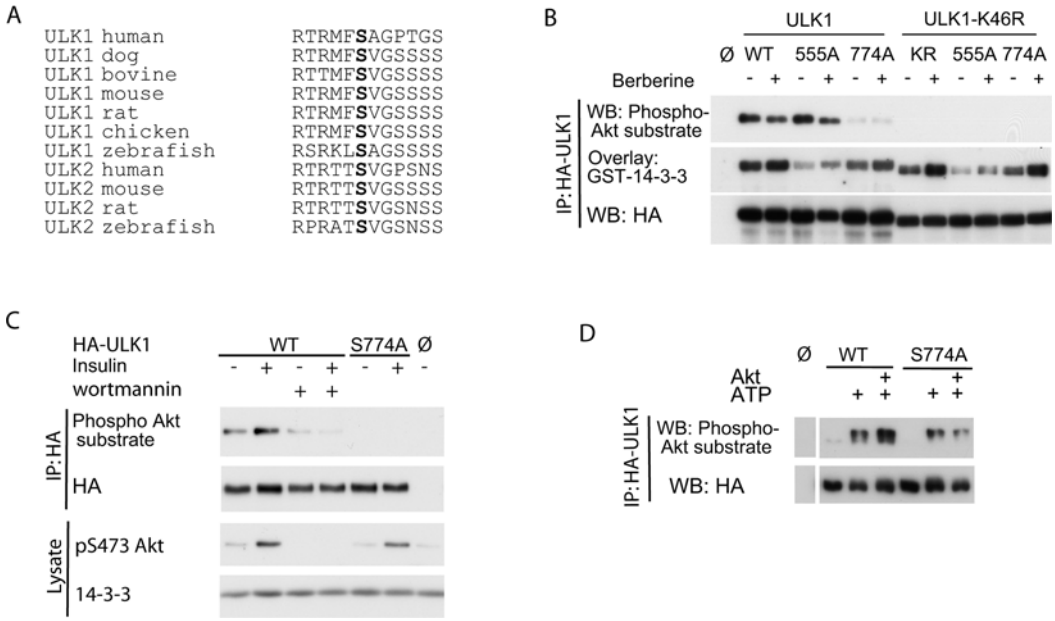

Figure 3 Phosphorylation of ULK1 at Ser^{774} is insulin-dependent

(**A**) Conservation of the putative phosphorylation site Ser^{774}. (**B**) Ser^{774} in ULK1 is recognized by the anti-PAS antibody. WT ULK1, ULK1^{K46R} or point mutants ULK1^{S555A}, ULK1^{S774A}, ULK1^{K46R,S555A} and ULK1^{K46R,S774A} were expressed in HEK-293FT cells. Cells were starved in KRP for 2 h, with or without berberine (10 μM) for 30 min. ULK1 was detected after immunoprecipitation (IP) as in Figure 1 by Western blotting (WB) with GST–14-3-3, anti-HA or anti-PAS antibody. (**C**) Phosphorylation at Ser^{774} is insulin-responsive and wortmannin-sensitive. WT ULK1 or ULK1^{S774A} was transfected into insulin-responsive HEK-293E cells. Cells were incubated for 2 h in DMEM and treated with or without wortmannin for 15 min before insulin treatment for 5 min. HA–ULK1 was visualized by Western blotting with either anti-HA or anti-PAS antibodies after immunoprecipitation (IP). Western blots for pSer^{473} Akt and 14-3-3 in the cell lysates are shown in the bottom panels. (**D**) *In vitro* phosphorylation of ULK1 by Akt. WT ULK1 or ULK1^{S774A} were immunoprecipitated (IP) from HEK-293FT transfected with ULK1 constructs together with Atg13 and incubated with DMEM without FBS for 2 h. Immunoprecipitates were left untreated or incubated with ATP alone or with ATP and Akt for 90 min at 37 °C. Immunoprecipitates were subjected to SDS/PAGE and Western blotting (WB) with either anti-HA or anti-PAS antibodies.

mobility, presumably due to phosphorylation, there was no visible effect on complex formation. This indicates that the mammalian ULK1–Atg13–FIP200 complex formation is independent of phosphorylation.

DISCUSSION

Regulation of autophagy is tightly associated with the cellular metabolic state. Anabolic signals, such as insulin, switch autophagy off, whereas catabolic signals tend to enhance autophagy. mTOR, which is activated by insulin and antagonized by AMPK, is thought to be the major regulator of autophagy by mTOR-dependent phosphorylation of ULK1 [18,21,23]. In the present study, we have shown that the RTK and AMPK pathways may have a direct effect on ULK1 activity independently of mTOR.

AMPK is involved in the regulation of both energy homoeostasis at the cellular level as well as metabolic control of whole organisms. This is underscored by the variety of substrates that are directly phosphorylated by AMPK. These include ACC (acetyl-CoA carboxylase), TBC1D4 [TBC (Tre-2/Bub2/Cdc16) domain family, member 4]/AS160 (Akt substrate of 160 kDa), PFK2 (phosphofructose kinase 2), mTOR and eNOS (endothelial nitric oxide synthase), which are involved in the regulation of fatty acid oxidation, glucose transport, glycolysis, protein synthesis and cardiovascular homoeostasis respectively [40]. Although AMPK has been implicated in autophagy in mammalian cells, no autophagy-specific targets had been identified until fairly recently [24–26,41–44]. In the present study, we have shown that ULK1 is a novel AMPK substrate and this leads to 14-3-3 binding to Ser^{555}. Similar findings have been described by others while the present paper was under review [24–26]. Also, in yeast, Atg1 suppresses glycogen storage defects caused by the lack of Snf1 [41] suggesting a functional link between the AMPK analogue Snf1 and Atg1.

AMPK and Akt have been shown to reciprocally regulate the phosphorylation of a range of substrates, including eNOS, AS160 and TSC2, and this usually has a reciprocal effect on function. On the basis of the present study, we suggest that ULK1 represents another example of this reciprocal mode of regulation. ULK1 phosphorylation was insulin-dependent as indicated by increased labelling of ULK1 with the anti-PAS antibody in response to insulin and this was blocked by the PI3K (phosphoinositide 3-kinase) inhibitor wortmannin. Mutation of ULK1 at the Ser^{774} Akt consensus site inhibited insulin-dependent phosphorylation of ULK1. Furthermore, WT ULK1 phosphorylation was responsive to Akt activity, whereas ULK1^{S774A} was not. These data suggest that ULK1 might be a direct Akt substrate. Moreover, insulin was shown to regulate autophagy independently of mTOR (Figure 4). This leads to a model whereby AMPK and Akt antagonistically regulate autophagy both upstream and downstream of mTOR. Both kinases phosphorylate TSC2, and AMPK also phosphorylates raptor, whereas Akt phosphorylates PRAS40, collectively regulating mTOR activity. Conversely, phosphorylation of ULK1 by both kinases would represent regulation downstream of mTOR and thus provide profound regulation of the autophagy pathway.

Intriguingly, some suggestions for partial mTOR-independent regulation of autophagy exist in the literature. For example, a role for Akt in the regulation of ULK1, independent of mTOR, is in accordance with a study showing synergistic effects of rapamycin and Akt inhibitors on autophagy [45]. In addition, mTOR inhibition alone results in only partial activation of autophagy compared with starvation, indicating the requirement for further regulatory steps independent of mTOR [21]. On the

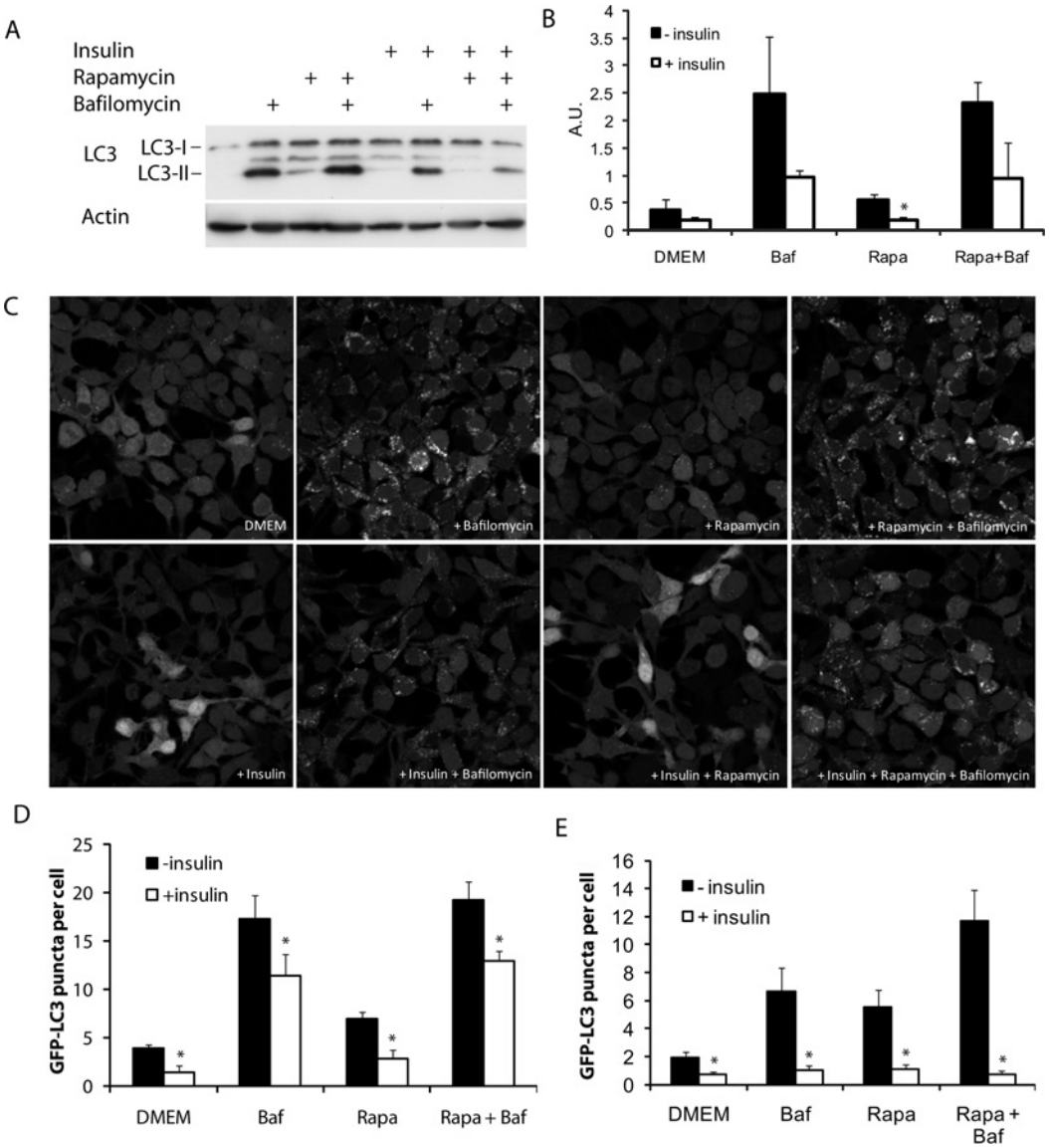

Figure 4 Insulin attenuates induction of autophagy by rapamycin

(**A**) HEK-293E cells were incubated for 2 h in DMEM without FBS in the absence or presence of 500 nM rapamycin, 350 nM insulin and/or 100 nM bafilomycin as indicated. Cell lysates were immunoblotted for endogenous LC3 and β-actin. (**B**) Quantification of LC3-II levels compared with β-actin (**A**). ($n = 3$, means $\pm$ S.E.M., $^*P < 0.05$). (**C**) HEK-293E cells stably expressing GFP–LC3 were incubated as indicated in the absence or presence of 500 nM rapamycin, 350 nM insulin and/or 100 nM bafilomycin for 2 h in DMEM without FBS. Cells were fixed and imaged using fluorescence microscopy. (**D**) Quantification of GFP–LC3 puncta in HEK-293E cells from (**C**) (30 cells/condition per experiment, $n = 3$, means $\pm$ S.E.M., $^*P < 0.05$). (**E**) Quantification of GFP–LC3 puncta in 3T3-L1 fibroblasts from Supplementary Figure S3 at http://www.BiochemJ.org/bj/440/bj4400283add.htm (30 cells/condition per experiment, $n = 3$, means $\pm$ S.E.M., $^*P < 0.05$). GFP–LC3-expressing 3T3-L1 fibroblasts were incubated overnight in DMEM without FBS and for 2 h in the absence or presence of 500 nM rapamycin (Rapa), 350 nM insulin and/or 100 nM bafilomycin (Baf) as indicated.

other hand, rapamycin only enhances ULK1 activity up to 1.5-fold [18]. Together with the recently described role of ULK1 in an Atg5-independent novel autophagy pathway [46], these data suggest regulation of autophagy at a level downstream of mTOR potentially through ULK1.

Many kinases, including members of the AGC kinase family and AMPK, contain an activating phosphorylation site in their kinase loop [47]. Phosphorylation can either occur by autophosphorylation, as in the case of PKA (protein kinase A), or by an independent kinase. For a number of AGC kinases, such as Akt, PKC (protein kinase C), S6 kinase and RSK (ribosomal S6 kinase), this upstream activation loop kinase seems to be PDK1. We have shown in the present study that the predicted homologous Thr^{180} in the activation loop of the catalytic centre of ULK1 is necessary for ULK1 kinase activity, as the T180A mutation leads to a functionally inactive kinase. It remains to be shown whether Thr^{180} is autophosphorylated or whether an upstream activation loop kinase for ULK1 exists. Recently, a homologous site in yeast Atg1 has been described and suggested to be autophosphorylated and shown to be required for autophagy induction in yeast [38].

It has recently been found that ULK1 can be phosphorylated by both AMPK and mTOR [24–26]. Egan et al. [24] found that AMPK leads to phosphorylation of ULK1 at Ser^{467}, Ser^{555}, Thr^{574} and Ser^{637}, whereas Kim et al. [25] observed phosphorylation at Ser^{317} and Ser^{777}. In addition, they also reported mTOR-dependent phosphorylation of ULK1 at Ser^{757}. AMPK-dependent phosphorylation was found to increase upon starvation, whereas mTOR phosphorylation at Ser^{757} inhibited ULK1 binding to

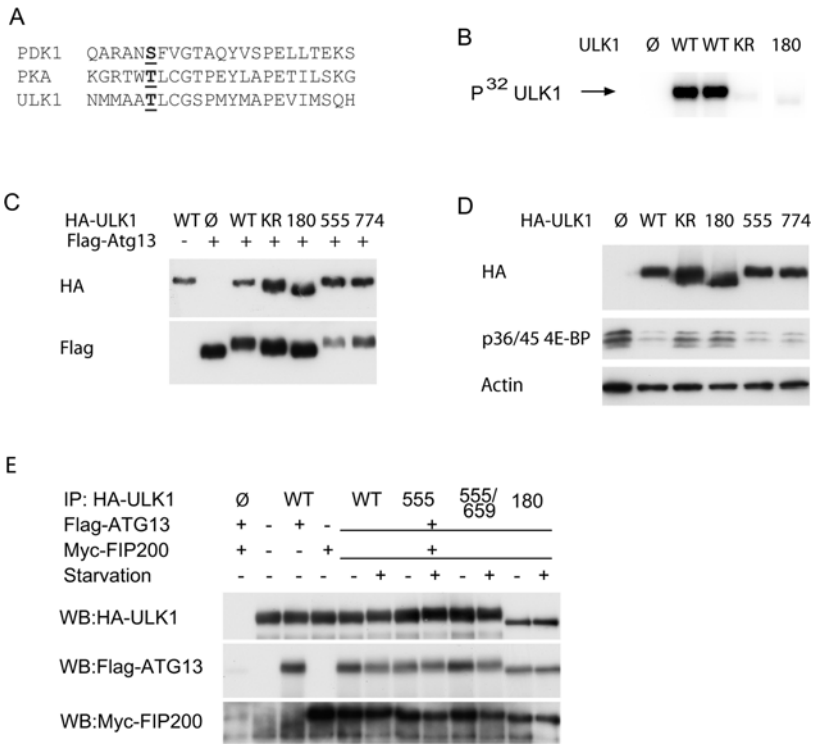

Figure 5 Thr^{180} in the conserved kinase-activation loop is necessary for ULK1 autophosphorylation activity, but not for ULK1–mAtg13–FIP200 complex formation

(**A**) Sequence comparison of ULK1 with PDK1 and PKA showing alignment of the conserved phosphorylation site in the kinase-activation loop. (**B**) Autophosphorylation of ULK1 is inhibited by mutation of Thr^{180}. WT ULK1, ULK1^{K46R} and ULK1^{T180A} mutants were immunoprecipitated from transfected HEK-293FT cells. Autophosphorylation was tested by incubating the ULK1 constructs in the presence of [γ-^{32}P]ATP. After SDS/PAGE, phosphorylation was visualized by autoradiography. (**C**) ULK1^{S180A} does not cause a mobility shift in co-expressed Atg13. WT HA–ULK1, ULK1^{K46R}, ULK1^{T180A}, ULK1^{S555A} and ULK1^{S774A} mutants were co-transfected with FLAG–Atg13 into HEK-293FT cells. Cell lysates were analysed by SDS/PAGE and Western blotting using anti-HA or anti-FLAG antibodies. (**D**) ULK1^{S180A} shows the characteristics of the kinase-inactive K46R mutant with respect to retrograde inhibition of mTOR signalling. WT ULK1, ULK1^{K46R}, ULK1^{T180A}, ULK1^{S555A} and ULK1^{S774A} mutants were transfected into HEK-293FT cells and incubated in DMEM and FBS for 2 h. Cells were lysed in 2% SDS and HES buffer and lysates were analysed by SDS/PAGE and Western blotting using anti-HA or anti-(pSer^{36}/pSer^{45} 4E-BP) antibodies. (**E**) ULK1–Atg13–FIP200 complex formation is independent of ULK1 phosphorylation at Thr^{180}, Ser^{555} or Thr^{659}. HA–ULK1, FLAG–Atg13 and Myc–FIP200 were co-expressed in HEK-293FT cells. Cells were incubated in either DMEM (−) or KRP (+) buffer for 2 h. ULK1 was immunoprecipitated (IP) with an anti-HA antibody. After SDS/PAGE, immunoprecipitates were analysed by Western blotting (WB) using anti-HA, anti-FLAG and anti-Myc antibodies.

AMPK, thereby preventing activation of ULK1. In contrast, Shang et al. [26] found a decrease in phosphorylation at Ser^{637} upon starvation. The regulation is complicated further by differences in cell types in their dependency on ULK1/2 during glucose starvation [48]. In the present study, we have found additional evidence for AMPK-dependent regulation of ULK1 at Ser^{555} and show that phosphorylation at this site is required for the ULK1 interaction with 14-3-3. Moreover, we describe two additional phosphorylation sites in ULK1 at Ser^{774} and Thr^{180}. We propose that phosphorylation of ULK1 does not regulate the formation of the Atg13–ULK1–FIP200 complex itself, but is involved in the regulation of other protein–protein interactions, such as the binding of 14-3-3 and possibly the association with the mTOR complex [21]. In addition, we propose that phosphorylation regulates the activation of the ULK1 kinase complex via both conformational changes and by regulating its intracellular localization. Taken together, our results are consistent with a working model by which ULK1 could form a signalling node that receives and processes the input of a number of signalling pathways implicated in autophagy regulation.

AUTHOR CONTRIBUTION

Markus Bach and Georg Ramm performed the experimental work. Mark Larance contributed to the experimental work. Georg Ramm and David James supervised the work and wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

The serine/threonine kinase ULK1 is a target of multiple phosphorylation events

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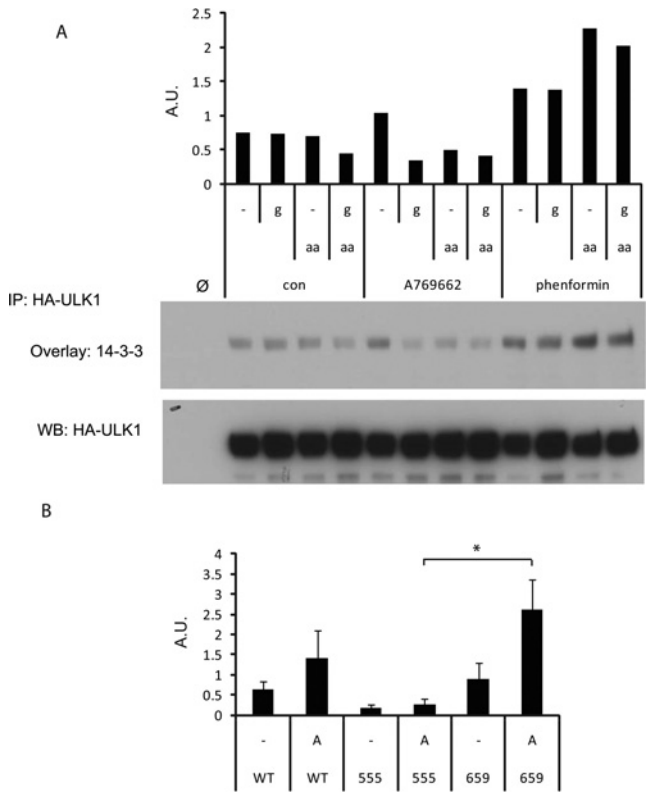

Figure S1 AMPK agonists increase 14-3-3 binding to Ser^{555} of ULK1

(**A**) HA-tagged ULK1 was expressed in HEK-293FT cells. Cells were starved for 2 h in KRP supplemented with glucose (g) and/or regulatory amino acids (aa) in the absence or presence of 50 μM A769662 or 2 mM phenformin for 30 min. ULK1 was immunoprecipitated (IP) using an anti-HA antibody and subjected to SDS/PAGE and Western blotting (WB) using either an overlay with GST-14-3-3 followed by an anti-GST antibody or anti-HA antibody. (**B**) Quantification of Figure 1(D) of the main text. GST–14-3-3 binding was quantified relative to total protein level (anti-HA) ($n = 3$, means $\pm$ S.E.M., $^*P < 0.05$). A.U., arbitrary units.

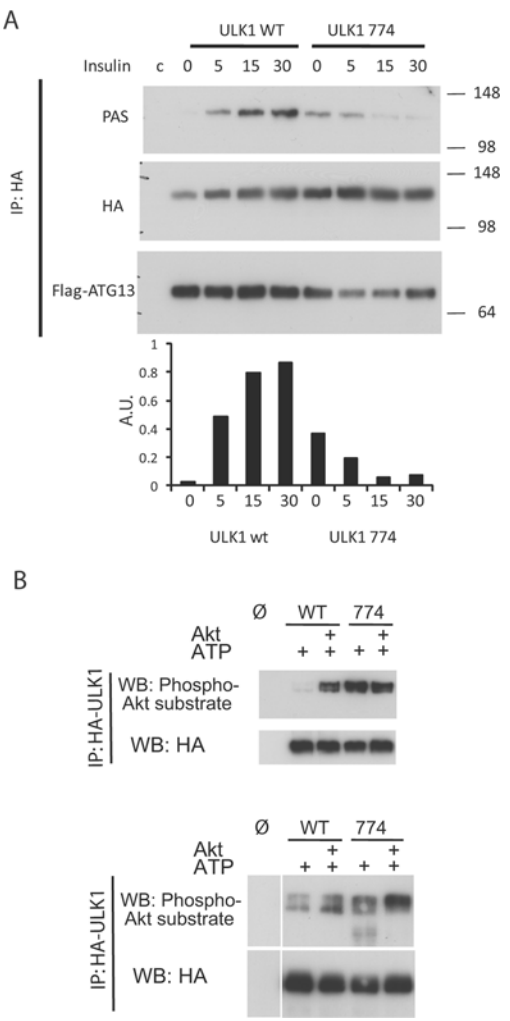

Figure S2 Insulin and Akt-dependent binding of PAS to ULK1

(**A**) Time-dependent phosphorylation of ULK1 in response to insulin. HA-tagged ULK1 or ULK1^{S774A} was co-expressed with FLAG-tagged Atg13 in HEK-293E cells. Cells were incubated for 2.5 h in DMEM and for the last 5, 15 or 30 min with 350 nM insulin. ULK1 was immunoprecipitated (IP) using an anti-HA antibody and subjected to SDS/PAGE and Western blotting with anti-PAS, anti-HA and anti-FLAG antibodies. PAS binding over total protein level is shown in the histogram. A.U., arbitrary units. (**B**) *In vitro* phosphorylation of ULK1 by Akt. WT ULK1 or ULK1^{S774A} were immunoprecipitated (IP) from HEK-293FT cells transfected with ULK1 constructs together with Atg13 and incubated with HBSS for 2 h. Immunoprecipitates were incubated with ATP alone or with ATP and Akt for 90 min at 37 °C. Immunoprecipitates were subjected to SDS/PAGE and Western blotting (WB) with either anti-HA or anti-PAS antibodies.

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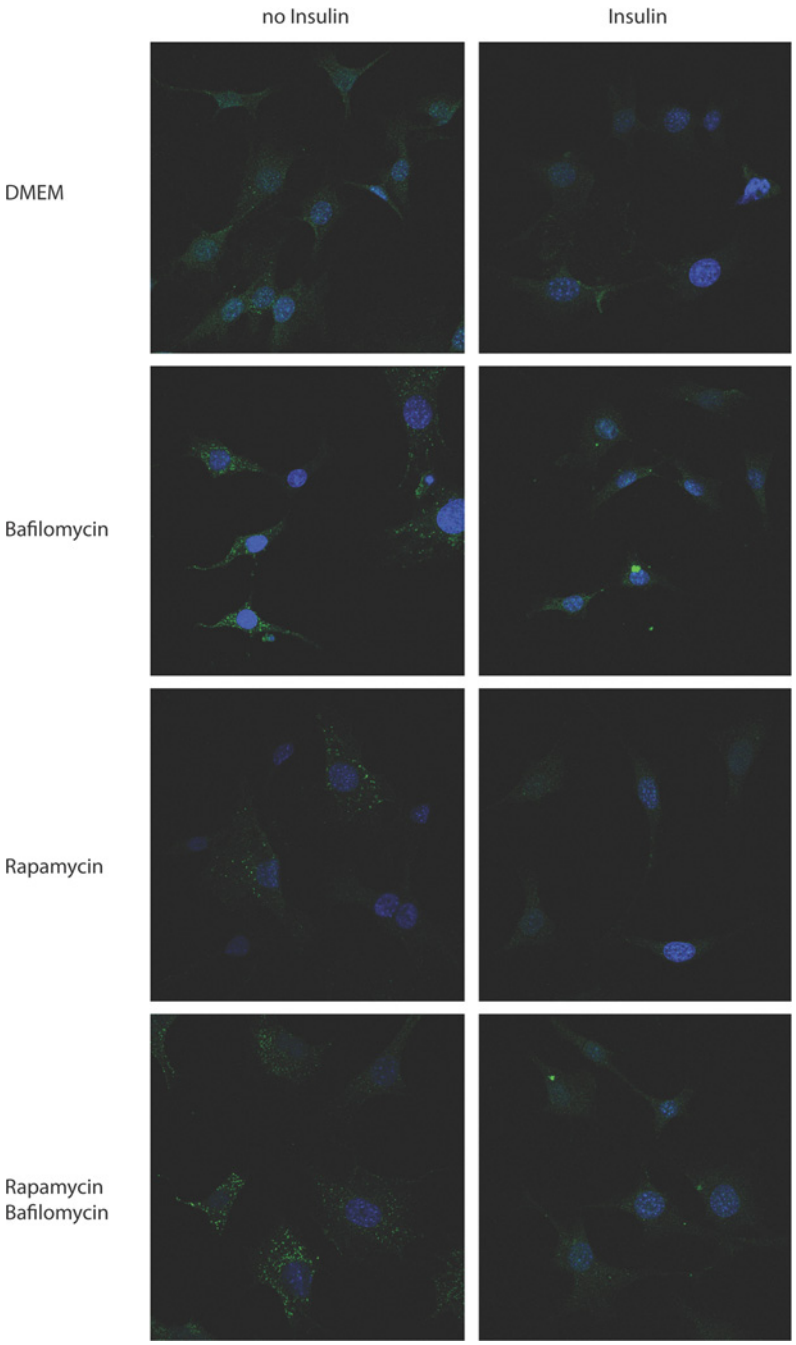

Figure S3 Insulin attenuates induction of autophagy by rapamycin in 3T3-L1 fibroblasts

GFP–LC3-expressing 3T3-L1 fibroblasts were incubated overnight in DMEM without FBS and for 2 h in the absence or presence of 500 nM rapamycin, 350 nM insulin and/or 100 nM bafilomycin as indicated.

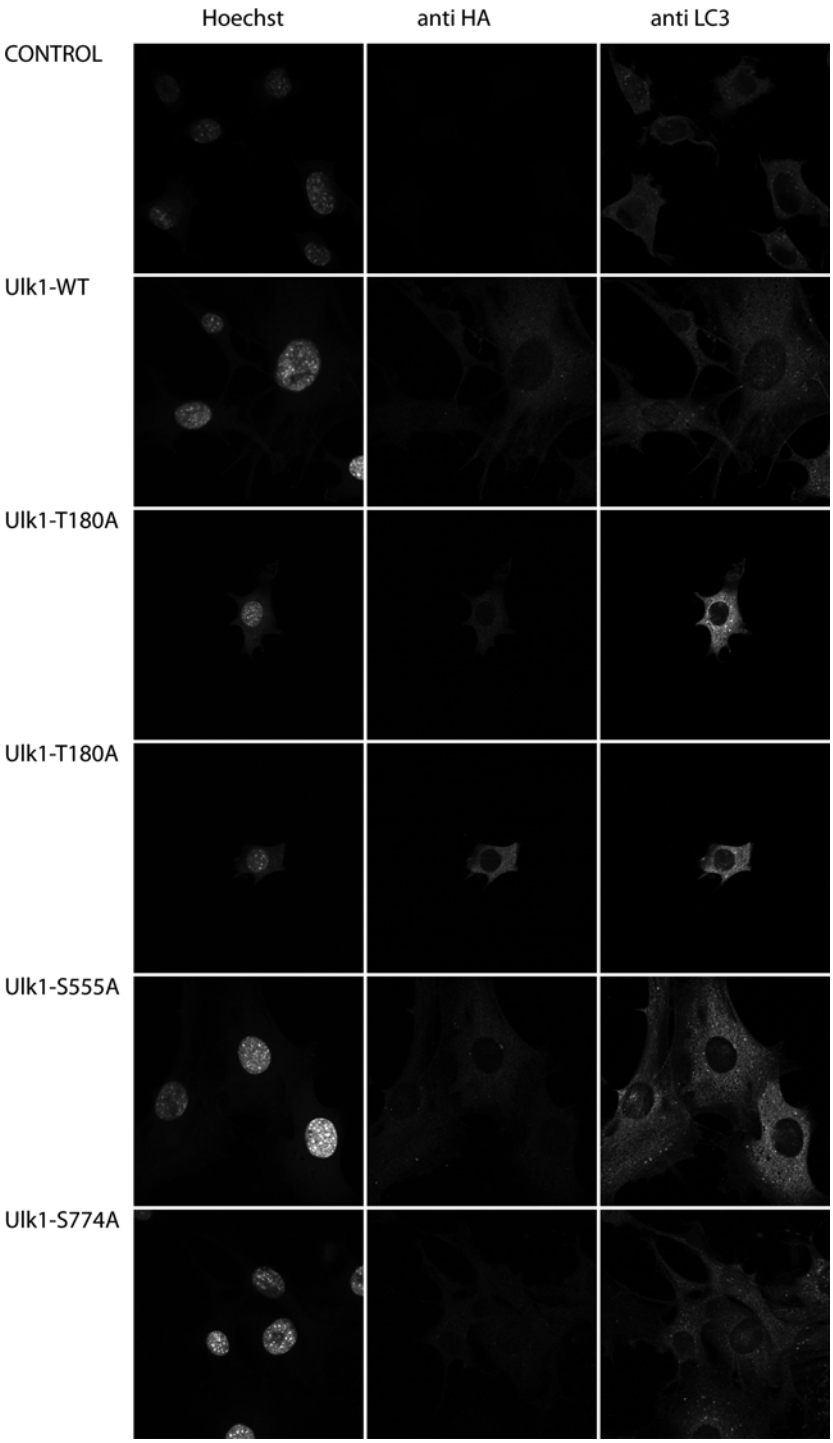

Figure S4 ULK1 puncta formation in 3T3-L1 fibroblasts in response to starvation in HBSS (no glucose) for 2 h

WT ULK1, and ULK1^{T180A}, ULK1^{S555A} and ULK1^{S774A} mutants were retrovirally expressed in 3T3-L1 fibroblasts. Cells were starved for 2 h, fixed, labelled with anti-HA and anti-LC3 antibodies followed by secondary antibodies coupled to Alexa Fluor® 488 or Alexa Fluor® 568 and imaged using fluorescence microscopy.

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