Introduction

Cells consume a tremendous amount of metabolic energy for survival, most of which is used in peptide chain elongation. The rate of peptide chain elongation is modulated by EEF2 which in turn is regulated by EEF2K.1,2 Under stress or starvation conditions, in order to reduce energy consumption, EEF2K is normally activated by phosphorylation at Ser398 via Ca2+/calmodulin or AMPK/AMP-activated protein kinase,3-6 The activated EEF2K in turn phosphorylates its target EEF2 at Thr56. 7 This phosphorylation inactivates EEF2 resulting in termination of peptide elongation by decreasing the affinity of the elongation factor toward the ribosome.7,8 Other stimuli such as stress and growth factors that promote protein synthesis must inhibit the activity of EEF2K. Recent studies demonstrate that signaling pathways such as those modulated by RPS6KA1/p90 (ribosomal protein S6 kinase, 90 kDa, polypeptide 1), MAPK13/p38δ/SAPK4 (mitogen-activated protein kinase 13), MTOR (mechanistic target of rapamycin) and RPS6KB (ribosomal protein S6 kinase, 70 kDa, polypeptide) can directly phosphorylate EEF2K at specific sites that inactivate EEF2K leading to increased protein translation.9-11

Silencing of EEF2K (eukaryotic elongation factor-2 kinase) reveals AMPK-ULK1-dependent autophagy in colon cancer cells

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Keywords: elongation factor-2 kinase, autophagy, colon cancer, AMPK, ULK1, ATG, MTOR, ROS, cancer growth, cell survival

Abbreviations: AMPK, AMP-activated protein kinase; AMPKα1 and PRKAA2; ATG, autophagy-related genes; CHX, cycloheximide; DCFDA, 2′,7′-dichlorofluorescein diacetate; EEF2, eukaryotic translation elongation factor 2; EEF2K, eukaryotic elongation factor-2 kinase; MAP1LC3/LC3, microtubule-associated protein 1 light chain 3; MAPK13/p38δ/SAPK4, mitogen-activated protein kinase 13; MEFs, mouse embryonic fibroblasts; MTOR, mechanistic target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PRKA, protein kinase A; ROS, reactive oxygen species; RPS6KA1/p90/RSK1, ribosomal protein S6 kinase, 90 kDa, polypeptide 1; RPS6KB, ribosomal protein S6 kinase, 70 kDa, polypeptide; SEM, standard error of the mean; ULK1, unc-51 like autophagy activating kinase 1
Apart from regulating the activity of EEF2K, AMPK can activate autophagy. Autophagy is a self-degradative process by the removal of damaged proteins and organelles to promote a cell survival response to nutritional starvation or stress conditions. Certain examples have demonstrated that AMPK is activated by an elevated AMP/ATP ratio due to cellular and environmental stress such as nutritional deprivation. Autophagy can also be activated in response to many forms of cellular stress beyond nutritional deprivation including reactive oxygen species (ROS) and DNA damage. Recent studies have shown that autophagy could also act as an apoptosis-independent programmed cell death. The precise role of autophagy in cellular responses to stress is far from fully elucidated.

Previous studies have demonstrated that EEF2K-mediated EEF2 phosphorylation at Thr56 could induce autophagy as a survival mechanism in glioma cells and breast cancer cells, and inhibition of EEF2K could potentiate the efficacy of anticancer agents against cancers. Silencing of EEF2K markedly increased but did not reduce the amount of LC3-II levels in both HT-29 and HCT-116 cells, suggesting that the increased protein synthesis can induce autophagy (Fig. 1A). The same result was obtained using multiple siRNAs targeting different regions of EEF2K (Fig. 1B). These findings were further substantiated by the increase of LC3 dots accumulation in EEF2K-depleted cells (Fig. 1C). As shown in Figure 1C, EEF2K silencing significantly increased LC3 puncta accumulation in both the cytoplasm and nucleus, and most of these LC3 puncta were concentrated in the nucleus. The amount of LC3 dots per cell was significantly increased by more than 6-fold in EEF2K knockdown cells as compared with the control group (Fig. 1D). Furthermore, to distinguish between induction of autophagy and inhibition of autophagic vesicles degradation in EEF2K silenced cells, we analyzed autophagic flux in EEF2K-silenced cells in the absence or presence of lysosomal protease inhibitors E64d and peptatin A. As shown in Figure 1E, protease inhibitors could further increase both LC3-II and mammalian autophagy-specific substrate SQSTM1/p62 levels in EEF2K-silenced cells when compared with vehicle treatment, suggesting that LC3-II accumulation in EEF2K-silenced cells was attributable to promotion of autophagy but not to impairment of autophagic degradation. Taken together, these results indicate that knockdown of EEF2K induces autophagy in human colon cancer cells.

**BECN1 and ATG7 are required for autophagy in response to EEF2K silencing**

A series of autophagy-related (ATG) genes are involved in the process of autophagy. We would like to know whether autophagy induced by silencing of EEF2K contributes to regulation of specific proteins of the ATG family. ATG5 and ATG7 (a ubiquitin-activating enzyme homolog), are required for initiation of autophagy. BECN1 is required for the initiation of autophagosome formation. Previous studies show that autophagy can be induced through ATG5-, BECN1-, or ATG7-dependent or independent signaling pathways. To determine whether induction of autophagy by EEF2K silencing is related to ATG5, BECN1, or ATG7, we first analyzed the expression levels of ATG5, BECN1, and ATG7 separately by western blot. As shown in Figure 2A, knockdown of EEF2K significantly increased the protein levels of BECN1 and ATG7, but not ATG5. The increase in BECN1 and ATG7 levels in EEF2K-depleted cells is attributed to protein synthesis but not to transcriptional increase (Fig. 2B). In order to further validate the increased BECN1 and ATG7 due to protein synthesis, we blocked protein degradation by MG132. The result showed that protein levels of BECN1 and ATG7 were significantly accumulated in EEF2K-depleted cells after exposure to MG132, suggesting EEF2K silencing does not block protein degradation of BECN1 and ATG7 (Fig. 2C). Taken together, the increase of both BECN1 and ATG7 in EEF2K knockdown cells is not due to blockage of degradation but to protein synthesis. We silenced BECN1 using siRNA in HT-29 cells. The result showed that knockdown of BECN1 could significantly block

**Results**

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Previous studies have shown that EEF2K is effective in inducing autophagy in glioma and breast cancer cells. We have therefore investigated whether EEF2K could also induce autophagy in human colon cancer cells. As shown in Figure 1A, silencing of EEF2K using a single siRNA could completely block its downstream target EEF2 phosphorylation at Thr56 in human colon cancer HT-29 and HCT-116 cells, consistent with the fact that reduction of EEF2K activity can reduce the phosphorylation of EEF2 at Thr56. However, silencing of EEF2K markedly increased but did not reduce the amount of LC3-II levels in both HT-29 and HCT-116 cells, suggesting that the increased protein synthesis can induce autophagy (Fig. 1A). The same result was obtained using multiple siRNAs targeting different regions of EEF2K (Fig. 1B). These findings were further substantiated by the increase of LC3 dots accumulation in EEF2K-depleted cells (Fig. 1C). As shown in Figure 1C, EEF2K silencing significantly increased LC3 puncta accumulation in both the cytoplasm and nucleus, and most of these LC3 puncta were concentrated in the nucleus. The amount of LC3 dots per cell was significantly increased by more than 6-fold in EEF2K knockdown cells as compared with the control group (Fig. 1D). Furthermore, to distinguish between induction of autophagy and inhibition of autophagic vesicles degradation in EEF2K silenced cells, we analyzed autophagic flux in EEF2K-silenced cells in the absence or presence of lysosomal protease inhibitors E64d and peptatin A. As shown in Figure 1E, protease inhibitors could further increase both LC3-II and mammalian autophagy-specific substrate SQSTM1/p62 levels in EEF2K-silenced cells when compared with vehicle treatment, suggesting that LC3-II accumulation in EEF2K-silenced cells was attributable to promotion of autophagy but not to impairment of autophagic degradation. Taken together, these results indicate that knockdown of EEF2K induces autophagy in human colon cancer cells.

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Figure 1. Silencing of EEF2K induces autophagy in human colon cancer cells. (A and B) HT-29 or HCT-116 cells were transfected with nontargeting control siRNA (sicTL), a single siRNA duplex targeting EEF2K (siEEF2K; A) or multiple siRNAs targeting different regions of EEF2K (siEEF2K; B) for 48 h. EEF2K, phospho-EEF2 (Thr56; p-EEF2), EEF2, LC3, and ACTB/β-actin were analyzed by western blot. Representative western blot and densitometric analysis normalized to ACTB demonstrating the effect of EEF2K silencing on LC3-ii levels. (C and D) HT-29 or HCT-116 cells were transfected with control siRNA or a single siRNA duplex targeting EEF2K for 48 h. (C) Representative immunofluorescent images showing redistribution of autophagic marker LC3 in EEF2K knockdown cells were taken on a confocal microscope. Cells were fixed with 3.5% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and stained with LC3 antibody and DAPI. Scale bar: 10 µm. (D) The average number of LC3 dots per cell was counted in more than 5 fields with at least 100 cells for each group. (E) Representative western blot and densitometric analysis normalized to ACTB demonstrating the effect of lysosomal protease inhibitors E64d plus pepstatin A on EEF2K silencing induced LC3-ii accumulation. HT-29 cells were transfected with nontargeting control siRNA or EEF2K siRNA. At 3 h after transfection, cells were treated with 10 μg/ml E64d and pepstatin A (Pep A) for 45 h. All quantitative data shown represent the means ± SEM of at least 3 independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, vs. the sicTL group (A, B, and D) or vehicle treatment only (E).
the accumulation of LC3-II in EEF2K-silenced cells (Fig. 2D). Similar to the effect of BECN1 knockdown, silencing of ATG7 also markedly attenuated the accumulation of LC3-II (Fig. 2E). Moreover, the number of LC3 dots per cell was significantly reduced after silencing of BECN1 or ATG7 in EEF2K knockdown cells (Fig. 2F).

Taken together, these results indicate that the upregulation of BECN1 and ATG7 is responsible for autophagy induced by EEF2K silencing.

**EEF2K silencing-induced autophagy functions to promote colon cancer cell survival**

It has been reported that targeting EEF2K by siRNA reduces cancer growth in glioma and breast cancer cells.17,21 Contrary to this, silencing of EEF2K significantly promoted colon cancer cell viability and colony formation, suggesting that EEF2K negatively regulates cell proliferation in human colon cancer cells (Fig. 3A, B, and D). These findings were confirmed by the decrease of cell viability and colony formation in EEF2K-overexpressing cells as compared with control (Fig. 3A, C, and E).

In order to further validate the observation on cell survival in EEF2K-silenced cells, we analyzed cell size and cell number in EEF2K knockdown cells as well as in EEF2K-overexpressed cells. Both cell size and cell number in EEF2K-depleted cells were significantly increased compared with control (Fig. 3F and H), while the cell size and cell number were decreased in EEF2K-overexpressing cells as well as in EEF2K-silenced cells. These findings are further substantiated by the result that EEF2K silencing significantly attenuated the antitumor efficacy of oxaliplatin against colon cancer cells (Fig. 3J).

**Figure 2.** BECN1 and ATG7 are required for autophagy in response to EEF2K silencing. (A) Silencing of EEF2K upregulates the protein levels of BECN1 and ATG7, but not ATG5. HT-29 or HCT-116 cells were transfected with nontargeting control siRNA (siCTRL) or a single EEF2K siRNA duplex (siEEF2K) for 48 h. ATG5, BECN1, ATG7, and ACTB were analyzed by western blot. Data shown are representative of more than 3 independent experiments. (B) Silencing of EEF2K does not change the mRNA levels of ATG5, BECN1, and ATG7. Cells were transfected as in (A). The mRNA levels of ATG5, BECN1, and ATG7 were analyzed by RT-PCR. (C) Effect of MG132 on the protein levels of ATG5, BECN1, and ATG7 in EEF2K knockdown cells. HCT-116 cells were transfected with nontargeting control siRNA (siCTRL) or a single EEF2K siRNA duplex (siEEF2K) for 48 h. Before harvested for western blot, cells were treated with MG132 (10 µM) for 12 h. (D and E) Representative western blot and densitometric analysis normalized to ACTB demonstrating the effects of BECN1 siRNA (D) and ATG7 siRNA (E) on LC3-II levels induced by EEF2K silencing. HT-29 cells were transfected with nontargeting control siRNA, EEF2K siRNA, BECN1 siRNA (siBECN1), ATG7 siRNA (siATG7), siEEF2K plus siBECN1, or siEEF2K plus siATG7 for 48 h. All quantitative data shown represent the means ± SEM of at least 3 independent experiments. *P < 0.05 and ^P < 0.01, vs. the siEEF2K group. (F) The effects of BECN1 siRNA and ATG7 siRNA on LC3 dots accumulation induced by EEF2K silencing. HT-29 cells were transfected with nontargeting siRNA, EEF2K, BECN1 siRNA (siBECN1), ATG7 siRNA (siATG7), siEEF2K plus siBECN1, or siEEF2K plus siATG7 for 48 h. Cells were fixed, stained for LC3, and imaged. The average number of LC3 dots per cell was counted in more than 5 fields with at least 100 cells for each group and expressed as the means ± SEM of 3 independent experiments. *P < 0.001, vs. the EEF2K siRNA group (siEEF2K).
Our findings in colon cancer cells are in accordance with other reports that knockdown of EE2F2 by siRNA as well as reduction of EE2F phosphorylation at effective concentrations by the EE2F2 inhibitor A-484954 has little inhibitory effect on cancer cell growth in certain cancer cells including lung cancer and prostate cancer under both serum and serum-free conditions.23 In addition, overexpression of EE2F2 could significantly enhance the antitumor efficacy of oxaliplatin against colon cancer cells, indicating that increase of EE2F2 activity can be used to treat colon cancer (Fig. 3K).

Furthermore, given the fact that intracellular autophagy promotes cell survival or induces programmed cell death, we investigated the role of autophagy in EE2F2 knockdown colon cancer cells. Previous studies report that inhibition of EE2F2-mediated autophagy by silencing of BECN1 can significantly enhance the efficacy of anticancer agents against glioma and
Figure 3 (See previous page). EEF2K silencing promotes cell survival in human colon cancer cells. (A) Representative western blot demonstrating the knockdown efficiency of EEF2K siRNA and the overexpression efficiency of EEF2K in both HT-29 and HCT-116 cells. Cells were transfected with control siRNA (siCTL), EEF2K siRNA (siEEF2K), empty vector (Vector), or EEF2K plasmids (EEF2K) for 48 h. (B and C) The effects of EEF2K knockdown and EEF2K overexpression on cell viability. HT-29 or HCT-116 cells were transfected as in (A), and then assessed by MTT assay. (D and E) The effects of EEF2K knockdown and EEF2K overexpression on colony formation. HT-29 or HCT-116 cells were transfected as in (A). After 48 h transfection, cells were seeded into 6-well plates at the density of 150 cells per well for control siRNA and EEF2K siRNA groups (D) and 200 cells per well for the empty vector and EEF2K overexpression groups (E), incubated at 37 °C for 12 to 14 d, stained with crystal violet (0.5% w/v) and imaged. Colonies with 50 or more cells were counted. (F and G) The effects of EEF2K knockdown and EEF2K overexpression on cell size. HT-29 or HCT-116 cells were transfected as in (A). After 48 h transfection, cells were imaged using a Nikon fluorescence microscope. Scale bar: 20 μm. Cell size was analyzed using the MetaMorph software. The amounts of cell size in more than 50 cells for each group were quantified. (H and I) The effects of EEF2K knockdown and EEF2K overexpression on cell number. HT-29 or HCT-116 cells were transfected as in (A). Cell number was quantified after 48 h transfection. (J) The effect of EEF2K siRNA on oxaliplatin induced apoptosis. HCT-116 cells were transfected with control siRNA or EEF2K siRNA for 24 h, and then treated with vehicle (0.1% DMSO) or oxaliplatin (25 μM) for 48 h. Cells were stained with ANXA5-PL. The percentage of apoptotic cells (ANXA5+) was analyzed by flow cytometry. (K) The effect of EEF2K overexpression on oxaliplatin-induced apoptosis. HT-29 cells were transfected with empty vector (Vector), or EEF2K plasmids (EEF2K) for 24 h, and then treated with vehicle (0.1% DMSO) or oxaliplatin (25 μM) for 48 h. Cells were stained with ANXA5-PL and analyzed by flow cytometry as in (J). All quantitative data shown represent the means ± SEM of at least 3 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001, vs. the siCTL group (B, D, F, and H), the vector group (C, E, G, and I), or the oxaliplatin treatment only (J and K).

breast cancer cells, suggesting autophagy functions as a cell-survival mechanism.26,27 In line with this finding, we found that disruption of autophagy by knockdown of BECN1 or ATG7 reduced cell viability and clonogenicity in EEF2K-silenced colon cancer cells (Fig. 4A and B). Similar to the effect of BECN1 and ATG7 knockdown, the blockage of autophagic flux by protease inhibitors E64d and pepstatin A attenuated the increase of cell viability induced by EEF2K silencing (Fig. 4C). These results indicate that the increase of cell viability by EEF2K silencing in colon cancer cells is attributed to induction of the cell-survival mechanism of autophagy. Considering the fact that autophagy induced by EEF2K silencing acts as a cell-survival mechanism in colon cancer cells, upregulation of EEF2K as an anticancer approach might be feasible in human colon cancer.

Silencing of EEF2K cannot potentiate the anticancer efficiency of MK-2206 against colon cancer cells

AKT is an important anticancer target. The AKT inhibitor MK-2206 has been well studied for its role in promoting autophagy through activation of EEF2K and inactivation of EEF2 in glioma cells, as indicated by the increase of LC3-II.16 Consistent with this finding, MK-2206 at effective concentrations such as 0.1–5 μM also significantly promoted autophagy as indicated by the accumulation of LC3-II levels in human colon cancer cells, while EEF2 phosphorylation at Thr56 was not markedly increased in cells treated with MK-2206 at the same concentration range (Fig. 5A). These results indicate that autophagy induced by MK-2206 in colon cancer cells could not be completely attributed to activation of the EEF2K. In order to further validate the effect of EEF2K on AKT inhibition-induced autophagy in colon cancer cells, cells were transfected with siRNA against EEF2K before exposure to MK-2206. As shown in Figure 5B, knockdown of EEF2K could not block the autophagic response triggered by the AKT inhibitor MK-2206 in human colon cancer cells, suggesting that EEF2K does not correlate with MK-2206-induced autophagy. This result contradicts the conventional notion that inhibition of AKT by MK-2206 activates EEF2K-dependent autophagy. Although MK-2206 at 10 μM increased EEF2 phosphorylation at Thr56, knockdown of EEF2K could not potentiate the anticancer efficacy of MK-2206, implying that EEF2K cannot serve as an anticancer target for colon cancer therapy (Fig. 5A and C). EEF2K has been demonstrated to play a critical role in induction of autophagy in glioma cells in response to cellular stress such as AKT inhibition by MK-2206 and nutrient deprivation. However, the underlying molecular mechanisms by which EEF2K controls autophagy remain unknown. Our findings on the role of EEF2K in autophagic response induced by AKT inhibition add new insight to the AKT-mediated autophagy pathway. In addition, our results also suggest that upregulation of EEF2K activity might constitute a therapeutic option for the treatment of certain cancers including human colon cancer.

The AMPK-ULK1 pathway is required for autophagy in response to EEF2K silencing

Knockdown of EEF2K stimulates protein synthesis, which results in ATP consumption and then leads to an increase of AMP/ATP ratio.30 In line with this finding, ATP was markedly reduced in colon cancer cells after EEF2K silencing (Fig. 6A). Previous studies show that an increased AMP/ATP ratio can activate AMPK by phosphorylation at Thr172.29 Consistent with this finding, AMPK was significantly activated in both EEF2K-depleted HT-29 and HCT-116 cells (Fig. 6B). It has been demonstrated that AMPK can activate autophagy by activation of ULK1 or by inactivation of MTOR.30,31 In this study, we found that ULK1 was activated by phosphorylation at Ser555 and dephosphorylation at Ser757, but MTOR was not inactivated in EEF2K-depleted cells (Fig. 6B). These findings suggest that AMPK may enhance autophagy through activation of ULK1 but not via inhibition of MTOR pathway. In order to validate that EEF2K silencing leads to protein synthesis, which depletes ATP levels and then activates AMPK-ULK1-mediated autophagy, we blocked protein synthesis by cycloheximide in EEF2K-depleted cells and then detected the active form of ULK1 and LC3 levels. As shown in Figure 6C, cycloheximide could completely block both ULK1 phosphorylation at Ser555 and LC3-II accumulation induced by EEF2K silencing. This result indicated that protein synthesis induced by EEF2K silencing is responsible for ULK1 activation and autophagy accumulation. In order to further validate whether AMPK and its downstream target ULK1 are involved in autophagy induced by EEF2K knockdown, the effects of PRKAA1 and PRKAA2 (AMPKα)
siRNA and ULK1 siRNA on autophagy in EEF2K-depleted cells were analyzed. As shown in Figure 6D, AMPKα siRNA could block ULK1 phosphorylation at Ser555 and significantly reduce LC3-II accumulation in EEF2K knockdown cells, suggesting that AMPK is responsible for EEF2K knockdown-induced autophagy. Furthermore, silencing of ULK1 significantly reduced LC3-II levels, indicating that ULK1 is also involved in autophagy induced by EEF2K silencing (Fig. 6E). These findings were further substantiated by quantification of the amount of LC3 dots per cell, showing that knockdown of AMPKα or ULK1 could completely block LC3 dots accumulation induced by EEF2K silencing (Fig. 6F). In addition, we found that knockdown of AMPKα and ULK1 could significantly inhibit cell growth in EEF2K-depleted human colon cancer cells (Fig. 6G). ROS production is one form of cellular stress that plays a critical role in the induction of autophagy. ROS levels were therefore analyzed using DCFDA staining in HT-29 cells after EEF2K silencing, followed by fluorescence microscopy and flow cytometry. Our results demonstrated that ROS levels were not significantly increased in EEF2K-depleted cells as compared with control or H2O2 treatment, suggesting that ROS production is not involved in autophagy induced by silencing of EEF2K (Fig. 6H and I). Taken together, these results indicate that autophagy induced by silencing of EEF2K is attributed to activation of AMPK-ULK1 pathway, independent of the suppression of MTOR activity and stimulation of ROS production.

**Discussion**

EEF2K is well known for its role in the negative regulation of protein translation through inactivation of EEF2 by phosphorylation at Thr56. Previous studies report that the activated EEF2K can induce autophagy in glioma and breast cancer cells. However, the effect of EEF2K on growth of colon cancer cells as well as the underlying mechanism involved is not understood. In this study, we demonstrate that silencing of EEF2K induces autophagy in colon cancer cells and the AMPK-ULK1 pathway is required for this autophagy.

Previous studies report that knockdown of EEF2K by siRNA abrogates autophagy and then results in inhibition of tumor growth, augmentation of apoptosis, and sensitization of glioma or breast cancer to the anticancer agents doxorubicin or MK-2206.16,17 In contrast, silencing of EEF2K by siRNA enhances autophagy instead of blocking autophagy in human colon cancer cells. The anticancer efficiency of MK-2206 is not further enhanced in colon cancer cells after silencing of EEF2K.
This finding in colon cancer cells is in line with accumulating reports that knockdown of EEF2K by siRNA does not inhibit cell growth of lung cancer and prostate cancer cells under both serum and serum-free conditions. In addition, mice lacking EEF2K do not exhibit delays in development and reproduction, indicating that disruption of EEF2K is not sufficient for the inhibition of cell growth. Knockdown of EEF2K can activate EEF2 by reduction of EEF2 phosphorylation at Thr56 resulting in promotion of protein synthesis. It has also been reported that inhibition of EEF2 rapidly arrests protein synthesis and leads to cancer cell growth inhibition. It is therefore conceivable that activation of protein synthesis by silencing of EEF2K does not arrest cancer growth in colon cancer cells. Taken together, EEF2K performs 2 apparently opposite functions in either promoting or inhibiting both autophagy and cancer growth in cell type-dependent manners.

Besides the cytoplasmic LC3-positive autophagosomes during autophagy, recent studies have demonstrated that LC3 punctate signals can also concentrate in the nucleus. For example, the picornavirus foot-and-mouth disease virus can induce LC3 puncta signal to concentrate close to the nucleus in >95% of the cells within 2 h after infection of CHO cells. C2-mercaptoethanol, temozolomide, and arsenic trioxide can induce cytoplasmic and nuclear localization of LC3B in glioblastoma cells U373-MG.
Figure 6. For figure legend, see page 1502.
 autophagy in carcinogenesis has not been completely understood. The role of autophagy in cancer in general is quite complex and is likely dependent on the tumor tissue of origin, stage, and the constellation of genetic mutations and epigenetic changes. Resistance of cancer cells to treatment can be associated with both autophagy and inhibition of the more common apoptotic cell death pathway. Under nutrient-deprivation condition, high levels of EEF2K could be activated to block translation elongation to adapt to the stress condition, suggesting that activated EEF2K functions to promote cell survival. Inhibition of EEF2K potentiates the anticancer efficacy of the AKT inhibitor MK-2206 in glioma cells. According to these findings, blockage of EEF2K could represent a treatment option for breast cancer and glioblastoma. Contrary to this finding, we found that inhibition of EEF2K by knockdown could activate autophagy and then promote cell survival under nutrient condition or in the presence of the antitumor drug oxaliplatin, indicating that EEF2K plays an important role in negatively regulating cell growth in human colon cancer cells. Our finding is consistent with the effect of EEF2K inhibitor A-484954 on cell growth in lung and prostate cancer cells. Cancer cells grow and divide much more rapidly than normal cells, thus they have a much higher demand for nutrients and oxygen than nutrient deprivation. Therefore, upregulation of EEF2K could represent an approach to treat certain cancers such as human colon cancer. Knowledge of the mechanisms and molecules involved will help us to understand the role of EEF2K on the growth and survival of different cancers. Taken together, modulation of EEF2K activity in different cancers might constitute a feasible therapeutic method for cancer treatment.

The signaling pathways that lead to autophagy under nutrient-deprivation conditions have been clearly characterized. MTOR is a central cell growth regulator that links nutrient signals and autophagy. Under starvation conditions, MTOR, which functions as a critical negative regulator of autophagy, is inhibited. However, mammalian cells rarely experience nutrient deprivation under normal physiological conditions. Inactivation of MTOR is not necessary for autophagy under nutrient conditions. Guo et al. report that lipopolysaccharide could induce autophagy via activation of AMPK but not via inhibition of MTOR. Consistent with this finding, autophagy induced by silencing of EEF2K is attributed to activation of AMPK, independent of MTOR inhibition in colon cancer cells under normal nutrient condition.

It appears that EEF2K plays opposite roles in either inducing or inhibiting autophagy in different cancer types. The signaling pathway downstream of EEF2K-EEF2 in controlling autophagy remains unknown. In this study, we report for the first time that silencing of EEF2K enhances autophagy-related genes and promotes cell survival via the AMPK-ULK1-dependent pathway (Fig. 7). This finding indicates that the increase of EEF2K activity might reduce the expression of autophagy-related genes such as BECN1 and ATG7, and inactivate the autophagic AMPK-ULK1 pathway. These, in turn, attenuate autophagy and block the growth of human colon cancer cells. We also report that increase of EEF2K activity can suppress autophagy.
and enhance the efficacy of drugs against colon cancer cells. In addition, it has been demonstrated that EEF2K is downregulated in human colorectal carcinoma patients (Fig. S1). Therefore, upregulation of EEF2K can be a novel strategy for the treatment of human colon cancer. Considering multiple autophagy pathways regulated by EEF2K, attenuation of autophagy by direct increase of EEF2K activity would be a better method than directly targeting a single autophagy pathway in human colon cancer cells. The approach of targeting against EEF2K has gained some attention for treating glioma and breast cancer. However, the general applicability of this approach is questionable in view of our findings. Cancer tissue typing in terms of its autophagic response toward EEF2K inhibition should be performed to assess whether a specific cancer would benefit from this approach. According to our findings, upregulation of EEF2K activity may be developed as a novel approach for the treatment of human colon cancer.

Materials and Methods

Reagents and antibodies
Alexa Fluor 488 donkey anti-rabbit IgG (A21206) antibody was purchased from Life Technologies Corporation. Fetal bovine serum (16000-044) was purchased from Gibco Invitrogen. McCoy’s 5A medium (M4892), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; M2128), cycloheximide (C7698), MG132 (M7449), pepstatin A (P5318) and anti-LC3B antibody (L7543) were obtained from Sigma. Anti-BECN1 (sc-10086), anti-AMPKα (D14E11) (#8441), anti-ULK1 (Ser555) (#8054), anti-phospho-ULK1 (Thr65 and Ser757) (#8485), anti-phospho-ULK1 (Ser555) (D1H4) (#5869) and anti-EEF2K antibody were purchased from Cell Signaling Technology. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; M2128), cycloheximide (C7698), MG132 (M7449), pepstatin A (P5318) and anti-LC3B antibody (L7543) were obtained from Sigma. Anti-BECN1 (sc-10086), anti-AMPKα (D14E11) (#8441), anti-ULK1 (Ser555) (D1H4) (#5869) and anti-EEF2K antibody were purchased from Cell Signaling Technology.

Overexpression of human EEF2K
A plasmid pDONR223-EEF2K containing full-length of human EEF2K coding region was obtained from Addgene (Addgene plasmid 23726, USA). Amplification of the coding region was performed by PCR using GeneAmp High Fidelity Enzyme Mix (Life Technologies, 4328216). The PCR conditions were denaturation at 94 °C for 3 min, followed by 20 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min 30 s, with a final extension step at 72 °C for 10 min. The products were purified using the QIAquick Gel Extraction Kit (Qiagen, 28706) and then inserted into a pcDNA3.1/V5-His TOPO TA expression vector (Life Technologies, K4800-01). The resultant construct encompassing EEF2K with V5 and polyhistidine epitope tags was confirmed by sequencing.

Cell culture
The human colon cancer HT-29 (HTB-38) and HCT-116 (CCL-247) cells purchased from American Type Culture Collection (ATCC) were cultured in complete McCoy’s 5A medium (supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin). All experiments were performed in HT-29 or HCT-116 cells between passage 10 and 20.

Small interfering RNA (siRNA) transfection
ON-TARGETplus SMART pool against human EEF2K siRNA (L004950-00-0005) was obtained from Dharmaco, Inc. Other siRNAs were purchased from GenePharm. EEF2K silencing was performed using an siRNA duplex targeting the following sequence: 5′- AAGCUGCAGAC CAGAAUGCUCA A-3′. BECN1 was silenced using siRNA duplexes targeting the following sequences: 5′-GAUACCGACU UGUUCCUAU-3′ and 5′-CUAAGGACGU GCCGUUAUA-3′. ATG7 was targeted with siRNA duplexes targeting the following sequences: 5′-CCAACACACUGACUGCUUU-3′ and 5′-GCCACAGAU GGAUGAGCA-3′. PRKA1 and PKRKA2AMPKα were silenced with siRNA duplexes targeting the following sequences: GAGAGAGCU GCCGUUAUA and GCUGUUUGGU GUAGGAUA, respectively. ULK1 was targeted with siRNA duplexes targeting the following sequences: 5′-UUGGCCCUCUGU ACCACUUC GAAAU-3′ and 5′-GAGCAAGAC ACACGGAAA-3′. A nontargeting siRNA was used as a control with sense (5′-UCUACAGGAG ACGACCUUU-3′) and antisense (5′-AAGUCUCUGU CCUGUGA-3′). In brief, cells were transfected in McCoy 5A medium with 90 nM of each siRNA duplex using DharmaFECT transfection reagent according to the manufacturer’s protocol.

Immunofluorescence staining
Cells were grown on slides and transfected with siRNAs. After 48 h transfection, cells were washed 3 times with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.46 mM KH2PO4, pH 7.4), fixed with 3.5% formaldehyde in PBS for 10 min, washed once with PBS, permeabilized with 0.1% Triton X-100 (USB, 22686) in PBS for 10 min, and blocked with 0.5% BSA (Sigma, A2153) in PBS for 15 min. Cells were incubated with LC3 antibody (1:150) for 2 h at room temperature, followed by incubation with Alexa Fluor 488 antibody (1:200) for 1 h at room temperature. All antibodies were diluted with 0.5% BSA in PBS. Slides were mounted with Vectashield mounting medium and images were taken with an Olympus FV1000 confocal microscope (Olympus, PA, USA) using a 60 × 1.35 NA oil objective.

RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR)
Total RNA was isolated by Trizol (Life Technologies; 15596018). RT-PCR (reverse transcription polymerase chain reaction) was performed with PrimeScript™ RT reagent Kit (Takara; RR037A) according to the manufacturer’s instructions. To detect the mRNA levels of ATG5 and BECN1, primers used were as follows: ATG5 forward, 5′-AGCAACTCTG GATGGGATTG-3′; reverse, 5′-CAGTCAGAG GTGTCTCCA-3′; BECN1 forward, 5′-GGCCACAATAG...
ATGGGTCTGA-3; reverse, 5'-GCTTTTGTCC
ACTGCTCCTC-3; EFG7 forward, 5'-ACCCAGAGA
AGCTGAAAGA-3; reverse, 5'-GCGAAAGTGC
AAGAGGAGGA-3; EEF2K forward, 5'-ATGACTCGC
AGTTCGCT-3; reverse, 5'-TGCATCGGTCACTAC-3;
GAPDH forward, 5'-AAGGGAGAAG TCAAAGATT-3;
reverse, 5'-CATGCCGTTG AACATATTG-3. GAPDH was
used as internal control.

Clonogenic assay
For clonogenic assay, cells were transfected with control siRNA
(siCTL), EEF2K siRNA (siEEF2K), BECN1 siRNA (siBECN1),
ATG7 siRNA (siATG7), siBECN1 plus siEEF2K, siATG7 plus
siEEF2K, empty vector (Vector), or EEF2K plasmids (EEF2K)
for 24 h, and then the cells were seeded out in appropriate
dilutions into 6-well plates, followed by incubation at 37 °C for
12 to 14 d. Colonies were fixed with glutaraldehyde (6.0% v/v),
stained with crystal violet (0.5% w/v) and imaged. Colonies with
50 or more cells were counted.

ANXA5 (annexin V) and propidium iodide (PI) staining
Cells were transfected with siRNA, EEF2K siRNA
(siEEF2K), empty vector (Vector), or EEF2K plasmids (EEF2K)
for 24 h, and then the cells were treated with vehicle (0.1% DMSO)
or oxaliplatin (Sigma, O9512) for 48 h, washed with PBS, incubated in the binding buffer (10 mM HEPES, 140 mM
NaCl, 2.5 mM CaCl2, 0.1% BSA, pH 7.4) containing ANXA5-
FITC for 15 min.9 Cells were immediately exposed to 2 µg/ml
PI (Sigma, P4170) before the analysis on a FACScan flow
cytometer (Becton Dickinson, San Jose, CA).

Western blot analysis
Cell extracts were prepared for western blot. In brief, cells
were lysed with lysis buffer (20 mM TRIS-HCl, pH 7.4, 150
mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium
pyrophosphate, 1 mM DTT, 1 mM sodium orthovanadate
[Sigma, S6508], 1 µg/ml leupeptin [Sigma, L2884], 1 mM
phenylmethylsulfonyl fluoride [Sigma, P7626]) for 1 h on ice.
The total proteins were analyzed on a 12% gel by SDS-PAGE,
transferred onto a nitrocellulose membrane, blocked with 5%
BSA in TBST buffer (Tris-buffered saline [50 mM TRIS, 150
mM NaCl, pH 7.5, containing 0.1% Tween-20), incubated with
primary antibodies at 4 °C overnight, washed 3 times for 15 min
each in TBST at room temperature, incubated with horseradish
peroxidase-conjugated secondary antibodies for 1 h at room
temperature, and washed 3 times for 15 min each in TBST.
The bands were detected using the Chemiluminescence HRP
Substrate (Millipore, WBKLS0500). The relative band intensity
was quantified using the AlphaEaseFC software version 6.0.0.

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Measurement of cellular ATP
Cells were seeded into 96-well plates at 4.0 × 10^3 cells per
well and transfected with nontargeting control siRNA or siRNA
targeting EEF2K for 48 h. ATP contents were measured using
the ATPlite Luminescence Assay Kit (PerkinElmer, 6016943)
according to the manufacturer’s protocol. The relative ATP level
was normalized to control samples.26

ROS analysis
ROS generation in cells after EEF2K silencing was analyzed.
In brief, cells were transfected with nontargeting control siRNA
or EEF2K siRNA for 48 h, and then stained with 20 µM
2',7'-dichlorofluorescein diacetate (DCFDA; Sigma, D6883)
for 30 min in the dark. For morphological study, the cells
were imaged under a Nikon TE2000 fluorescence microscope
(Nikon, Melville, NY, USA). For quantifying the ROS levels, the cells
were analyzed using a flow cytometer (Becton-Dickinson, CA,
USA) in FL1 channel.

Statistical analysis
Statistical analysis was performed using the 2-tailed
Student t test for comparison of 2 groups or one-way analysis
of variance for comparison of more than 2 groups followed by
the Tukey multiple comparison test. For multiple testing,
the P values were determined using a 2-way analysis of
variance with Bonferroni post-test. All statistical analyses were
performed using the GraphPad Prism software version 5.01
(GraphPad, San Diego, CA). Data were expressed as mean ±
standard error of the mean (SEM) of at least 3 independent
experiments. A P value < 0.05 was considered statistically
significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here:
www.landesbioscience.com/journals/autophagy/article/29164
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