A non-canonical MEK/ERK signaling pathway regulates autophagy via regulating Beclin 1

Jianrong Wang‡§1, Mary W. Whiteman§, Huiqin Lian§, Guangxin Wang§, Amit Singh§, Dongyang Huang‡, Ted Denmark§

From the ‡Medical College Shantou University, Guangdong 515041, China; §Treman Research Institute, Ithaca, NY 14850, USA

Correspondence: wangjr@stu.edu.cn

Running title: Signaling regulation of autophagy via Beclin 1

Autophagy is an evolutionally-conserved machinery involving the degradation and turnover of cytoplasmic material in lysosomes. Autophagy plays a role in cellular homeostasis (1), antiaging (2, 3, 4), development (1, 5), protection of the genome (6) and regulation of cell size (7). Autophagy may act as a means of defense against bacterium and virus invasion and link to various diseases including cancer (8-10), cardiomyopathy (11) and neurodegenerative disorders (12).

Autophagy starts with the formation of an autophagosome, enclosed within a double membrane that engulfs part of the cytoplasm. During periods of autophagy stimuli, cells respond to either maintain the metabolism essential for survival or execute cell death. Autophagy-essential proteins (Atg) are the molecular basis of autophagy machinery. About thirty Atg proteins in yeast and ten in mammals have been identified. In yeast, the protein kinase target of rapamycin (TOR) mediates autophagy via Atg1-Atg13 kinase complex. Atg1 interacts with multiple components of the autophagic machinery, through direct association, phosphorylation, and/or intracellular localization (13, 14).

In mammalian systems, autophagosomes fuse with lysosomes to generate autophagolysosomes, which undergo a maturation process by fusing with endocytic compartments and lysosomes (15). Since it is not known how the Atg1 homolog acts in mammals, a different mechanism may be involved in regulating autophagy. Beclin 1/Atg6, microtubule-associated protein 1 light chain 3 (LC3)/Atg8, Atg5, Atg12 and Atg 13 are essential for autophagosome formation in mammalian species (5, 16-20). Atg7 and Atg3 are required in the conjugation reaction between Atg12 and Atg5, and in the lipidation of LC3. LC3 is lipidated via a ubiquitylation-like system (17, 21), generating a soluble form LC3-I. LC3-I is further modified to a membrane-bound form, LC3-II, which is subsequently localized to...
autophagosomes and autolysosomes until being degraded by the lysosome.

Beclin 1 was initially isolated as a B-cell lymphoma-2 (Bcl2)-interacting tumor suppressor in mammalian cells (22). Overexpression of Bcl2 attenuates the formation of the kinase complex Beclin 1-class III PtdIns 3-kinase (PI3KC3) essential for the formation of autophagosomes (23). The UV radiation resistance-associated gene tumor suppressor (UVRAG) and the activating molecule in Beclin 1-regulated autophagy protein 1 (Ambra1) were identified as new Beclin 1-binding partners that also regulate autophagy by regulating the Beclin 1-PI3KC3 kinase complex. Association of Beclin1 with PI3KC3 is negatively regulated by Bcl2 (22) and positively regulated by UVRAG and Ambra1 (24, 25). Beclin 1 is homoallelically deleted in many human tumors. Decreased Beclin 1 level causes defective autophagy and breast cancer, but restoration of Beclin 1 induces autophagy and inhibits tumorigenicity of human breast cancer cells (18). These reports evidence the dependency on Beclin 1 for a functional autophagy mechanism.

Diverse signaling pathways have been reported in the regulation of autophagy in mammalian cells (26, 27). In contrast to yeast, mammalian cells regulate autophagy via both class I and class III PI3K. Class I PI3K plays an inhibitory role, while class III PI3K kinase complex, which includes Beclin 1, plays a stimulatory role in autophagy by promoting the nucleation of autophagic vesicles (28, 29). Recent study also indicates that hVps15 is required in regulation of class III PI3K in mammalian cells (30). However, the signaling mechanisms controlling autophagy-essential proteins, in particular the Beclin 1, and the opposing consequences of autophagy remain to be resolved.

Our present studies identify and position a non-canonical MEK/mitogen activated protein kinase kinase/ERK/extracellular signal-regulated kinase pathway downstream of AMPK (AMP activated protein kinase) and upstream of TSC (tuberous sclerosis complex) and mTOR (mammalian target of rapamycin). This MEK/ERK module regulates autophagy via regulating Beclin1 level through AMPK-MEK/ERK-TSC-mTOR pathway. Moderately enhanced Beclin 1 by transient or moderate activation of MEK/ERK and subsequent inhibition on mTORC1 (mTOR complex 1) or mTORC2 (mTOR complex 2) individually causes protective autophagy. Strongly pronounced Beclin 1 by sustained or strong activation of MEK/ERK followed by dual inhibition on mTORC1 and mTORC2 causes destructive autophagy. Our results thus reveal interesting Beclin 1 thresholds in regulating autophagy.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Antibody and reagent sources were the following: LC3, Beclin 1, PI3KC3 (Abgent), Akt, Raptor, Rictor, GβL, β-actin, Raf1, non-phospho or phospho-MEK1/2, ERK1/2, p90RSK, Elk, AMPKα, TSC1, TSC2, mTOR, p90RSK, Elk, 4EBP1 and S6K (Cell Signaling), vitamin D3 (Cayman Chemical), rapamycin and bafilomycin A1 (Calbiochem), pepstatin A (Sigma), PD98059 and AICAR (Toronto Research Chemicals), and compound C (Merck).

**Cell culture**—Human erythroleukemia K562 cells were grown in IMEM and Rat hepatoma H4IIE cells were grown in DMEM. The cells were starved for amino acid by culturing in Dulbecco’s Phosphate Buffered Saline (D-PBS) with 10% of fetal calf serum, or starved for serum by culturing in IMEM (K562) or DMEM (H4IIE).

**Mice**—C57BL/6 mice were used to test physiological response of the autophagic signaling. Heart and liver tissues were surgically excised, immediately frozen in liquid nitrogen, ground in liquid nitrogen with a mortar and pestle, and homogenized briefly with an electric blender in 5 M guanidinium thiocyanate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, and 25 mM sodium citrate.

**DNA constructs and transfection**—Constitutively active MEK (218D222D) was ordered from Addgene (31), constitutively active MEK1 mutant with disrupted ERK docking site motif (11A40A99A204A) was created from the MEK1(218D222D) by mutagenesis, wt-ERK1 and dominant negative ERK1 (T202AY204F) was provided by Melanie Cobb (32), constitutive active ERK1 (T217DY221D) was created from the wt-ERK1 by mutagenesis, constitutively active Akt was created as previously described (33). Rat AMPKα2 kinase-dead construct
was made by mutating its cDNA at the lysine residue critical for ATP binding and hydrolysis with lysine 45 changed to arginine, resulting in a cDNA encoding a kinase-dead protein (34). The mutated cDNA was subcloned into a mammalian expression vector with GFP marker. GFP-LC3 plasmid was from Addgene. The above DNA constructs were transfected into H4IIE cells with DEAE-Dextran or K562 cells with electroporation, followed by selection against G418. Transfectants with a green fluorescent protein (GFP) marker were further sorted by flow cytometry.

RNA interference—RNA interference was performed by transfection of the cells with 20 nanomolars of desalted, purified and annealed double-stranded siRNA against the designated genes using DEAE-Dextran transfection for H4IIE cells or electroporation for K562 cells. Human siRNA sources: MEK1/2, Raf1, ERK1/2, mTOR, TSC2 (Cell Signaling), Beclin1, p90RSK, Elk, Raptor and Rictor (Santa Cruz).

Kinase assay—mTOR kinase assay was performed as previously described. Radioactivity in GST-4EBP1 was quantitated by Molecular Dynamics PhosphorImager and ImageQuant software (35).

Autophagy assay—Autophagy was determined by detection of the processing of autophagy marker LC3 by autophagic flux assay (36), fluorescence microscopic detection of the formation of the autophagosomes in the cells transfected with GFP-LC3 as described (37).

Immunoblotting and co-immunoprecipitation—Western blotting and co-immunoprecipitation were performed as described (38).

RESULTS

Autophagy response is MEK/ERK-dependent—To explore the role of MEK/ERK in the induction of autophagy, we examined whether MEK/ERK is activated in response to autophagy stimuli using rat hepatoma H4IIE and human erythroleukemia K562 cells. Autophagic response involves the cleavage of cytosolic LC3-I into a lipidated LC3-II, which is then recruited to the autophagosomal membrane (19, 21 and 39). Thus, processing of LC3 and punctuate GFP labeled LC3 pattern represents autophagosomes or autophagic responses. Autophagy stimuli including rapamycin or vitamin D3 treatment, amino acid or serum starvation caused autophagic responses shown by the processing of LC3-I into LC3-II. These autophagic activities coincided with MEK/ERK activation in both H4IIE (Fig. 1A) and K562 cells (Fig. 1B). To test whether the above coincidence is a causal event, the rat and human cell lines were treated with autophagy stimuli together with MEK inhibitor PD98059. Inhibiting MEK/ERK by PD98059 abolished the autophagic response (Fig. 1A, B), indicating that induction of autophagy depends on MEK/ERK activation. Similar results were observed in human myeloid leukemia HL-60 cells and human breast cancer MCF-7 cells (data not shown). To test if activation of MEK/ERK is sufficient to trigger autophagy, H4IIE cells were transfected with constitutively active MEK or ERK. The results show that constitutive expression of active MEK or ERK triggered autophagic responses indicated by autophagic flux assay (Fig. 1C).

To visually observe the dependency of autophagy on a MEK/ERK signal, we transfected H4IIE or K562 cells with a GFP-LC3 construct followed by autophagy stimuli with or without PD98059 treatment. The fluorescence microscopic results show that inhibiting MEK/ERK inhibited punctuate GFP-LC3 pattern caused by autophagy stimuli (Fig. 1D), further indicating that the induction of autophagy requires MEK/ERK signals. These data suggest that induction of autophagic responses may ubiquitously depend on MEK/ERK activation.

Expression of Beclin 1 is MEK/ERK-dependent—The immediate question is how autophagy is regulated by MEK/ERK signaling. Beclin 1 is ubiquitously and constitutively expressed at basal level in mammalian cells despite its frequent downregulation in some cancer cells. Beclin 1 expression level coincides with autophagy activity and tumorigenesis suppression (8, 18 and 40). Defective Beclin 1 expression or depleting Beclin 1 cripples autophagy (18, 37 and 41), but its overexpression triggers autophagy (18, 42). Since autophagy response depends on both Beclin 1 and activation of MEK/ERK, we asked whether constitutive Beclin 1 is regulated by MEK/ERK. Inhibiting the basal MEK/ERK
activity by PD98059 or knockdown of MEK by RNA interference resulted in the depletion of constitutive Beclin 1 (Fig. 1E), indicating that expressing or maintaining the constitutive Beclin 1 depends on the basal activity of MEK/ERK. However, knockdown of Raf1 by RNA interference or constitutive expression of the Raf1 CR3 domain, the active Raf1, which was proven functional previously (38), hardly affected the basal activity of MEK/ERK (Fig. 1E, F), suggesting that basal MEK/ERK activity conferring the constitutive Beclin 1 may largely be independent of Raf1. We then asked if MEK/ERK activation in response to autophagy stimuli upregulates Beclin 1. Indeed, the increase in Beclin 1 by autophagy stimuli coincided with MEK/ERK activation and inhibiting MEK/ERK activation abolished Beclin 1 upregulation in response to autophagy stimuli in both H4IIE and K562 cells (Fig. 1G). Despite MEK/ERK activation by autophagy stimuli, depleting Beclin 1 by RNA interference abolished autophagic responses (Fig. 1H). These data thus suggest that both basal Beclin 1 and upregulation of Beclin 1 are MEK/ERK-dependent and MEK/ERK regulates autophagy through regulating Beclin 1 level.

**MEK can bypass ERK to trigger autophagy**—Previous studies suggested that ERK is required in autophagy; however, the autophagy dependency on ERK was largely concluded from pharmacological inhibition with a MEK inhibitor (43, 44). To better understand the role of ERK in regulating autophagy, we examined the consequences on autophagy by depleting the ERK1/2 canonical downstream effectors, p90RSK and Elk, by RNA interference in K562 cells. MEK1/2 and ERK1/2 activation triggered by amino acid starvation coincided with the phosphorylation of p90RSK (Fig. 2A) and Elk (Fig. 2B). Knockdown of either p90RSK or Elk did not affect the autophagic response to amino acid starvation (Fig. 2A, B), suggesting that ERK may cross-talk with other pathways or use non-canonical downstream effectors to regulate autophagy, or MEK may bypass ERK to regulate autophagy. To answer this question, ERK1/2 was knocked down by RNA interference in K562 cells and the cells were starved for amino acids. Depleting ERK1/2 partially inhibited autophagic responses (Fig. 2C), suggesting that although ERK is involved in the regulation of autophagy, MEK may play more critical role in regulating this cellular cascade by bypassing ERK. To confirm this, K562 cells were transfected with constitutively active MEK1, or co-transfected with both constitutively active MEK1 and dominant negative ERK1. MEK activation upregulated Beclin 1 and triggered LC3 processing, and inhibition on ERK by dominant negative ERK expression attenuated, but did not block, the autophagic response by constitutively active MEK shown by the autophagic flux assay (Fig. 2D). The above data propose that despite the role of ERK in regulating autophagy to some extent, MEK can bypass ERK to induce autophagy.

**AMPK is an upstream regulator of MEK in regulating autophagy**—An early study reported that activation of AMPK by addition of the cell-permeable nucleotide analogue AICA riboside (AICAR) in hepatocytes inhibits autophagy (45). But, a recent study using different mammalian cell types and compound C, a specific AMPK inhibitor, showed that AMPK is required for autophagy (46). Our results show that starvation-caused MEK activation as well as AMPK activation and autophagic response were inhibited by compound C in H4IIE cells (Fig. 3A), while inhibition of MEK by PD98059 did not inhibit AMPK activation in response to the starvation (Fig. 3B), suggesting that AMPK may be an upstream regulator of MEK in modulating autophagy.

To understand how MEK links AMPK, H4IIE cells were treated with AICAR to trigger autophagic responses. Inhibiting MEK did not inhibit AMPK activation by AICAR. AICAR activated MEK and triggered autophagic responses shown by elevated Beclin 1 level and processing of LC3. Inhibiting MEK blocked the autophagic response by AICAR (Fig. 3C). This result suggests that enhancing Beclin 1 and triggering autophagic responses by AMPK activation depends on MEK activation. Furthermore, co-immunoprecipitation results revealed that MEK interacts with AMPK but not Raf1 in response to autophagy stimuli (Fig. 3D), and this interaction is apparently caused by activation of AMPK and MEK signaling because activated MEK binds to activated AMPK, but hardly binds to Raf1 upon exposure to autophagy stimuli (Fig. 3E). In contrast, growth hormone treatment, which is known to activate the canonical Raf/MEK/ERK pathway.
in H4IIE cells (47), caused strong Raf1 binding to MEK (Fig. 3D, E). The results thus propose that MEK interacts with its upstream regulator AMPK, but not Raf1, in response to autophagy stimuli.

To confirm the role of AMPK as an upstream regulator of MEK in regulating autophagy proposed by the pharmacological data, we generated kinase-dead AMPKα2 H4IIE cell line by mutating rat AM PKα2 cDNA at the lysine residue critical for ATP binding and hydrolysis which was reported by an early study (34). The results show that in kinase-dead (KD) AM PKα2 cells, amino acid starvation, which is a strong autophagy stimulus, failed to activate MEK and cause autophagic precessing of LC3 (Fig. 3F), further supporting the notion that AMPK regulates autophagy via regulating its downstream effector MEK.

MEK/ERK binds to and activates TSC2 in regulating autophagy—AMPK activates TSC2 by phosphorylation (48) while activation of TSC2 suppresses mTOR signaling (49) and activation of AM PK downregulates mTOR activity (50), supporting a positive role of AMPK in regulating autophagy. Both H4IIE and K562 cells express a dominant TSC2 and a weak TSC1 (Fig. 4A). To examine whether MEK/ERK plays a role in TSC activation by AM PK, H4IIE cells were stimulated with AICAR, with or without PD98059. Activation of AM PK activated TSC2, but inhibiting MEK inhibited TSC2 activation by AM PK shown by the crippled phosphorylation of TSC2 (Fig. 4B), suggesting that activation of TSC2 by AM PK is MEK/ERK-dependent.

Upon activation by autophagy stimuli, MEK formed multiple complexes (Fig. 4C). TSC2 bound to the activated MEK and activated ERK (Fig. 4D), suggesting that TSC2 is the downstream effector of MEK/ERK in regulation of autophagy.

Physical interaction between TSC2 and ERK was previously reported (51). TSC2 binds to ERK through the ERK docking site (ERK D domain), consisting of a conserved L/I-X-L/I motif located three to five residues downstream of a cluster of basic amino acids (52). Since MEK activation can induce autophagy when ERK activation is inhibited (Fig. 2D), we asked if MEK can directly activate TSC through physical interaction. To test whether MEK interacts with TSC2 through the ERK D domain, the four ERK D domains were disrupted by mutating the four amino acids in the ERK docking site motif (L11A L40A I99A and I204A) of the active MEK1. In the absence of ERK by RNA interference, constitutively active wild-type MEK interacted with TSC2, but disruption of the ERK D domains of MEK1 attenuated this interaction (Fig. 4E), suggesting that MEK interacted with TSC2 through the ERK D domains. Constitutively active MEK phosphorylated TSC2 and caused LC3 processing, which was not affected by depleting ERK with RNA interference, but disrupting these docking sites of MEK1 crippled the phosphorylation of TSC2 and the processing of LC3 in the cells that express ERK (Fig. 4F). These results indicate that MEK binds to and activates TSC2 independent of ERK in regulating autophagy.

AMPK-MEK/ERK-TSC2 signaling functions in physiological response of autophagy—To test if this non-canonical MEK/ERK module physiologically regulates autophagy, we detected MEK/ERK signaling in neonatal mice, which adapt to sudden interruption of trans-placental nutrient by inducing autophagy until supply can be restored through milking (5). At 5h after birth without milking, the heart and liver samples displayed AMPK and MEK/ERK activation, Beclin 1 upregulation and LC3 processing, as well as MEK/ERK association with AMPK or TSC1 due to nutrient interruption. By contrast, at 120h after birth when the mice began suckling for several days, activation of AMPK or MEK/ERK or physical interaction between MEK/ERK and AMPK or TSC1 was not detected (Fig. 5A, B). These results suggest that the non-canonical MEK/ERK signaling plays a physiological role in the regulation of mammalian autophagy.

MEK/ERK regulates Beclin 1 through negatively regulating mTORC1 and mTORC2—Unlike positive regulation of autophagy by MEK/ERK, mTOR is a major negative regulator of autophagy. Activation of AMPK and TSC2 upregulates Beclin 1 (Fig. 3C, 4F, 5A) and negatively regulates mTOR activity (48, 49). Furthermore, AMPK activation of TSC2 depends on MEK/ERK (Fig. 4B). We thus hypothesize that MEK/ERK may upregulate
Beclin 1 and autophagy through downregulation of mTOR. To test this, we made transfectant H4IIE cells constitutively expressing active Akt, which is a known upstream positive regulator of mTOR (53). Although overexpressing active Akt did not inhibit MEK/ERK activation in response to autophagy stimulus AICAR, it activated mTOR and crippled LC3 processing (Fig. 6A). Activation of AMPK by AICAR enhanced Beclin 1 level, caused LC3 processing (Fig. 6A) and the punctuate GFP-LC3 distribution representing autophagy, but active Akt overexpression inhibited them (Fig. 6A, B). Thus, MEK/ERK activation fails to upregulate Beclin 1 and trigger autophagy when mTOR is activated. These data suggest that MEK/ERK regulates Beclin 1 and autophagy through downregulation of mTOR activity.

The role of MEK/ERK in regulating mTOR activity was further assessed by measuring mTORC1 activity using GST-4EBP1 as substrate. Overexpressing active Akt enhanced mTORC1 activity (Fig. 6C). Various autophagy stimuli inhibited mTORC1 activity (Fig. 6D). However, PD98059 released the suppression of mTORC1 activity by MEK/ERK activation (Fig. 6D), indicating that MEK/ERK upregulates autophagy possibly through downregulating mTORC1 activity. Surprisingly, depleting basal MEK/ERK activity by PD98059 almost completely abolished mTORC1 activity (Fig. 6D). These data suggest that basal MEK/ERK activity is needed for constitutive mTORC1 activity, but pronounced MEK/ERK activation inhibits mTORC1 activity and thus activates autophagy. Amino acid starvation caused MEK/ERK activation, ultimately leading to an autophagic response, attenuated the binding of mTOR to Raptor or Rictor, but not GβL (Fig. 6E), suggesting that assembly of mTOR complexes mTORC1 and mTORC2 is sensitive to MEK/ERK activation, and upregulation of Beclin 1 by MEK/ERK activation, which triggers autophagy, may require inactivation of both mTORC1 and mTORC2. Surprisingly, depleting MEK/ERK activity by PD98059, which depleted basal Beclin1 (Fig. 1E, 3C, 6E), disassembled both mTORC1 and mTORC2 (Fig. 6E), suggesting that assembly of mTORC1 and mTORC2 requires basal MEK/ERK activity. It thus appears that basal MEK/ERK activity confers basal mTOR activity by conferring the assembly of complexes mTORC1 and mTORC2 while activation of MEK/ERK inactivates mTOR by disassembling both mTORC1 and mTORC2.

To test whether downregulation of both mTORC1 and mTORC2 is required to upregulate Beclin 1 and trigger autophagy in response to MEK/ERK activation, mTOR, Raptor, or Rictor was knocked down individually or in combination by RNA interference using the K562 cells stably transfected with GFP-LC3. The mTOR is activated in the control cells in rich medium due to basal MEK/ERK activity (Fig. 6D). The mTOR activity phosphorylated p70S6K and the translation repressor protein, 4EBP1, in control cells (Fig. 6F). Efficient knockdown of mTOR caused hypophosphorylation of S6K and 4EBP1, enhanced Beclin 1 (Fig. 6F) and the punctuate distribution of the GFP-LC3 (Fig. 6G). Knockdown of mTOR plus amino acid starvation caused a more pronounced Beclin 1 and autophagic response. By contrast, Knockdown of Raptor or Rictor individually only caused a moderate enhanced Beclin 1 and weak autophagic responses shown by the LC3 processing determined with autophagic flux, but knockdown of Raptor and Rictor together caused an pronounced Beclin 1 and autophagic response, comparable to that caused by global mTOR knockdown (Fig. 6F, G). These data suggest that efficient upregulation of Beclin 1 attributed to the induction of autophagy requires inactivation of both mTORC1 and mTORC2 activities.

MEK/ERK activation inhibits mTORC1 via TSC2 but inhibits mTORC2 independent of TSC2—TSC2 mediates cellular energy response through regulating mTOR (49). To determine whether MEK/ERK downregulation of mTOR kinase activity and upregulation of Beclin 1 and autophagy involves TSC1/2, the dominant form of TSC, TSC2, was knocked down by RNA interference using the H4IIE cells transfected with constitutively active MEK or ERK. Constitutive expression of active MEK or active ERK caused dephosphorylation of both 4EBP1 and p70 S6K, the two direct downstream effectors of mTORC1, which coincided with Beclin 1 upregulation and LC3 processing, while knockdown of TSC2 crippled the hypophosphorylation of 4EBP1 and S6K as well as the upregulation of Beclin1 and processing of LC3 (Fig. 7A). It thus appears that the
downregulation of mTORC1 activity by MEK/ERK, upregulation of Beclin 1, and induction of LC3 processing requires TSC2. Knockdown of TSC2 did not cripple activation of MEK/ERK in response to amino acid starvation, but crippled the autophagic response (Fig. 7B), further confirming that TSC2 is in the downstream of MEK/ERK in the signaling pathway that regulates autophagy.

Autophagy stimuli caused MEK/ERK activation (Fig. 1) and attenuated mTOR binding to Raptor or Rictor (Fig. 7C). Knockdown of TSC2 crippled Raptor, but not Rictor, disassociation from mTOR in response to autophagy stimuli that triggers MEK/ERK activation (Fig. 7C). These data suggest that MEK/ERK inhibition of mTORC1 activity is dependent on TSC2 but MEK/ERK inhibition of mTORC2 is independent of TSC2.

Beclin 1 modulates a protective or destructive autophagy—To explore the signaling mechanisms by which a protective or destructive autophagy is modulated, H4IIE cells were stimulated with amino acid or serum starvation, or treated with rapamycin, in combination with or without autophagy inhibitor bafilomycin A1. Autophagic response increased from 3 to 12 h of amino acid starvation shown by the number of autophagic vacuoles per cell or by the percentage of the cells with GFP-LC3 vacuoles (Fig. 8A, the upper panel). At 3h, the amino acid starved cells had a decreased cell viability of 88.3% compared to the control cells, which had 97.2% viability. Inhibiting autophagy by bafilomycin A1 further reduced cell viability to 77.5%, suggesting that autophagy played a protective role at 3h of amino acid starvation. At 6h, cell viability dropped to 70.3% in the starved cells, and 69.2% in the starved cells treated with bafilomycin A1, suggesting a balance exists between protective and destructive autophagy. At 12h, cell viability further dropped to 40.3% while treatment with bafilomycin A1 increased the cell viability to 48.3%, suggesting destructive autophagy occurs in the late stage of the starvation (Fig. 8A, the lower panel). Compared with amino acid starvation, serum starvation caused slower and less intense autophagy (Fig. 8B, the upper panel). Inhibiting autophagy by bafilomycin A1 reduced cell viability at 24h and did not change the viability at 48h, but increased the viability at 72h compared to the control cells (Fig. 8B, the lower panel), suggesting that autophagy by serum starvation is protective at 24h, neutral at 48h, and destructive at 72h. By contrast, rapamycin caused weak autophagy (Fig. 8C, the upper panel). Inhibiting autophagy on the rapamycin-treated cells decreased cell viability at 24, 48 and 72h, suggesting that rapamycin causes protective autophagy (Fig. 8C, the lower panel). These data suggest that transient or less intense autophagy stimuli cause protective autophagy, but sustained or intense autophagy stimuli cause destructive autophagy.

Since we observe that MEK/ERK activation triggers autophagy by regulating Beclin 1, we ask whether Beclin 1 regulates both protective and destructive autophagy, and if so, how. The results show that MEK/ERK activation at the three designated time points remained unchanged, but Beclin 1 level was increased along with the starvation, suggesting that less-enhanced Beclin 1 causes protective autophagy, moderately-enhanced Beclin 1 causes neutral autophagy, and strongly-pronounced Beclin 1 causes destructive autophagy (Fig. 8D). The similar pattern that Beclin 1 level determines autophagic consequences was also revealed by serum starvation, however, serum starvation triggers a slower and less intense autophagy than amino acid starvation (Fig. 8E). By contrast, rapamycin caused much less enhanced Beclin 1, which was unchanged throughout the protective autophagy response (Fig. 8F). These data thus suggest that the cellular consequences of autophagy may depend on the longevity of MEK/ERK activation and the enhanced Beclin 1 level.

**DISCUSSION**

The results presented here demonstrate that a non-canonical MEK/ERK module regulates autophagy by regulating Beclin 1 level through the AMPK-MEK/ERK-TSC-mTOR signaling pathway (Fig. 9). Basal MEK/ERK activity confers constitutive Beclin 1 that is insufficient to trigger autophagic response. MEK/ERK activation by AMPK upon various autophagy stimuli inactivates mTOR by disassembling the functional complexes mTORC1 and/or mTORC2, which results in the upregulation of Beclin 1 to different thresholds leading to autophagy of opposing consequences, suggesting that Beclin 1 may play a central role in integrating signals downstream of mTOR.
Our studies thus provide the first mechanistic link between a signaling pathway and autophagy-essential proteins in mammalian system.

**Mechanism of the non-canonical MEK/ERK module regulating autophagy—**

ERK is a widely conserved family of serine/threonine protein kinases implicated in many cellular programs such as cell proliferation, differentiation and apoptosis. It can be activated by a wide variety of oncogenes and extracellular stimuli including mitogens, growth factors, cytokines and chemokines. An increasing number of studies have suggested that ERK plays a role in modulating autophagy (27, 43, 44, 54-57). However, the role of ERK in autophagy was primarily concluded from the pharmacological blockade of autophagy by compound PD98059, an MEK-specific inhibitor. Since this chemical indirectly blocks ERK via inhibiting its canonical upstream regulator MEK, it is not surprising that ERK was exclusively credited in regulating autophagy because ERK has been believed to be the solely known substrate of MEK.

We demonstrated in this study that basal MEK/ERK activity maintains the activity of mTORC1 and mTORC2 by protecting them from being disassembled (Fig. 6E the lower panel), thus maintains basal Beclin1 level incapable of triggering autophagic response (Fig. 1E, 3C, 6E the upper panel). Unexpectedly, we found that although ERK is involved in the regulation of autophagy because constitutive expression of active ERK causes autophagy response (Fig 1C, 7A) while specific inhibition on ERK by RNA interference (Fig. 2C) or dominant negative ERK (Fig. 2D) partially inhibits autophagy, knockdown of the canonical effectors of ERK, p90RSK and Elk, by RNA interference, did not cripple autophagic response (Fig. 2A, B). This unusual observation suggests that ERK may use non-canonical downstream effectors in the context of autophagic signaling. Coincidentally, MEK can bypass ERK (Fig. 2D) and interact with its downstream effector other than ERK in the context of autophagy stimuli (Fig. 4D, 5B), indicating that ERK is not the sole downstream effector of MEK and MEK plays a more important role than ERK in regulating autophagy. Interestingly, MEK regulates Golgi fragmentation in mitosis independent of ERK1/2 (58, 59), and the nucleation of autophagy by Beclin 1-PI3KC3 kinase complex is executed at the trans-Golgi network in mammalian cells (60), both of which support the unusual role and the non-canonical module of MEK/ERK in regulating Beclin 1 and autophagy.

Recent studies show that ERK upregulates starvation-caused autophagy by downregulating Akt/mTOR/S6K (27), but the molecular link between this pathway and the basis of autophagy machinery remains largely unclear. We observed here that activation of MEK/ERK coincides with upregulation of Beclin 1 and autophagic responses, and MEK/ERK is activated by AMPK but not Raf1 in response to autophagy stimuli. AMPK has been proposed as a physiological cellular energy sensor because AMP is the most sensitive indicator for a cellular energy status. Cellular ATP concentration is much higher than AMP and an insignificant decrease in ATP levels can result in an extraordinary increase in AMP levels that are sensed by and stimulate AMPK (24). MEK/ERK activation by AMPK thus confers the sensitivity and accuracy of MEK/ERK in regulating autophagic activity. TSC is known to be regulated by cellular energy levels. Activation of AMPK by energy starvation results in direct phosphorylation of TSC (49). Our study shows that AMPK activation of TSC depends on ERK/ERK. Furthermore, activated MEK interacted with TSC, and the physical interaction between activated MEK and TSC requires the ERK D docking site sequence motif contained in both MEK and TSC, consistent with the notion that stable binding of substrates to MAPK kinase involves recognition of a docking site on the substrate (2). Furthermore, this non-canonical MEK/ERK signaling pathway functions not only in human and rat cell lines but also in neonatal mice, indicating a physiological significance. Our findings thus position this unusual MEK/ERK module downstream of AMPK and upstream of TSC in response to autophagy stimuli.

Our present study shows that mTOR regulates autophagy caused both by starvation and non-starvation stimuli that activate MEK/ERK, consistent with the report that AMPK regulates autophagy through mTOR also at non-starved conditions (61), suggesting a possible universal mechanism in the regulation of autophagy through mTOR. Since constitutive expression of Beclin 1 is due to the basal activity
of MEK/ERK and enhanced Beclin 1 is attributed to the enhanced MEK/ERK activation in both human and rat cells, we postulate that the dependency of Beclin 1 on MEK/ERK signals may also be a universal mechanism in regulating autophagy.

**Beclin 1 plays a central role in the mechanisms determining a protective or destructive autophagy**—Our previous study shows that autophagic activity depends on the Beclin 1 level (41). This study finds that the non-canonical MEK/ERK module regulates mutually antagonistic processes by regulating Beclin 1 levels. Basal activity of MEK/ERK protects mTORC1 and mTORC2 from being disassembled, thus conferring basal mTOR activity and basal Beclin 1 level incapable of triggering autophagy. MEK/ERK activation by AMPK upregulates Beclin 1 through downregulation of mTOR activity. Transient or moderate activation of MEK/ERK causes inhibition of mTORC1 or mTORC2, resulting in a moderate Beclin 1 increase and an ultimate cytoprotective autophagy, but sustained or strong MEK/ERK activation completely inhibits both mTORC1 and mTORC2, resulting in a strongly pronounced Beclin 1 and an ultimate cytodestructive autophagy. The requirement of an enhanced Beclin 1 level by MEK/ERK activation for autophagy thus appears to represent interesting threshold effects. The degree of enhanced Beclin 1 level may determine the level of autophagic activity and the degree of autophagy elicited may play a role in cell fate decisions, with low levels serving a homeostatic role and high levels promoting cell death. Our findings thus establish the molecular connection between MEK/ERK and mTOR in the regulation of mammalian autophagy—essential proteins and the opposing consequences of autophagy.

Mammalian cells undergoing autophagy result from exposure to nutritional, chemical, physical autophagy stimuli or extreme physiological conditions such as birth. Despite the ubiquitous and constitutive expression of Beclin 1 in mammalian cells, our data demonstrate that autophagy is not evident until Beclin 1 is elevated, and autophagic activity is not detectable at a constitutive Beclin 1 level under normal growth conditions in cancer cells. In the brain, autophagic activity is very low even under starvation conditions (20). These suggest that either there is no constitutive autophagy or autophagy can only occur at extremely low levels under normal growth conditions when Beclin 1 is maintained at a basal level. Therefore, we doubt that autophagy plays a housekeeping role in all types of mammalian cells. Constitutive or basal Beclin 1 may be implicated in other cellular processes. Our recent study shows that constitutive Beclin 1 inhibits activation of apoptosis and confers differentiation capability in leukemia HL-60 cells (37, 41). Since autophagy can be regulated by several proteins capable of binding Beclin 1, including Bel2, UVRAG and Ambra1 (22, 24 and 25), Beclin 1 may play a central role in integrating signals by various stimuli.

Studies on Beclin 1 demonstrate its increasing importance in the mammalian system, because mammalian species fail to survive in the absence of Beclin 1, develop cancers with low Beclin 1 levels, suppress tumorigenesis with normal Beclin 1 levels, secure a protective autophagy at moderately-enhanced Beclin 1 levels, and suffer from a destructive autophagy when too much Beclin 1 is induced. Thus, our present study adds new understanding of Beclin 1 in the regulation of autophagy.

**ACKNOWLEDGEMENTS**

We thank Samantha Spindel and Ariel A Kauss for their comments on this manuscript. This work was funded by the grants from Shaotou University and Unimed Foundation.

**REFERENCES**

6. Karantza-Wadsworth, V., Patel, S., Kravchuk, O., Chen, G., Mathew, R., Jin,

**FIGURE LEGENDS**

**FIG. 1. Autophagic activity and Beclin1 expression is MEK/ERK-dependent.** (A) and (B) Autophagic response coincides with MEK/ERK signaling. H4IIE and K562 cells were treated with various autophagy stimuli (rapamycin 10 μM for 48h, serum depletion for 48h, amino acid depletion for 6h, 50nM vitamin D3 for 72h), with or without 2μM PD98059. (C) Constitutive expression of active MEK or ERK triggers autophagic responses. H4IIE cells were transfected with constitutively active MEK or active ERK and determined for LC3 processing in the presence of lysosomal protease inhibitor pepstatin A. (D) Fluorescence microscopy of GFP-LC3 transfectant cells undergoing the designated treatments. The percentage of cells with punctuate pattern is indicated as means ± SDs (n=3). (E) Basal MEK/ERK activity confers constitutive Beclin 1 expression. Depleting basal phospho-MEK1/2 but not Raf1 by siRNA depleted basal Beclin 1. (F) Constitutive expression of CR3
domain of Raf1 does not trigger autophagic responses. (G) Autophagy stimuli-enhanced Beclin 1 is diminished by inhibition of MEK/ERK. H4IIE and K562 cells were starved for amino acids for 6h or serum for 48h or treated with 10μM rapamycin, together with or without 2μM PD98059. (H) Depleting Beclin 1 abolishes autophagy in response to MEK/ERK activation. Beclin 1 knockdown H4IIE cells were starved for amino acids, with or without 2μM PD98059. Shown in this figure from A to G are representative Western blots or fluorescence microscopy of each three independent experiments.

**FIG. 2. MEK can bypass ERK to trigger autophagy.** (A) and (B) Knockdown of p90RSK or Elk did not abolish autophagy. K562 cells were transfected with siRNA against p90RSK or Elk, and determined for LC3 processing in response to amino acid starvation. (C) Knockdown of ERK did not abolish autophagy. K562 cells were transfected with siRNA against ERK1/2 and determined for LC3 processing in response to starvation. (D) MEK can trigger autophagic response independent of ERK. K562 cells were transfected with constitutively active MEK (caMEK) or caMEK together with dominant negative ERK (dnERK), and determined for LC3 processing by autophagic flux assay. Shown in this figure from A to D are the representative Western blots of each three independent experiments.

**FIG. 3. MEK/ERK is positioned downstream of AMPK in autophagic signaling.** (A) Inhibiting AMPK inhibited MEK activation in response to autophagy stimuli. H4IIE cells were starved for amino acids and treated with 10μM compound C. (B) Inhibition of MEK did not inhibit AMPK activation in response to autophagy stimuli. H4IIE cells were starved for amino acids and treated with or without 2μM PD98059, and determined for AMPK activation. (C) Upregulation of Beclin 1 and induction of autophagy by AMPK activation depends on MEK. H4IIE cells were treated with 1mM AICAR, with or without 2μM PD98059, and determined for activation of AMPK and MEK, expression of Beclin 1, as well as LC3 processing and Beclin 1 binding to PI3KC3. (D) and (E) MEK interacts with AMPK but not Raf1 in response to autophagy stimuli. H4IIE cells were starved for amino acids or treated with 1mM AICAR and determined for physical interaction between MEK and AMPK, between Raf1 and MEK, or between AMPK and Raf1 by using total (D) or phospho-antibody (E). Growth hormone treatment was served as a positive control for canonical Raf/MEK/ERK activation and interaction in H4IIE cells. (F) Specific inhibition on AMPK by kinase-dead AMPK crippled activation of MEK and autophagic activity in response to autophagy stimuli. H4IIE cells stably transfected with either Rat AMPKα2 kinase-dead construct or vector control was starved for amino acids for 6 hours. Expression of total AMPKα, total and phospho-MEK and processing of LC3 were determined. Shown in this figure from A to F are the representative Western blots or co-IP blots of each three independent experiments.

**FIG. 4. MEK/ERK is positioned upstream of TSC in autophagic signaling.** (A) TSC2 is the dominant form of TSC1/2 in both H4IIE cells and K562 cells. (B) AMPK activation of TSC2 is MEK/ERK-dependent. H4IIE cells were stimulated by 1mM CICAR, with or without 2μM PD98059, and determined for activation of AMPK or TSC2. (C) Activated MEK forms multiple complexes in response to autophagy stimuli. K562 and H4IIE cells were starved for amino acids and determined for formation of the complexes including MEK by non-denaturing Western blotting. (D) MEK and ERK associates with TSC2 in response to autophagy stimuli. H4IIE cells were starved for amino acids or treated with AICAR and determined for TSC2 binding to MEK or ERK. (E) MEK binding to TSC2 requires ERK D domain. H4IIE cells were transfected with constitutively active MEK (caMEK) or caMEK mutant with the ERK D domain disrupted, and determined for TSC2 binding to active MEK or MEK mutant without ERK D domain. (F) Disruption of the association between MEK and TSC2 crippled TSC2 activation and the autophagic response. H4IIE cells transfected with caMEK or caMEK together with ERK siRNA, or caMEK mutant with the ERK D domain disrupted. Shown in this figure from A to F are the representative Western blots or co-IP blots of each three independent experiments.
Fig. 5. AMPK-MEK/ERK-TSC signaling functions in physiological response of autophagy in mice. (A) AMPK-MEK/ERK signaling responds to autophagy stimuli in neonatal mice. Western blotting was performed to determine activation of AMPK, MEK and ERK, expression of Beclin1 and processing of LC3 with cell lysates prepared from the hearts and livers of mice at 5 or 120 hours after birth. (B) MEK/ERK interacts with AMPK or TSC1 in response to autophagy stimuli in neonatal mice. MEK or ERK co-immunoprecipitation was performed to examine their interactions with upstream regulator or downstream effector in response to autophagy or non-autophagy stimuli with the cell lysates from the hearts and livers of the mice 5 or 120 hours after birth. Shown in this figure are the representative Western blots (A) or co-IP blots (B) of each three independent experiments.

Fig. 6. MEK/ERK upregulates Beclin1 and autophagy by downregulating mTOR activity. (A) MEK/ERK fails to upregulate Beclin 1 and autophagy when mTOR is activated. H4IIE cells were transfected with constitutively active Akt, with or without 1μM CICAR, and determined for MEK/ERK or mTOR activation, Beclin 1 expression, and LC3 processing. (B) Fluorescence microscopy of the GFP-LC3 cells transfected with caAkt with or without autophagy stimuli. (C) Overexpressing active Akt enhances mTORC1 activity using GST-4EBP1 as a substrate. The $^{32}$P incorporated into GST-4EBP1 is shown in a bar graph. (D) MEK/ERK activation inhibits mTORC1 activity. H4IIE cells were stimulated with designated autophagy stimuli or transfected with caMEK or caERK, with or without 2μM PD98059, and determined for mTORC1 activity as described in (C). (E) Inhibiting MEK/ERK disassociates both mTORC1 and mTORC2. H4IIE cells were starved for amino acids, with or without 2μM PD98059, determined for Beclin 1 expression and LC3 processing, as well as mTOR binding to Raptor, Rictor or GβL. (F) Inhibiting mTORC1 or mTORC2 individually causes a mild increased Beclin 1 and autophagic response, but inhibiting both mTORC1 and mTORC2 causes strongly pronounced Beclin 1 levels and autophagic responses. H4IIE cells were transfected with siRNA against mTOR, or Raptor or Rictor individually or in combination, and determined for activation of mTOR effectors, Beclin 1 expression and LC3 processing by autophagic flux assay. The Beclin 1 levels were quantified by density scanning. (G) Fluorescence microscopy of GFP-LC3 transfectant H4IIE cells with Raptor or Rictor knockdown individually or in combination. Shown in this figure from A to G are the representative Western or co-IP blots or quantified data (means ± SDs) of each three independent experiments.

Fig. 7. MEK/ERK inhibition on mTORC1 is TSC2-dependent but its inhibition on mTORC2 is TSC2-independent. (A) Depleting TSC2 increases mTOR activity and cripples Beclin 1 upregulation and LC3 processing by MEK/ERK activation. H4IIE cells stably transfected with constitutively active MEK or ERK were transiently transfected with siRNA against TSC2. (B) Depleting TSC2 does not affect MEK/ERK activation, but cripples Beclin 1 upregulation and LC3 processing in response to autophagy stimuli. H4IIE cells were transfected with siRNA against TSC2, and starved for amino acids. (C) Depleting TSC2 cripples the disassembly of mTORC1 but not mTORC2 in response to autophagy stimuli. H4IIE cells were transfected with siRNA against TSC2 and starved for amino acids and determined for mTOR binding to Raptor or Rictor or GβL. The binding was quantified by density scanning. Shown in this figure from A to C are the representative Western or co-IP blots or quantified data (means ± SDs) of each three independent experiments.

Fig. 8. Moderately enhanced Beclin 1 causes cytoprotective autophagy, but prominently enhanced Beclin 1 causes cytodestructive autophagy. (A) Amino acid starvation causes a protective autophagy at early stage and a destructive autophagy at late stage. H4IIE cells were starved for amino acids, and autophagic activity represented by LC3 dots (upper panel) and cell viability (lower panel) were determined at the designated time points. (B) Serum starvation causes a protective autophagy at early stage and a destructive autophagy at late stage, determined at the designated times as in (A). (C) Rapamycin (10 μM) causes a protective autophagy throughout, determined at the designated times as described in (A). Data shown in (A), (B) and (C) are means ± SDs (n = 3). *: P < 0.05, **: p < 0.01. (D) Amino acid starvation causes a sustained high MEK/ERK activation and an increasingly pronounced Beclin 1 expression. Beclin1 expression and ERK activation are quantitated by density scanning. (E) Serum starvation causes a sustained high MEK/ERK activation and
increasingly pronounced Beclin 1 expression. (F) Rapamycin causes a sustained moderate MEK/ERK activation and moderately-enhanced Beclin 1 determined as in (D). Shown in this figure from D to F are the representative Western blots and quantified data of each three independent experiments.

**FIG. 9. A diagram for the non-canonical MEK/ERK signaling pathway regulating autophagy via regulating Beclin1.** Basal MEK/ERK activity maintains the activity of mTORC1 and mTORC2 by protecting them from being disassembled, thus maintains basal Beclin1 level incapable of triggering autophagic response. The non-canonical MEK/ERK module is activated by upstream regulator AMPK upon autophagy stimuli. Activation of MEK/ERK upregulates Beclin 1 level through disassembling mTOC1 and/or mTORC2, with significantly sustained and elevated Beclin1 level causing cytodestructive autophagy, moderately elevated Beclin1 causing cytoprotective autophagy. Disassembling mTORC1 by MEK/ERK depends on TSC, whereas disassembling mTORC2 does not depend on TSC2. Although ERK is implicated in the regulation of autophagy, MEK can bypass ERK to trigger autophagic response.
Fig. 1 Autophagic activity and Beclin 1 expression is MEK/ERK-dependent
Fig. 2 MEK can bypass ERK to trigger an autophagic response.
Fig. 3 MEK/ERK positions downstream of AMPK in autophagic signaling
Fig. 4 MEK/ERK positions upstream of TSC in autophagic signaling
Fig.5 AMPK-MEK/ERK-TSC signaling functions in physiological response of autophagy in mice
Fig. 6 MEK/ERK upregulates Beclin 1 and autophagy by downregulating mTOR activity.
Fig. 7 MEK/ERK inhibition on mTORC1 is TSC2-dependent, but its inhibition on mTORC2 is TSC2-independent.
Fig. 8 Moderately enhanced Beclin1 causes protective autophagy, but strongly pronounced Beclin1 causes destructive autophagy.
Fig. 9 An illustration of the model for the non-canonical MEK/ERK signaling pathway regulating autophagy via regulating Beclin 1.
A non-canonical MEK/ERK signaling pathway regulates autophagy via regulating Beclin 1
Jianrong Wang, Mary W. Whiteman, Huiqin Lian, Guangxin Wang, Amit Singh, Dongyang Huang and Ted Denmark

J. Biol. Chem. published online June 11, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.026013

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts