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Activated CRH receptors inhibit autophagy by repressing conversion of LC3BI to LC3BII

Lai Jin*, Yuanyuan Qian, Jun Zhou, Li Dai, Chunxuan Cao, Chao Zhu, Shengnan Li*

Department of Pharmacology, Nanjing Medical University, Nanjing 211116, PR China

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ABSTRACT

Clinical studies have elucidated the negative correlation between microtubules-associated protein 1 light chain 3-B (LC3B) protein expression and overall survival of breast cancer patients. Our previous data demonstrated corticortropin-releasing hormone family (CRHs) suppressed migration of breast cancer cells via CRH receptors (CRHRs). Here, we showed that the activation of CRHRs (CRHR1 and CRHR2) remarkably reduced the conversion of LC3BI to LC3BII and hence repressed macroautophagy/autophagy, resulting in migration inhibition. By means of RT-4 cells (expressing higher CRHR1) with stable CRHR1 silence which was constructed by lentivirus with short hairpin RNA, we further confirmed CRH-inhibited LC3BII conversion. Using CRHRs agonists and antagonists, we found CRHRs triggered a marked reduction in the number of LC3B dots in both RT-4 and Hela cells(expressing higher CRHR2) which stably express RFP-GFP-LC3B. Of note, this decreased amount of autophagosome was associated with activation of Phospholipase C β (PLCβ)-Inositol triphosphate (IP_3)-mTOR signaling. Earle's Balanced Salt Solution (EBSS) decreased the expression of the key focal adhesion protein, paxillin, which was recovered by CRHRs ligands (CRH and UCN2). The effect of CRHRs ligands on paxillin resulted in the suppression of cell migration. Altogether, these data reveal a new link between CRHRs signaling and autophagy, and may help to envisage therapeutic strategies in cancer cell invasion.

1. Introduction

Autophagy is a catabolic process for degrading damaged organelles, protein aggregates and the intracellular recycling of metabolites [1] that are utilized by tumor cells to survive nutrient stress, hypoxia and attack of immune cells [2]. Autophagy has been shown to play indispensable roles in cell proliferation, apoptosis, invasion, cancer stem cell viability and differentiation, epithelial-to-mesenchymal transition (EMT) and so on [3]. Therefore, autophagy is regarded as a potential therapeutic target on cancers. Recent studies indicate that the expression of LC3B, an autophagy marker, is positively correlated with the mortality [4] and can be used as an independent prognostic marker for relapse free and total survival rates in patients with breast cancer [5,6].

Corticortropin-releasing hormone (CRH) family is composed of CRH, Urocortin 1 (Ucn1), Urocortin2 (Ucn2) and so on. CRH receptors (CRHRs) known as type-1 (CRHR1) and type-2 (CRHR2) receptors that belong to G protein-coupled receptors (GPCRs) superfamily [7] are first identified in central nervous system (CNS) [8] and recently found in extracranial tissues [9]. CRHRs' natural ligands include CRH with high affinity to CRHR1, Ucn2 with high affinity to CRHR2, and Ucn1 with similar affinity to both [10]. In CNS, CRHRs regulate stress response such as behavioral, endocrine, immune and autonomic response [7,11]. And stress is also the major inducer for autophagy, pointing to the idea that CRHRs may engage autophagy during stress [12]. In periphery, emerging evidence demonstrates that CRHRs are involved in carcinogenesis such as apoptosis, migration, proliferation and angiogenesis [13–15]. We have recently reported that activated CRHRs suppressed TGFβ1-induced Epithelial-Mesenchymal Transition (EMT) via Smad2/3-Snail1/Slug signaling in breast cancer cells [14,16]. Diminished expressions of Snail1 and Slug were further shown to be associated with reservation of E-cadherin, inhibition of cell migration and invasion [14]. There is enough evidence that autophagy and EMT are linked in an intricate relationship as defects in the autophagic machinery restrain dissemination and metastatic spreading of cancer [17]. Marina et al. found autophagy promoted focal adhesion disassembly and cell motility through degradation of paxillin, which anchored junction of the cell to a non-cellular substrate [18]. Another study reported that autophagy was critical for EMT, in which TGF-β/Smad3-depdendent signaling was involved [19]. For the relationship between CRHRs and autophagy, CRH deficient mice showed sustained activation of base line autophagy [12] and Ucn1 inhibited Beclin1-mediated autophagic cell death [20]. However, the function and mechanism of CRHRs on autophagy in

* Corresponding authors.
E-mail addresses: ljin@njmu.edu.cn (L. Jin), junzhou@njmu.edu.cn (J. Zhou), lidai@njmu.edu.cn (L. Dai), czhu@njmu.edu.cn (C. Zhu), snli@njmu.edu.cn (S. Li).

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cancer cell migration has yet to be fully clarified.

Nutrients are major modulators of autophagy in a systemic manner by secreting hormones and neurotransmitters that regulate GPCRs [21]. The nutrient receptors including the amino acid responsive receptors, taste receptor type 1 and the calcium receptor induce autophagy in response to depletion of nutrients [22]. Different G protein-α-subunits control different signaling. Gq-PLC-IP_3 and Gi/Gs- cyclic AMP (cAMP) activation regulate a multitude of signaling molecules such as mTOR and the mitogen activated kinases (mainly ERK1/2) that modulate autophagy [21]. CRHRs are well known GPCRs which are activated by endocrine-, paracrine- or autocrine-secreted CRH or Ucn1/2/3 when the organism is in the stress [23]. The CRHRs are connected with different G protein-α-subunits including Gs, Gq, Gi and Go [24]. Thus, it is reasonable to hypothesize that activation of CRHRs linked to Gq could inhibit autophagy.

The current study aimed to elucidate the effects of CRHRs on autophagy and the mechanisms. The investigation confirmed that activation of CRHRs inhibited autophagy through blocking the conversion of LC3I to LC3II in RT-4, Hela, MDA-MB-231 and MCF-7 cancer cell lines. The PLCβ-IP_3-mTOR signal pathway was found to be indispensible for the migration of breast cancer cells. Collectively, these results broaden our understanding of the novel role of CRHRs in autophagy and indicate that CRH family peptides may be effective on controlling breast cancer metastasis in the clinic.

2. Materials and methods

2.1. Cells, virus and reagents

Human cell lines RT-4, Hela, MDA-MB-231, MCF-7 and HEK293FT cells were obtained from Zhongqiao New Boat Company (Shanghai, China). Cells of passages 5–25 were used for experiments. These cell lines were routinely cultured in our lab according to the instructions from American Type Culture Collection (ATCC). Lentiviruses containing the GFP-LC3 and RFP-GFP-LC3 vectors were provided by Genechem (Shanghai, China).

CRH, Urocortin (Ucn1), Urocortin2 (Ucn2), Astressin (Ast) were from ChinaPeptides (Shanghai, China) and Bafilomycin (BAfA1), U73122, 2-Aminoethoxydipheny Borate (2-APB), Polybrene, Puromycin were from MedChem Express (MCE, USA). Antibodies of LC3, phospho-mTOR (Ser2488), mTOR, phosphor-ERK1/2 (Thr202/Tyr204), ERK1/2 were from Cell Signaling Technology (1:1000 dilution, CST, USA). GAPDH (1:5000 dilution), Paxillin (1:1000 dilution) and TGFβ1 (1:1000 dilution) antibodies were obtained from Proteintech (USA). 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and Cell Counting Kit-8 (CCK-8) were from KeyGEN BioTECH (Nanjing, China). Lipofectamine 2000 transfection reagent and RNA isolation kit (TRIzol) were from Invitrogen and SYBR Green PCR Supermix without ROX was from Vazyme biotech. (Nanjing, China). The 24-well tranwell chamber was provided by Corning (USA). Earle's Balanced Salt Solution (EBSS) and all the cell culture medium were from Gibco (USA). PLCβ and IP3 ELISA kits were from YIFEIXUE BIO TECH (Nanjing, China). T4 DNA Ligase and High Fidelity restriction enzymes (*Eco*RI & *Age*I) were from NEW ENGLAND BioLabs (NEB, USA).

2.2. Plasmid construction, virus package and infections

PLKO.1 and Lentivirus Plasmids (pMD2.G & psPAX2) were kindly provided by Weibo Luo. Briefly, three sequences of shRNAs targeting CRHR1 were referred to Sigma. The sense and antisense primers were as follows: CRHR1-1, forward, 5′-CCGGGACATGGGAATGAATTGAAA TCTCGAGATTTCAATTCATTCCCATGTCTTTTT-3′, reverse, 5′-AATTAA AAAGACATGGGAATGAATTGAAATCTCGAGATTTCAATTCATTCCCAT GTC-3′; CRHR1–2, forward, 5′-CCGGTCCTGGTCCTGCTGATCAATTCT CGAGAATTGATCAGCAGGACCAGGATTTTT-3′, reverse, 5′-AATTAAAA ATCCTGGTCCTGCTGATCAATTCTCGAGAATTGATCAGCAGGACCA GGA-3′; CRHR1–3, forward, 5′-CCGGTCTATGGTGTCCGCTACAATACT CGAGTATTGTAGCGGACACCATAGATTTTT-3′, reverse, 5′-AATTAAAA ATCTATGGTGTCCGCTACAATACTCGAGTATTGTAGCGGACACCAT AGA-3′ (GENEray, Shanghai, China). After annealing, shCRHR1 were fused into restriction enzyme digested pLKO.1 vector, which were confirmed by DNA sequencing. Then the shCRHR1-pLKO.1 and Lentiviral particles were packaged in HEK293FT cells with Lipofectamine 2000.

The cells were transduced with lentiviruses containing CRHR1 shRNA, nonsilencing shRNA, GFP-LC3, RFP-GFP-LC3 using polybrene (8 μg/ml) (Maycotte, et al. 2012). After puromycin selection (1 μg/ml) for about 2 weeks, single clone was isolated and validated for CRHR1 knockdown and screened for green and red fluorescence.

2.3. Migration assay

10^5 (MDA-MB-231) or 10^6 (MCF-7) cells in EBSS were seeded on transwell filters (8-μm pores, 24 wells). Complete medium was added in the lower chamber. After 24 h, cells on the upper surface of the filter were wiped using a cotton swab. Cells that migrated onto the lower surface were fixed with 100% methanol and stained with DAPI. The cells were visualized under a fluorescence microscope (OLYMPUS, Japan). The cell number was counted in ten random visual fields and calculated by the ImageJ software.

2.4. Protein isolation and western blots

After treatment, cells were lysed with RIPA buffer contained protease and phosphatase inhibitors (Roche), and the amount of protein was determined by Bradford method. Briefly, 20 μg of protein lysate was loaded in SDS-polyacrylamide and then electrically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Then the membranes were blocked and incubated with primary antibodies overnight at 4 °C, and incubated with secondary HRP-conjugated IgG for 1 h. Finally, the bands were visualized by Chemiluminescence gel imaging system (SYNGENE) and the density was analyzed by GELPRO4 software.

2.5. Real-time PCR assay

RNA isolation was operated using TRIzol. The cDNA was synthesized with HiScript Reverse Tanscriptase (Vazyme, Nanjing, China) and amplified with SYBR green PCR mix (without ROX, Vazyme, Nanjing, China). The amplified products were detected by CFX Connect Real-Time System (BIO-RAD). The primers provided by TSINGKE (Nanjing, China) were as follows: CRHR1, forward, TCAGCCCCAGAAGGAGAAG, reverse, GGGATGTCCGTAGGACCC; CRHR2, forward, ATCCAAAATGG GCTCACACT, reverse, TCAACGGCGTCAAGTACAAC; GAPDH, forward, GGACCTGACCTGCCGTCTAG, reverse, GTAGCCCAGGATGCCCTTGA. Changes in CRHR1 and CRHR2 gene expressions were determined by comparative CT (ΔΔCT) method.

2.6. GFP-LC3 and RFP-GFP-LC3 redistribution evaluation

Cells were transduced with the lentiviral vector containing a GFR-LC3/RFP-GFP-LC3. After Puromycin selection, cells were plated on coverslips, treated and fixed with 100% methanol. For breast cancer cells, DAPI was added to stain the nuclei and visualized at 40× magnification using LSM710 confocal microscope (Zeiss, Germany). The green or orange dots were quantified by particle analysis of Image J 1.47v. Graphs showed the quantification of three independent experiments performed in duplicate and the representative images were displayed.

2.7. Enzyme-linked immunosorbent assay

For the quantitative determination of PLCβ and IP_3 levels in cells, the quantitative sandwich enzyme immunoassay technique was used with commercially available (YIFEIXUE BIO TECH). Briefly, the cells were harvested using RIPA buffer contained protease and phosphatase inhibitors (Roche), added to the 96-well plate provided by the enzyme-linked immunosorbent assay kit. Then the plate was incubated, washed and detected according to the manufacture's instruction.

2.8. Transmission electron microscope

The cells were collected, fixed with 4% glutaraldehyde and washed, and then fixed again with 2% osmic acid at 4 °C. After washes, the cells were dehydrated with acetone, soaked, repaired and embedded with embedding agent. Then the blocks were slice into 50–70 nm sections and stained with saturated uranyl acetate solution and lead acetate. Finally the ultra-thin sections were observed and photographed by transmission electron microscope.

3. Results

3.1. Activation of CRHRs inhibits autophagosome biogenesis in breast cancer cell lines

Autophagy is a dynamic process in which LC3B is cleaved and conjugated to phosphatidylethanolamine to form termed LC3BII during early periods of autophagy. LC3BII participates in elongation of autophagosome, yet it is degraded by the lysosomes during later periods of autophagy. Ucn1 was previously reported to inhibit autophagy in cardiac myocytes [20]. To determine whether activation of CRHRs inhibit autophagy of breast cancer cells, we used EBSS to induce autophagy [25]. We treated MCF-7 (mainly expressing CRHR1) or MDA-MB-231 (mainly expressing CRHR2) [14] cells with EBSS alone or along with 10^{-7} M Ucn1 for different time. As shown in Fig. 1A, EBSS starvation time-dependently induced LC3BII expression. And EBSS-induced LC3BII conversion was blocked by Ucn1, especially at early time points (2–6 h).

We then measured autophagic flux in both cell lines upon treatments by EBSS, Ucn1 or CRHRs antagonist, Astressin (Ast). Punctate GFP-LC3B staining provides a measure of autophagic flux. As expected, a marked increase in the number of GFP-LC3B puncta was observed in the cells with EBSS treatment compared with the control cells (Fig. 1B). Treatment with Ucn1 partially decreased the number of GFP-LC3B-labeled autophagosomes (Fig. 1B). Furthermore, pre-treatment with Ast reversed the number of LC3B puncta suppressed by Ucn1, suggesting that CRHRs engage an intracellular signaling pathway due to either inhibition of autophagosome biogenesis, or activation of lysosomal pathway. To distinguish between these two possibilities, we studied the effects of EBSS alone, or in combination with lysosomal inhibitor Bafilomycin (BAFA1), which induce accumulation of autophagosomes. As shown in Fig. 1C, combination of EBSS and BAFA1 treatments evoked a significant increase in the number of GFP-LC3B puncta compared with the EBSS treatment only. Furthermore, Ucn1 treatment showed more reduction in the number of LC3B dots when the cells were co-incubated with BAFA1 and EBSS. Finally, electron microscopy revealed that Ucn1 attenuated the number of autophagosome induced by EBSS&BAFA1 (Fig. 1D). The results above revealed that the effects of the CRHRs likely inhibited autophagic flux.

For further proof of CRHRs-evoked inhibition of autophagosome formation and exclusion of lysosomes roles, we performed Immunoblotting assay to detect LC3B expression in the presence of both EBSS and BAFA1. As shown in Fig. 1E, Ucn1 decreased LC3BII expression at 4 h and Ast pre-treatment abolished the effect of Ucn1. Taken together, these data indicated that activation of CRHR1 and CRHR2 inhibited EBSS-induced autophagy.

3.2. CRHRs-evoked inhibition of autophagy is relayed by LC3B

The CRHRs is composed of type1 (CRHR1) and type2 (CRHR2) receptors. In order to distinguish the effect of respective receptors and detect the influence of CRHRs in other cancer cell lines, we selected bladder cancer cell line RT-4 expressing high level of CRHR1 and Hela expressing high level of CRHR2 in this work (referred to database of human protein ATLAS). Meanwhile we used CRH (CRHR1 agonist) and Ucn2 (CRHR2 agonist) respectively to treat the RT-4 and Hela cells. Consistent with the observed effects in breast cancer cells, in RT-4 cells, CRH partially attenuated LC3BII expression induced by EBSS&BAFA1, and Ast completely abolished this effect (Fig. 2A upper). In addition, Ucn2 had no effect due to deficiency of CRHR2 in RT-4 cells. Similarly, in Hela cells, Ucn2 partially inhibited LC3BII production and Ast blocked this effect (Fig. 2A lower). CRH was negative control since Hela cells lack CRHR1. In line with above results, EBSS&BFAF1-induced RFP-GFP-LC3B dot formation was markedly decreased after incubation with CRH or Ucn2, and Ast reversed these effects (Fig. 2B).

We then constructed RT-4 cell line with CRHR1 deficiency to detect autophagy. Three different sequences of shRNA or scrambled shRNA were packed into viruses and infected RT-4 cells. Protein and mRNA expressions of CRHR1 were decreased by about 80% (Fig. 2C upper). CRH obviously inhibited expression of LC3BII in scramble cells, but the cells with CRHR1 knockdown showed no response to CRH on the condition of EBSS&BAFA1 treatment (Fig. 2C lower), further confirming that CRHR1 activated by CRH is required for inhibition of EBSS-induced autophagy, in which repression of LC3BII expression is involved.

3.3. The anti-autophagic effects of CRHRs depended on PLCβ-IP_3-mTOR signaling pathway

mTOR is an evolutionary conserved protein kinase and central regulator of cell growth. mTOR activity is inhibited during starvation-induced autophagy [26]. An increase in phospholipase C (PLC) activity elevates intracellular inositol triphosphate (IP_3) concentraion, which regulates a multitude of signaling molecules such as mTOR and activated kinases (MAPKs) ERK1/2 [21]. MTOR is the major signal molecule in autophagy inhibition. To detected whether activated mTOR is involved in CRHRs-mediated autophagy, we treated the cells with or without CRH/Ucn2 (10^{-7} M) for different time periods upon EBSS induction. As shown in Fig. 3, EBSS alone (control) supressed mTOR phosphorylation (p-mTOR) in a time-dependent manner, resulting in autophagy inhibition, consistent with previous data. In RT-4 cells (expressing higher CRHR1), CRH abolished EBSS-inhibited mTOR phosphorylation at the same time points, indicating that CRH activate mTOR to inhibit autophagy. Similarly, Ucn2 also phosphorylated mTOR to restrain autophagy in Hela cells, which expressing higher CRHR2. These results above demonstrated that activated CRHRs suppressed autophagy through mTOR on the starvation of EBSS.

To explore whether PLCβ-IP_3 signaling mediated mTOR-inhibited autophagy, mTOR phosphorylation as well as LC3B conversation were evaluated with PLCβ inhibitor (U73122) or IP_3 receptor inhibitor (2-APB). In RT-4 cells, 2-APB or U73122 alone prevented CRH-activated p-mTOR (Fig. 4A), suggesting that CRH induce mTOR phosphorylation through PLCβ and IP_3. Since PLCβ and IP_3 induce both mTOR and ERK1/2, p-ERK1/2 was tested. CRH also inhibited ERK1/2 in the presence of U73122 but not 2-APB (Fig. 4B). It indicated that some other molecules mediated CRH-inhibited ERK1/2, which might be in the downstream of PLCβ and upstream of IP_3. But neither 2-APB nor U73122 could change CRH-repressed accumulation of LC3BII (Fig. 4C) and RFP-GFP-LC3B puncta (Fig. 4E). It suggests there may be other 'replenishment' when the mTOR and ERK1/2 signaling pathways are inhibited.

To confirm the effect of PLCβ and IP_3 inhibitors, we then detected IP_3 levels and PLCβ activity using Elisa kits. As expected, IP_3 levels and

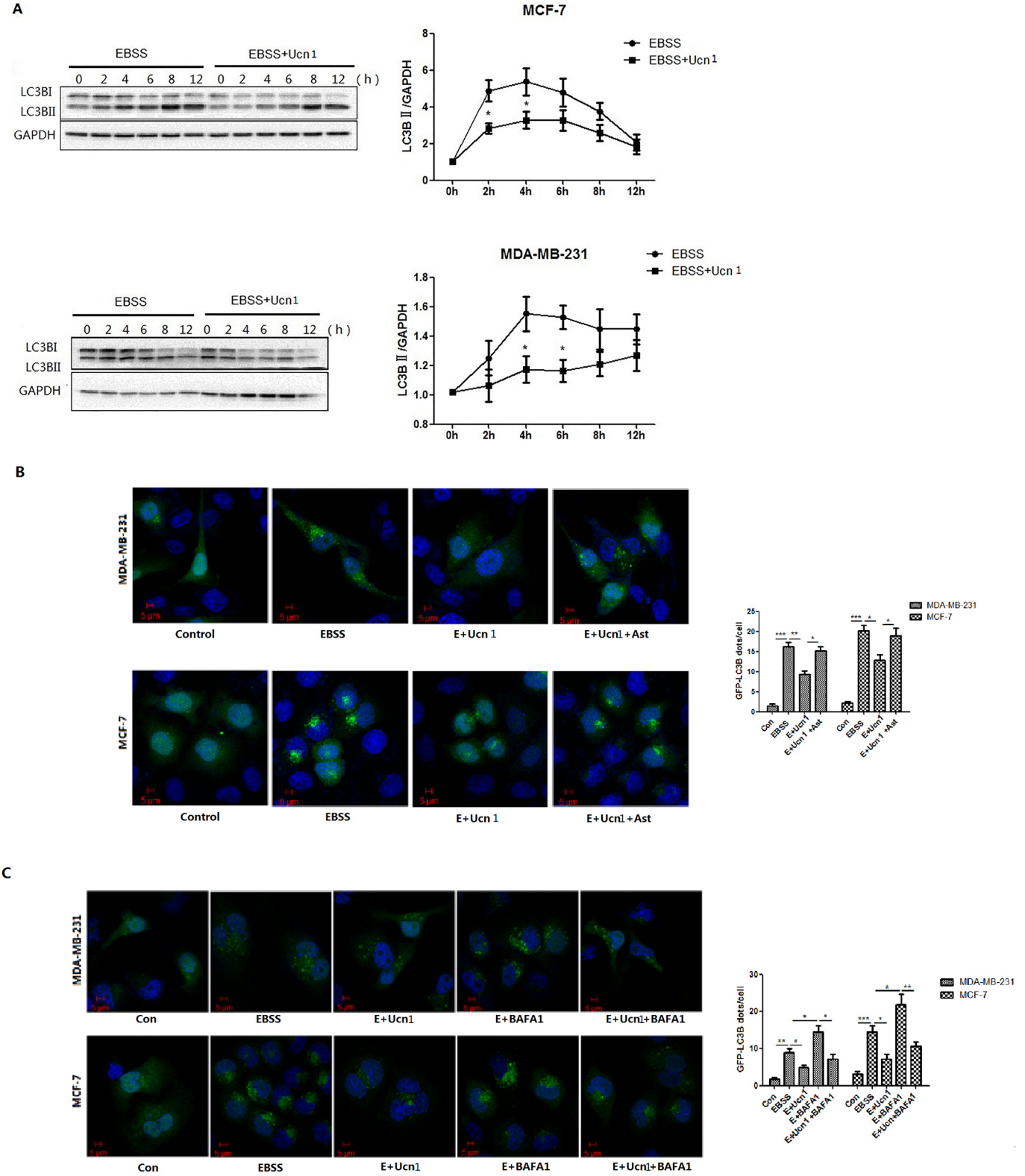
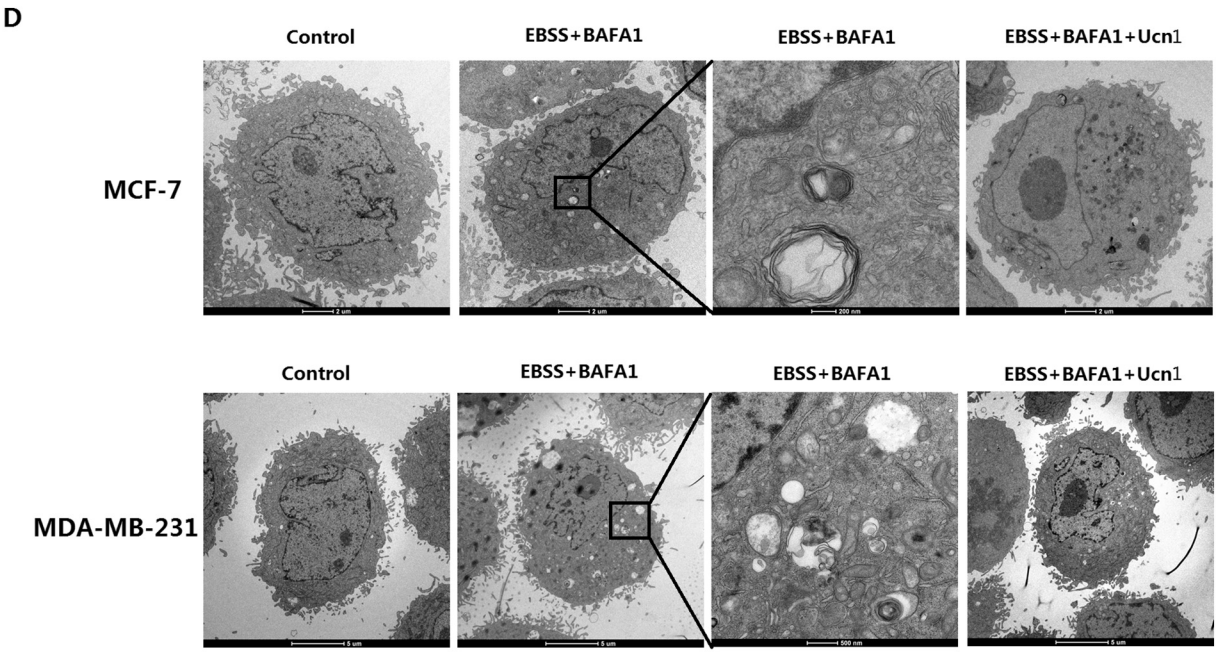


Fig. 1. Ucn1 inhibits autophagosome biogenesis of breast cancer cell lines: MCF-7 cells expressing CRHR1 or MDA-MB-231 cells expressing CRHR2. (A) Both cells were treated with EBSS alone or along with Ucn1 (10^{-7} M) for different time points (2, 4, 6, 8, 12 h), expression of LC3B were detected by Western blotting. (B) Both cells expressing fluorescent protein GFP-LC3B were treated (4 h) with Ucn1 (10^{-7} M) alone or along with Astressin (Ast, 10^{-6} M) upon EBSS starvation, as indicated. Cells were fixed and the number of GFP-LC3B fluorescent dots per cell was quantified by particle analysis of Image J 1.47v. Scale Bars, 5 μm. (C) Under the condition of EBSS induction, both cells expressing fluorescent protein GFP-LC3B were treated (4 h) with or without Ucn1 (10^{-7} M) and BAFA1 (50 ng/ml). Cells were fixed and the GFP-LC3B fluorescent dots per cell were quantified. Scale Bars, 5 μm. (D) Electron microscopy of both cells incubated with or without Ucn1 and BAFA1 upon EBSS starvation. Scale Bars, 2 μm on upper side, 5 μm on lower side. (E) Under condition of EBSS and BAFA1 co-incubation, both cells were treated (4 h) with or without Ucn1 (10^{-7} M) and Ast (10^{-6} M), LC3BII levels were detected. Data shown are profiles of a representative sample of each experimental group. Histograms show the mean ± S.E.M. of three independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$; ns, not statistically different.



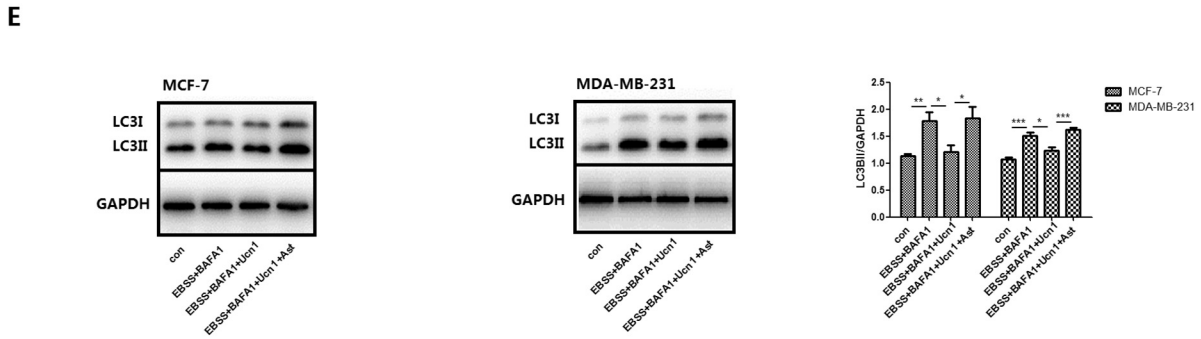


Fig. 1. (*continued*)

PLCβ activity were reduced after EBSS starvation (Fig. 4D). U73122 inhibited PLCβ activity and decreased IP_3 levels. 2-APB did not influence the IP_3 levels and PLCβ activity as it was the inhibitor of IP_3 receptor (Fig. 4D). These data confirmed that PLCβ was in the upstream of IP_3. Additionally, CRH activated PLCβ and increased IP_3 levels. U73122 but not 2-APB reversed the effects of CRH, consistent with the point that CRH induced PLCβ-IP_3-mTOR signaling.

We next tested the ability of CRHR2 using the same way above. Both U73122 and 2-APB treatments blocked the Ucn2-activated p-mTOR in Hela cells (Fig. 5A), suggesting that PLCβ and IP_3 were in the upstream of mTOR. Theoretically, both U73122 and 2-APB should block the effect of Ucn2 on autophagy inhibition. However, Ucn2 increased LC3BII accumulation when PLCβ was inhibited by U73122 (Fig. 5C & E), indicating autophagy induction. This data suggested that the effect of Ucn2 on autophagy altered if PLCβ activity is blocked, demonstrating the important role of PLCβ in Ucn2- inhibited autophagy. It is supposed that there may be other mechanism. Then we detected ERK1/2 phosphorylation. Ucn2 and U73122 treatments resulted in ERK1/2 activation and corresponding autophagy promotion, indicating ERK1/2 play relative dominant role after PLCβ inhibition (Fig. 5B, C, E). As shown in Fig. 5D, Hela cells exhibited higher levels of IP_3 with co-treatments of 2-APB/U73122 and Ucn2. Combined with the results above (Fig. 5B), we assumed that CRHR2 up-regulate IP_3 through PLCε to induced ERK1/2 phosphorylation [21] when the PLCβ is inhibited. Together, these data strongly demonstrated that activated CRHRs exerted anti-autophagic effect dependently of PLCβ-IP_3-mTOR signaling pathway.

3.4. Autophagy inhibition is required for CRH- and Ucn2-repressed migration

Emerging evidence shows that autophagy modulates tumor cell motility, invasion and epithelial-to-mesenchymal transition (EMT). Our previous studies indicated that CRHRs activated by CRH or Ucn1 inhibited TGF-induced EMT in breast cancer cells [14,16]. This work found activated CRHRs inhibited EBSS-induced autophagy. We therefore hypothesize that CRHRs-inhibited autophagy may partially be involved in migration suppression.

We tested cell viability after CRH treatment. Upon EBSS starvation, there was no significant decrease in cell growth with or without CRH/Ucn2 treatment (6 h, data not on shown). We next evaluated migration using the transwell assay. As shown in Fig. 6A, EBSS significantly stimulated the migration of the cells, and CRH/Ucn2 partially attenuated this effect, as previously reported [14,16]. For MCF-7 cells mainly expressing CRHR1, after EBSS incubation, 2-APB promoted migration and CRH partially blocked this effect. These data indicated that migration capacity was consistent with LC3BII expression, suggesting autophagy

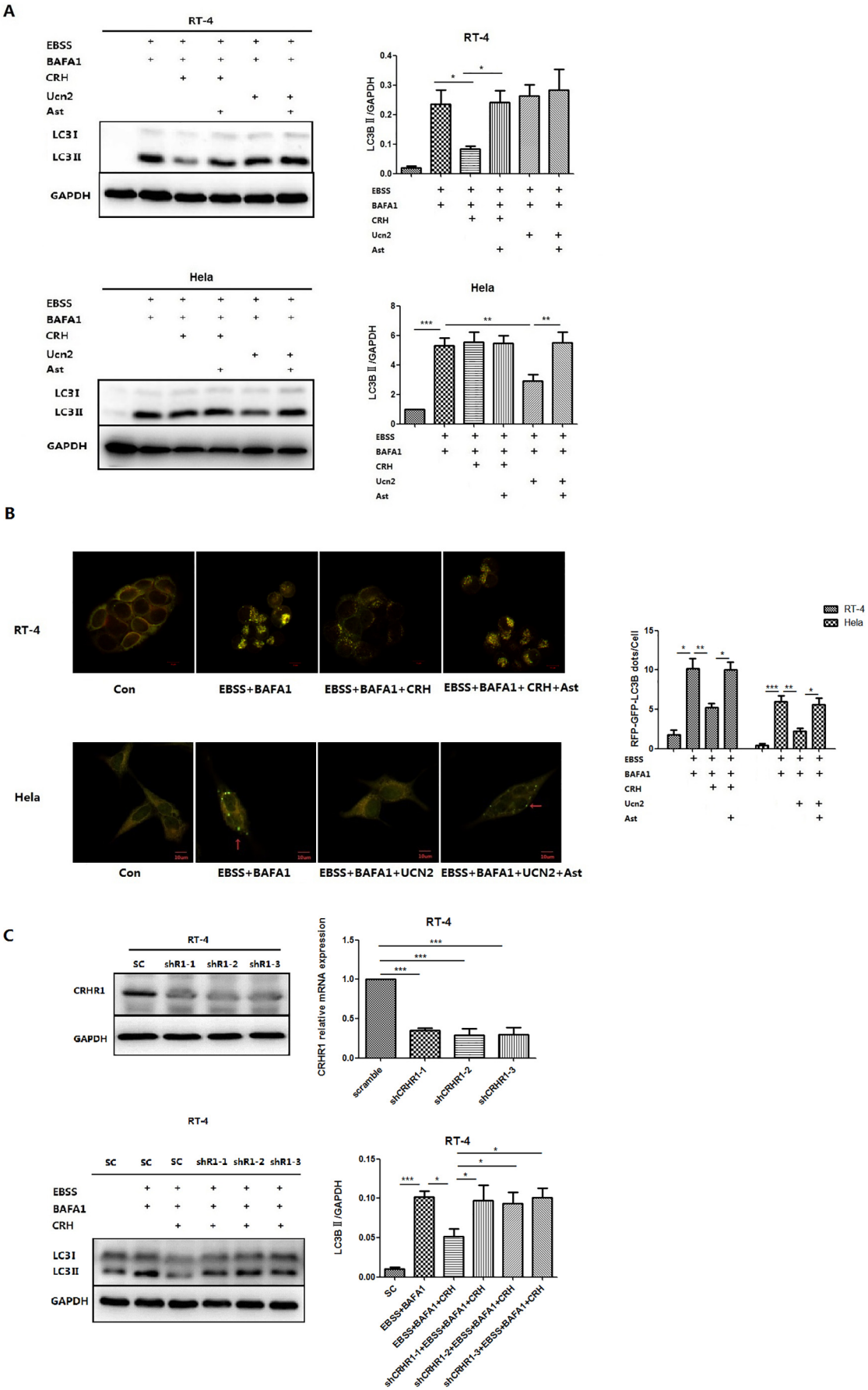


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Fig. 2. Activated CRHRs inhibit production of LC3BII. Upon treatment of EBSS and BAFA1, RT-4 cells expressing CRHR1 and Hela cells expressing CRHR2 were treated (RT-4 for 3 h, Hela for 6 h) with CRH/Ucn2 (10–7 M) alone or along with Ast (10–6 M), LC3BII expression (A) and fluorescent RFp-GFP-LC3BIIlevels were detected. Scale Bars, 10 μm (B). (C) Efficiency of CRHR1 knockdown (upper) were shown. And RT-4 cells expressing shCRHR1 were induced by CRH for 3 h, LC3BII expressions (lower) were tested. Histograms show the mean ± S.E.M. of three independent experiments. *P < .05; **P < .01; ***P < .001; ns, not statistically different.

play a role in CRHR1-inhibited migration. Due to the poor migrated ability of MCF-7, the cell number was small. For MDA-MB-231 and Hela cells mainly expressing CRHR2, Ucn2 partially inhibited EBSS-induced cell migration and it had no effect on migration toward 2-APB treatment, consistent with the effect on autophagy (LC3BII accumulation). These results above indicated that migrated cell number was negatively correlated with LC3BII expression, suggesting that CRHRs-blocked migration was partially due to autophagy inhibition.

Paxillin is a well-known focal adhesion protein and degraded by direct interaction with LC3, which promotes focal adhesion disassemble and cell motility [18]. To investigate whether paxillin is regulated by CRH-mediated autophagy, paxillin expression was detected. After EBSS treatment for 4 h, there was a decrease in total paxillin protein level in both cell lines (Fig. 6B), and CRH/Ucn2 abolished the effect of EBSS. 2-APB or U73122 failed to block CRH-induced paxillin accumulation in MCF-7 cells, which negatively correlated with LC3BII expression. And in MDA-MB-231 cells, paxillin levels were elevated in both 2-APB and 2-APB&Ucn2 treatments. U73122, on the contrary, significantly decreased paxillin expression and Ucn2 strengthened the effect of U73122, suggesting paxillin expression negatively associated with

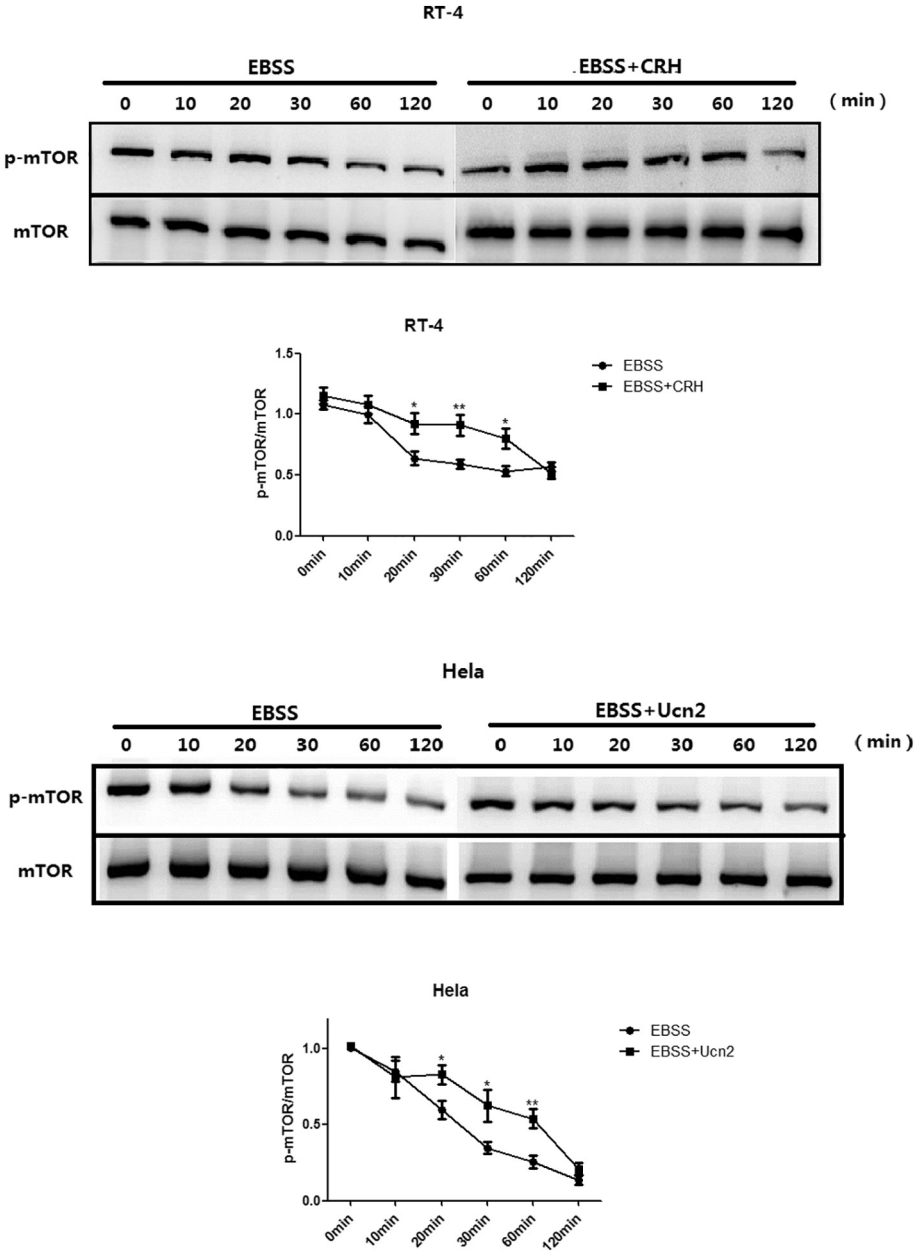

Fig. 3. CRH or Ucn2 phosphorylates mTOR in both RT-4 and Hela cells. Both cells were treated with EBSS for 10, 20, 30, 60 and 120 min in the presence or absence of CRH/Ucn2, p-mTOR levels were detected. Histograms show the mean ± S.E.M. of three independent experiments. *P < .05; **P < .01; ***P < .001; ns, not statistically different.

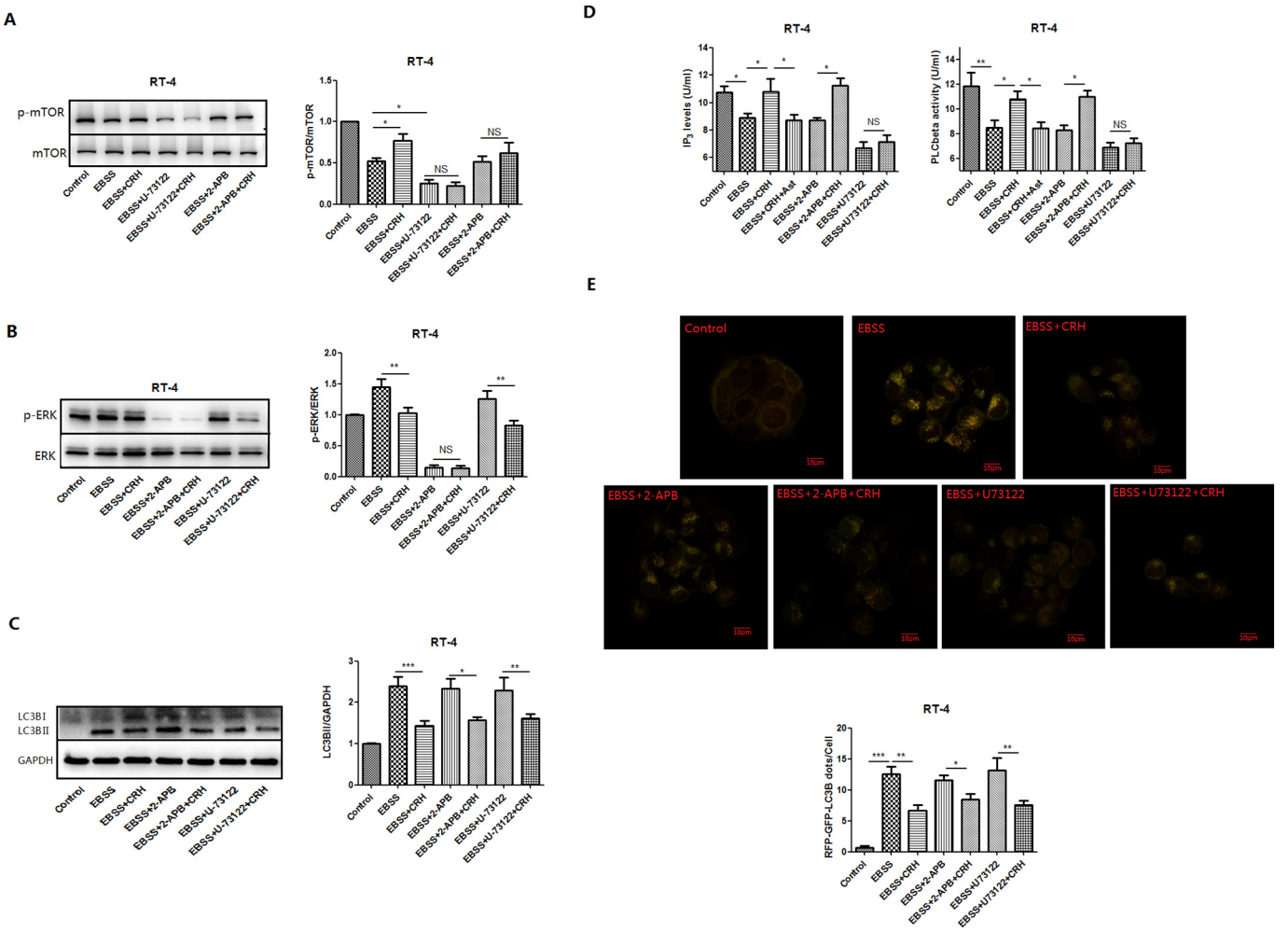

Fig. 4. PLCβ-IP3-mTOR signaling pathway is partly involved in CRH-inhibited autophagy starved by EBSS. Upon EBSS starvation, RT-4 cells were treated with 2-APB (100 μM)/U73122 (5 μM) in the presence or absence of CRH (10–7 M). P-mTOR (A), p-ERK (B), LC3BII (C), IP3 levels (D), PLCβ activity (D) and fluorescent RFP-GFP-LC3B expression (E) were tested. Scale Bars, 10 μm. Histograms show the mean ± S.E.M. of three independent experiments. $*P < .05$; $**P < .01$; $***P < .001$; ns, not statistically different.

LC3BII expression. Together, these results demonstrated that autophagy inhibition by activated CRHRs promoted paxillin accumulation to inhibit cell migration (Fig. 7).

4. Discussion

Data presented in this study provide a new link between CRHRs signaling and the autophagy. We found that activation of the two CRHRs, CRHR1 or CRHR2, inhibits the LC3BII accumulation to restrain autophagosome biogenesis. This inhibition is associated with increased PLCβ activity and subsequent high IP_3 level, which leads to mTOR activation. Furthermore, we demonstrated that activation of CRHRs abolished the effects of EBSS on paxillin reduction and cell migration. These data obtained in RT-4&MCF-7 cells (endogenously expressing CRHR1) and Hela&MDA-MB-231 cells (endogenously expressing CRHR2) suggest that modulation of autophagy by CRHRs may have a profound impact on the invasive properties of cancer cells.

Recent studies demonstrate that nutrients can modulate autophagy in a systemic manner by inducing hormones and neurotransmitters secretion, which regulate G protein coupled receptors (GPCRs) [21,27]. Different GPCRs includes different G protein α subunits such as Gs, Gq/G11, Gi, Go, and gustducin. Activated Gq subunits by amino acid leads to activation of PLCβ and thereby high level of downstream messenger, IP_3, which sufficiently inhibits autophagy through activating mTOR [28–30] while Gs activation increases intracellular cyclic AMP (cAMP) level to activate mitogen activated kinased (MAPKs) ERK1/2, which promotes autophagy [31]. CRH family peptides are major hormones in response to stress and correspondent CRHRs belong to GPCRs. The major G protein-α-subunits of CRHRs include Gs and Gq [32,33]. Thus, the effect of CRHRs on autophagy depends on the dominant signal pathway mediated by Gs or Gq. In the present study, both CRH and Ucn2 were found to inhibit autophagosome biogenesis through repressing conversion of LC3BI to LC3B II. As well known, Gq- PLCβ-IP3-mTOR is a classical autophagy regulating pathway. Therefore, our results highly suggest that CRH- and Ucn2-evoked inhibition of autophagy be modulated by Gq.

PLCβ and IP_3 are known to be downstream regulators of Gq. We used PLCβ and IP_3 receptor inhibitors U73122 and 2-APB separately to observe the activities of mTOR & ERK1/2 and LC3BII expression in RT-4 (expressing CRHR1) and Hela (expressing CRHR2) cells. In RT-4 cells, both U73122 and 2-APB blocked CRH-induced mTOR and CRH-inhibited ERk1/2 activities while neither U73122 nor 2-APB modified CRH-repressed LC3BII expression. This indicates that there must be other 'replenishment' in regulation of autophagy when Gq- PLCβ-IP_3-mTOR/ERk1/2 signal pathway was inhibited. Further work is needed to understand what other signal pathways participate in the autophagy inhibition after CRH treatment. Unlike RT-4 cells, Hela cells had increased autophagic activity induced by Ucn2 due to increased p-ERK1/

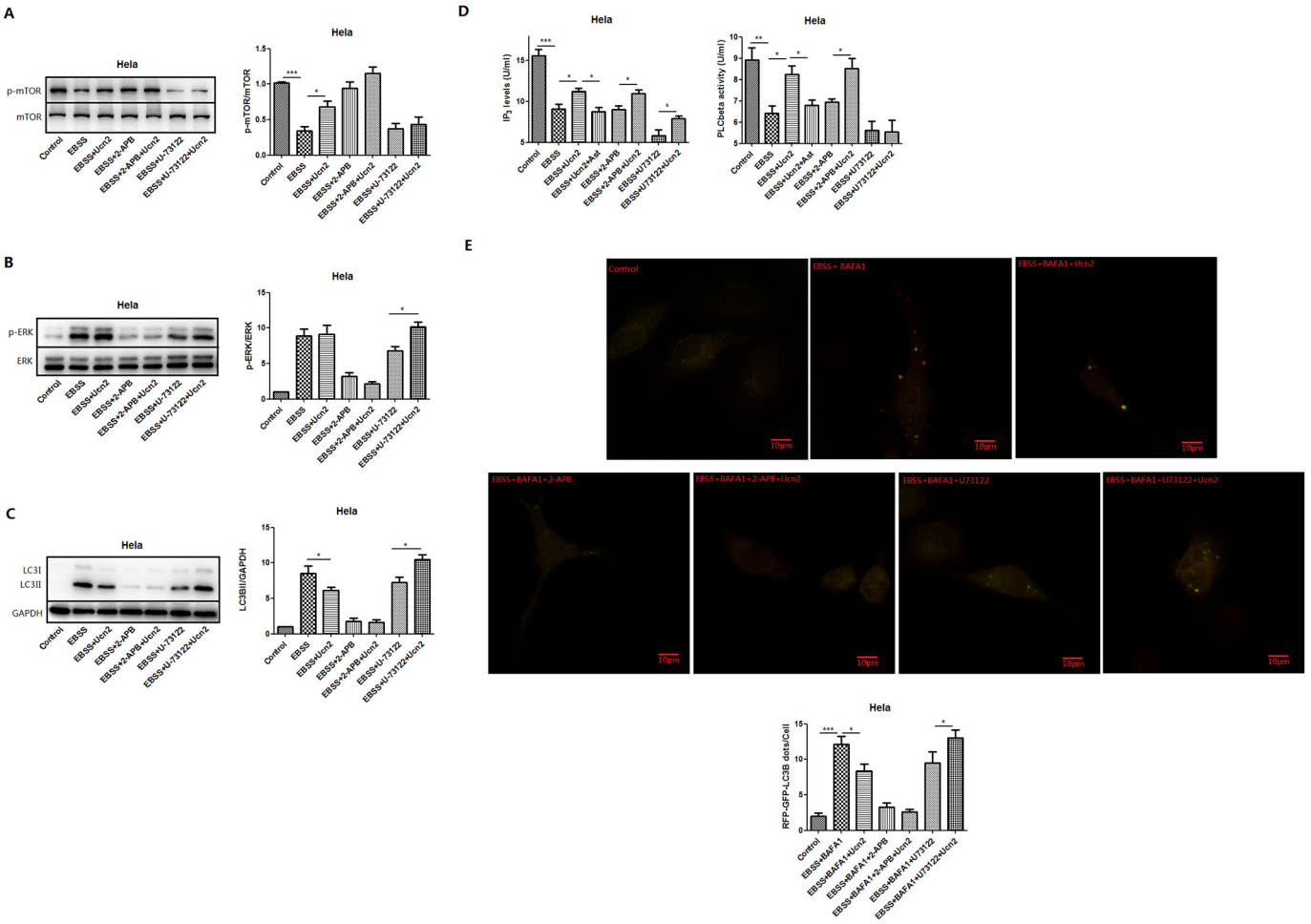

Fig. 5. Ucn2-evoked inhibition of autophagy depends on PLCβ-IP3-mTOR signaling pathway in Hela cells. Hela cells were treated with 2-APB (50 μM)/U73122 (2.5 μM) alone or along with Ucn2 (10–7 M) for 1 h in the presence of EBSS starvation. P-mTOR (A), p-ERK (B), LC3BII (C), IP3 levels (D), PLCβ activity (D) and fluorescent RFP-GFP-LC3B expression (E) were tested. Scale Bars, 10 μm. Histograms show the mean ± S.E.M. of three independent experiments. $*P < .05$; $**P < .01$; $***P < .001$; ns, not statistically different.

2 when PLCβ was blocked. It is worth mentioning that basal level of p-ERK1/2 was absolutely low in the treatment of 2-APB (Fig. 5B), emphasizing the major role of IP_3 in p-ERK1/2 activation. However, ERK1/2-induced autophagy was normally covered by mTOR activated by Ucn2, which showed autophagy inhibition. Since PLCβ is in the upstream of IP_3, we speculated that ERK1/2 is directly activated by IP_3, which is induced by PLCε [34]. These data suggest that CRHRs-inhibited autophagy mainly through PLCβ-IP_3–mTOR signal pathway.

Focal adhesion protein paxillin is a crucial scaffolding and signal integrator, which is reported to bind directly to LC3B to stimulate focal adhesion disassembly and metastasis [18,35]. Our previous studies have shown that CRH or Ucn repressed migration of breast cancer cell lines [14,16]. We therefore investigated whether active CRHRs had effects on autophagy-mediated cell migration and paxillin expression. We found in MCF-7 cells, 2-APB decreased Paxillin expression and CRH abolished this effect. Furthermore, the 2-APB-induced cell migration was inhibited correspondingly by CRH. However, in Hela cells, Ucn2 had no effect on cell migration and Paxillin reduction by 2-APB. There was no obvious significance although Ucn2 strengthened the effect of U73122 on paxillin expression. This was possibly due to low levels of paxillin after U73122 treatment. These data demonstrated that LC3BII expression positively correlated with cell migration and negatively correlated with paxillin expression after CRHRs activation, indicating that CRH- and Ucn2-evoked inhibition of autophagy restrain migration. The difference of Paxillin regulation between CRH and Ucn2 demonstrated again the different mechanism between CRHR1 and CRHR2 in autophagy. It should be noted that autophagy negatively correlated with Paxillin expression and positively with cell migration ability, consistent with the knowledge that LC3B is associated with proliferation, metastasis, and poor outcome [4–6].

The link between CRHRs and autophagy was first suggested on the model of ischaemia/reperfusion in neonatal and adult rat cardiac myocytes [20]. The researcher reported that Urocortin inhibited Beclin1-mediated autophagic cell death. Unfortunately, we could not find any change in Beclin1 protein expression and ATG3, ATG5, ATG7 ATG12 mRNA levels reported in another study [12]. This may be due to complexity for regulation of autophagy in different cell types. Data from Jun Li et al. demonstrated that starvation-induced autophagy induced the expression of EMT markers and invasion through a TGF-β/Smad3 signal-dependent manner [19]. Our previous data showed that activated CRHRs repressed TGF-β/Smad3- EMT [14,16]. EMT is the early phase of tumor metastasis. Thus, we can speculate that CRHRs activation may inhibit autophagy to eliminate TGF-β/Smad3- EMT. Further work is needed to prove it.

5. Conclusion

In summary, we report that, in addition to extracellular nutrients such as amino acids, CRHRs activated by CRH family peptides can significantly inhibit autophagy activity and hence restrain tumor cell

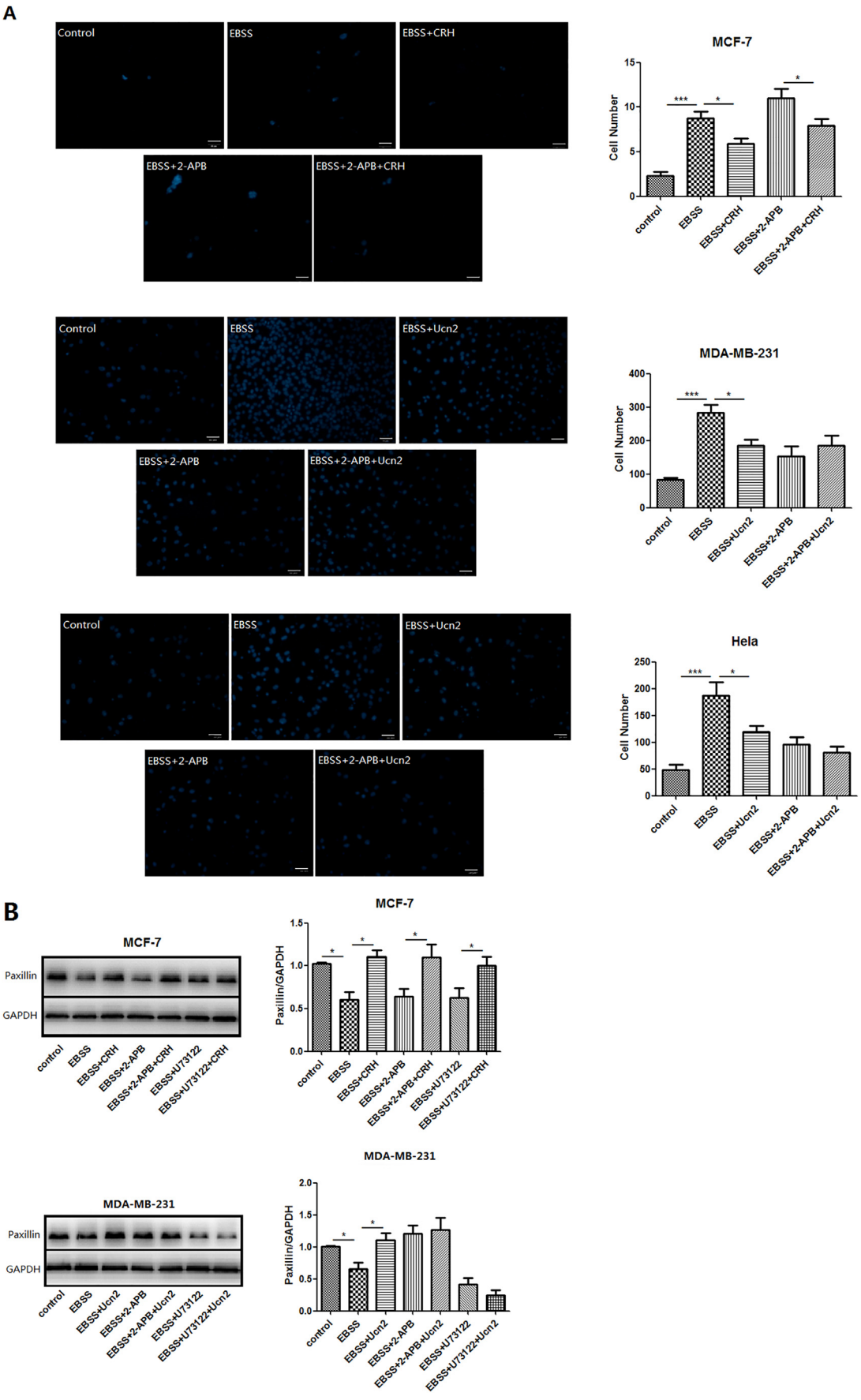

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Fig. 6. CRH- and Ucn2-inhibited migration depend on paxillin expression. (A) MCF-7 cells, MDA-MB-231 cells and Hela cells in EBSS were seeded per 8-μm pore cell culture insert, then treated with Ucn1 or/and 2-APB. Cells that migrated onto the lower surface of the membrane were fixed, stained and the number of cells was counted in ten random visual fields by analysis of Image J 1.47v. Scar bar, 50 μm. Data represent means ± S.E.M. (n = 3). (B) Paxillin expression was detected when MCF-7 and MDA-MB-231 cells were treated with 2-APB/U73122 in the presence or absence of CRH/Ucn2 (10–7 M) upon EBSS starvation. Histograms show the mean ± S.E.M. of three independent experiments. *P < .05; **P < .01; ***P < .001; ns, not statistically different.

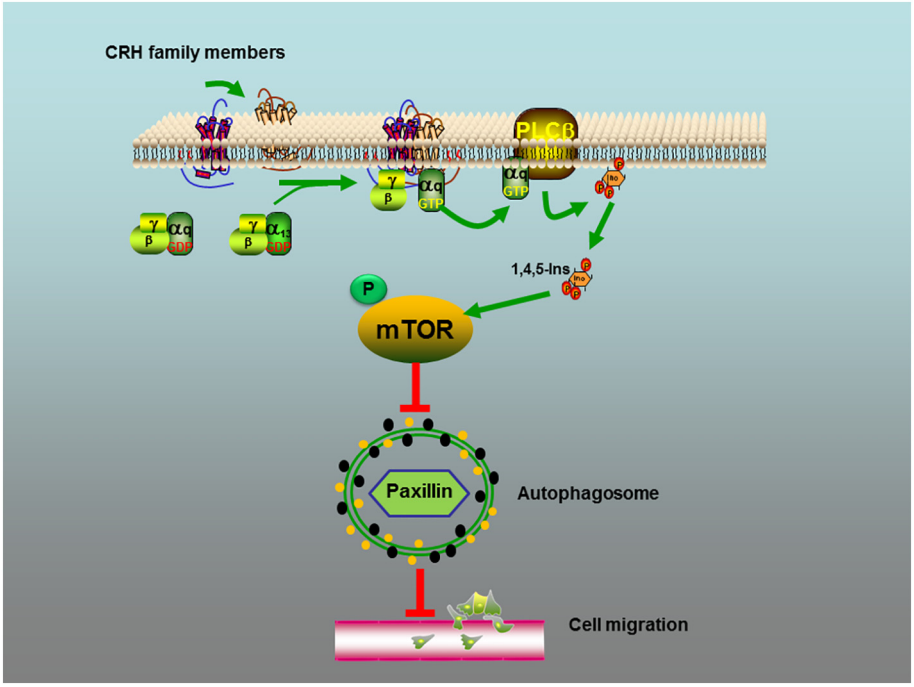

Fig. 7. Schematic diagram of signaling transduction pathway for this study.

migration. Based on the gradual increase of the studies highlighting a key role of the CRHRs in cancer metastasis [13,36–38], our work add a new insight in the influences of CRHRs on cancer development.

5.1. Statistics

The results were expressed as means ± S.E.M. Data was analyzed through GraphPad Prism 5.0 software by One-way ANOVA with Newman-Keuls for 2-group comparison tests. P < .05 was considered to be of statistical significance. Each experiment was repeated for at least three times.

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Declaration of interest

The authors declared that there were no conflicts of interest were disclosed.

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