Long noncoding RNA BCRP3 stimulates VPS34 and autophagy activities to promote protein homeostasis and cell survival

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Abstract

Background: Autophagy plays important roles in cell homeostasis and protein quality control. Long non-coding RNAs (lncRNAs) have been revealed as an emerging class of autophagy regulators, but the majority of them function in regulating the expression of autophagy-related genes. LncRNAs that directly act on the core autophagic proteins remain to be explored.

Methods: Immunofluorescence staining and Western blotting were used to evaluate the function of BCRP3 in autophagy and aggrephagy. RNA immunoprecipitation and in vitro RNA–protein binding assay were used to evaluate the interaction of BCRP3 with its target proteins. Phosphatidylinositol 3-phosphate ELISA assay was used to quantify the enzymatic activity of VPS34 complex. qRT-PCR analysis was used to determine BCRP3 expression under stresses, whereas mass spectrometry and Gene Ontology analyses were employed to evaluate the effect of BCRP3 deficiency on proteome changes.

Results: We identified lncRNA BCRP3 as a positive regulator of autophagy. BCRP3 was mainly localized in the cytoplasm and bound VPS34 complex to increase its enzymatic activity. In response to proteotoxicity induced by proteasome inhibition or oxidative stress, BCRP3 was upregulated to promote aggrephagy, thereby facilitating the clearance of ubiquitinated protein aggregates. Proteomics analysis revealed that BCRP3 deficiency under proteotoxicity resulted in a preferential accumulation of proteins acting in growth inhibition, cell death, apoptosis, and Smad signaling. Accordingly, BCRP3 deficiency in proteotoxic cells compromised cell proliferation and survival, which was mediated in part through the upregulation of TGF-β/Smad2 pathway.

Conclusions: Our study identifies BCRP3 as an RNA activator of the VPS34 complex and a key role of BCRP3-mediated aggrephagy in protein quality control and selective degradation of growth and survival inhibitors to maintain cell fitness.

Keywords: LncRNA, Autophagy, VPS34 complex, Protein quality control, TGF-β signaling, Cell death

Background

The removal of ubiquitinated protein aggregates is crucial for cell homeostasis and its impairment is associated with many pathological conditions, such as aging and neurodegenerative disorders [1, 2]. Although proteasome represents the main route for degradation of poly-ubiquitinated proteins, ubiquitinated protein aggregates...
are often resistant to proteasome degradation and are processed by macroautophagy (thereafter referred to as autophagy) [3, 4]. Autophagy is characterized by the formation of double-membrane vesicle structures in the cytoplasm, called autophagosomes. Cellular components are sequestered in the autophagosomes and subsequently degraded when autophagosomes fuse with lysosomes. The formation of double-membrane autophagosome requires the sequential actions of a number of ATG proteins [5]. Among them, the VPS34 complex, formed by class III phosphatidylinositol-3-kinase (PI3K) VPS34, together with the accessory proteins Beclin 1, VPS15, ATG14, and AMBRA1, is activated by the upstream kinases ULK1/2 and is responsible for generating phosphatidylinositol 3-phosphate (PI3P) in the nascent phagophores to promote the nucleation of autophagosomes [6]. Consistent with its important role in an early step of the autophagosome biogenesis process, this complex serves as a hub to integrate various signals and regulators that impact positively or negatively on the autophagy process [7].

The autophagy process for selective degradation of ubiquitinated substrates is called aggrephagy [8]. Similar to other types of selective autophagy, cargo selection is dependent on a set of cargo receptors the link cargos to the autophagic machinery. The major cargo receptor for aggrephagy is p62/SQSTM1 [9], although NBR1, Tollip, Optineurin, and TAXBP1 also participate in this process [10–13]. Notably, p62 controls both the assembly and degradation of protein aggregates. That is, the binding of polyubiquitinated proteins to the oligomeric p62 triggers a phase separation to concentrate the ubiquitinated proteins into larger condensates, where p62 facilitates the tethering of ubiquitin-positive condensates to the nascent autophagosome membrane through its LC3-interacting region (LIR) [9, 14–18]. The ATG8 family proteins LC3B and GABARAP are the first identified partners of p62. In addition, a recent study revealed the interaction of p62 LIR with FIP200, a subunit of the ULK1/2 complex, and that FIP200 and ATG8 family proteins compete for binding p62 [19]. Thus, the current model of aggrephagy indicates that p62 governs a sequential recruitment of upstream ATG proteins, such as ULK1 complex, VPS34 complex and ATG16L1, followed by a replacement of the ULK1 complex with the ATG8 family proteins for autophagosome expansion. Accordingly, blockage of VPS34 activity impairs aggrephagy to impede the clearance of ubiquitinated protein aggregates [20, 21].

LncRNAs, which comprise the largest part of mammalian transcriptome, have been found to participate in diverse cellular processes to influence on cell fitness. LncRNAs exert cellular effects by forming RNA–DNA, RNA–RNA or RNA–protein complex and this biochemical versatility makes mechanistic analysis rather challenging [22]. Recent studies have indicated lncRNAs as an emerging class of autophagy regulators and lncRNA-mediated regulations occur in various steps of the autophagosome formation and maturation process [23–25]. However, most lncRNAs function in regulating the expression of autophagic genes through mechanisms involving miRNAs [24, 25]. LncRNAs that bind and alter the activity/function of autophagic proteins have been scarcely reported, although lncRNA NBR2 fits this category by binding and activating AMPK for upregulating autophagy activity [26]. Here, we report lncRNA break point region pseudogene 3 (BCRP3) (https://www.ncbi.nlm.nih.gov/gene/?term=BCRP3) as an autophagy-stimulating factor by binding and activating VPS34 complex. The expression of BCRP3 is upregulated in response to proteotoxicity induced by proteasome inhibition and oxidative stress, thus facilitating an enhanced aggrephagy activity. We provide evidence that this mechanism is important in protein quality control and cell survival.

Methods

Plasmids

BCRP3 cDNA was amplified from the genomic DNA of BT474 cells using the primers: Fw: 5′ CCGGAATTC ACTCCGTAGTGCGACTTTG GT and Rv: 5′ACCGGT CGACTTTCGTCGAGAAATTTTTAATG. The cDNA was subcloned to the lentiviral vector pLAS5w.

Pneo. BCRP3 shRNAs were predicted via BLOCK-IT™ RNAi Designer (http://rnai.desi gner.thermosf isher.com/), generated by Purigo Biotechnology, Taipei, Taiwan, and cloned to pLKO.1. The shRNA targeting sequences are as follows: shLuc 5′TTACGCGTAGTACTTTCGA; shBCRP3#1 5′ATATTGGACGCTGCGACC; shBCRP3#2 5′GACCGCTGACCCCAGGCC. Plasmids encoding EGFP-2xFYVE, HA-Beclin 1, and FLAG-Beclin 1 were kindly provided by Guang-Chao Chen (Academia Sinica, Taipei, Taiwan). Plasmids encoding GFP-DFCP1, GFP-LC3, and RFP-p62 were kindly provided by Wei Yuan Yang (Academia Sinica). pcDNA4-VPS34-FLAG was purchased from Addgene, Watertown, MA, USA (#24398). The 4×SBE-Luc construct was obtained from Rik Derynck (University of California at San Francisco).

Cell culture and transfection

HCT116 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS). HeLa cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS, 1% PS, and 1 mM sodium pyruvate. BCRP3-deficient cells were generated by lentiviral transduction of BCRP3 shRNAs. For starvation, cells were incubated with Earle's Balanced Salt
Solution (EBSS). Plasmid transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions.

Lentiviral package and transduction
For lentiviral production, 293FT cells were co-transfected with package plasmid (pCMV8.91), envelop VSV-G plasmid (pMD.G), and BCRP3 cDNA or shRNA expressing plasmid at a weight ratio of 4:1:4. After 48 h of incubation, supernatant containing lentivirus was harvested and filtered through a 0.22 μm syringe filter. For BCRP3 overexpression, HeLa and HCT116 cells were transduced with lentivirus expressing BCRP3 and selected with 0.5 μg/ml G418 geneticin (Invitrogen, Thermo Fisher Scientific).

Antibodies and reagents
Antibodies used in this study were obtained from commercial sources and described in Additional file 1: Table S1. TβRI (type I TGF-β receptor) inhibitor SB431542, 5-fluorouracil (5-FU), and 3-methyladenine (3-MA) were from Sigma-Aldrich. MG132 was from Calbiochem, San Diego, CA, USA. As2O3 was a gift from Hsiu-Ming Shih (Academia Sinica).

Western blotting
Cells were lysed with NP-40 lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% NP-40, 1 mM PMSE, and protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The lysates were clarified by centrifugation at 15,490 × g for 15 min. The protein concentration was determined with Bradford reagent. Lysates with an equal amount of proteins were boiled with sample buffer, resolved by SDS-PAGE, and then transferred to PVDF membrane. After blocked with 5% non-fat milk and probed with the primary antibodies and subsequently with the secondary antibodies, membranes were proceeded for signal development using the Western Lightning Plus-ECL reagent (PerkinElmer, Waltham, MA, USA).

Immunoprecipitation
Cells were lysed with NP-40 lysis buffer or RIPA lysis buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.5, 1% NP-40, 0.1% SDS, 1 mM PMSE, and protease inhibitor cocktail). After centrifugation at 15,490 × g, the supernatant was pre-cleared with protein A beads for 1 h, followed by incubation with the desired antibodies for 1.5 h. The beads were washed three times, denatured with sample buffer, and proceeded for Western blotting analysis. For FLAG or GFP immunoprecipitation, anti-FLAG agarose beads (M2; Merck Millipore, Billerica, MA, USA) or GFP-Trap agarose beads (ChromoTek, Hauppauge, NY, USA) were used.

Immunofluorescence
Cells grown on coverslips in a 6-well plate were fixed with 4% paraformaldehyde at room temperature for 15 min, followed by permeabilization with chilled methanol on ice for 10 min. Cells were then blocked with 1% BSA and 10% goat serum in PBS for 1 h, followed by incubation with primary antibodies at 4°C for overnight. Next, fluorescence-tagged secondary antibodies were added and incubated at room temperature for 1 h together with DAPI for nuclear staining. Samples were mounted on glass slides using fluorescence mounting medium (DAKO, Carpinteria, CA, USA) and examined under an Olympus FV3000 confocal microscope (Olympus Co., Tokyo, Japan) with 60x/1.40 oil objective lens. For images analysis and cell counting, the ImageJ software (https://imagej.nih.gov/ij/download.html) was used.

Quantitative real-time PCR (qRT-PCR)
Total RNA was isolated from cells with Trizol reagent (Invitrogen, Thermo Fisher Scientific). 1 μg of RNA per sample was subjected to reverse transcription using the iScript cDNA Synthesis kit (Bio-Red, Richmond, CA, USA) according to the manufacturer’s protocol. qPCR was performed with the FastStart Universal SYBR Green Master reagent (Roche) on a LightCycler® 480 instrument II system (Roche). GAPDH was used as a reference gene. The sequences of PCR primers are listed in Additional file 1: Table S2.

Subcellular fractionation assay
1 × 10⁷ cells were lysed in 10 ml cytoplasmic lysis buffer (0.256 M sucrose, 8 mM Tris–HCl pH 7.5, 4 mM MgCl₂, 0.8% Triton X-100, and 0.25% PBS) with gentle pipetting and inverting, followed by rotation at 4°C for 10 min and incubation on ice for another 10 min. After centrifugation at 2,500 × g for 15 min, 250 μl (1/40 volume) of supernatant was transferred to a new tube and saved as the cytoplasmic fraction. The pellet was washed with 10 ml ice cold PBS twice and then lysed with 1 ml RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 0.5 mM dithiothreitol, 1:100 Supernatinal (Invitrogen, Thermo Fisher Scientific), and protease inhibitor cocktail). The lysate was sonicated using Qsonica (30 s ON, 30 s OFF for 15 min) and centrifuged at 13,000 rpm for 10 min. The supernatant was collected.
as the nuclear fraction. An equal portion (1/40 volume) of the cytoplasmic and nuclear fractions was subjected to RNA extraction with 1 ml Trizol reagent.

RNA fluorescence in situ hybridization (FISH)
RNA FISH was carried out using the BaseScope Assay kit (Advanced Cell Diagnostics, Newark, CA, USA) according to the manufacturer’s protocol. Briefly, HeLa cells cultured on slides were fixed with 4% paraformaldehyde at room temperature for 15 min, treated with hydrogen peroxide at room temperature for 10 min, and then digested with Protease III (1:15 dilution) at room temperature for 10 min. BCRP3-specific probes were custom produced by Advanced Cell Diagnostics (Cat#1055141-C1) and incubated with cells at 40°C for 2 h in a HybEZ™ Oven (Advanced Cell Diagnostics), followed by multistep signal amplification as instructed in the protocol. Finally, cells were mounted and imaged using an Olympus FV3000 confocal microscope with 60x/1.40 oil objective lens.

RNA immunoprecipitation
RNA immunoprecipitation was followed by a protocol described previously [27]. Briefly, cells were lysed with RIP buffer, kept on ice for 1 h, and then centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was pre-cleared with protein A beads for 1 h at 4 °C and then incubated with various antibodies for 2 h at 4 °C. The RNA-associated immunocomplexes were then captured by protein A beads for 1 h at 4°C. After washes, the beads were resuspended in 100 μl lysis buffer. 10 μl (1/10 volume) of resuspended mixture was saved for Western blotting analysis and the remaining 90 μl (9/10 volume) were proceeded for RNA extraction with Trizol reagent, followed by qRT-PCR analysis.

In vitro RNA–protein binding assay
The full-length BCRP3 cDNA was cloned to pcDNA3.1 in a sense direction. Biotin-labeled BCRP3 was synthesized by in vitro transcription using RNA Labeling Mix (Roche) and T7 RNA polymerase (Ambion, Austin, TX, USA), and purified by NucleoSpin® RNA Isolation kit (Macherey–Nagel, Bethlehem, PA, USA). Biotinylated BCRP3 was folded in RNA structure buffer (10 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM MnCl₂, and 50 μM ATP), 9 μl of 500 μM PI substrate, and 20 μl ddH₂O were added to the immunocomplex, together with the in vitro transcribed BCRP3 or control RNA. After incubation at 30°C for 30 min, the reaction was terminated by adding 12 μl 100 mM EDTA. The amount of PI3P produced was determined by a competitive ELISA assay. The quenched reaction was diluted and added to the PI3P-coated plate for competitive binding to the PI3P detector. The amount of PI3P was detected by reading the colorimetric changes at the absorbance of 450 nm. The concentration of PI3P was calculated as inversely proportional to the ELISA signal, and normalized to the amount of immunoprecipitated FLAG-VPS34.

Luciferase assay
Luciferase assay was conducted with the Dual-Glo Luciferase Reporter Assay System (Promega, Madison, WI, USA) followed by the manufacturer’s instructions. The Firefly luciferase activity was normalized to that of Renilla luciferase activity.

Apoptosis assay
Apoptotic cell death was measured using the Cell Death Detection ELISA plus kit (Roche) according to the manufacturer’s instructions. Briefly, 3 × 10⁵ cells were plated on 6 cm dishes for various treatments and were lysed using 250 μl lysis buffer. After centrifugation, 20 μl of supernatant was added onto the streptavidin-coated plate together with 80 μl Immunoreagent (biotin-conjugated anti-histone antibody and peroxidase-conjugated anti-DNA antibody) and the mixture was incubated for 2 h at 25 °C. Then, 100 μl of ABTS substrate solution was added to each well and the colorimetric changes were monitored at the absorbance of 405 nm.

BrdU incorporation and MTT assays
For assaying BrdU incorporation, cells were seeded on 96-well plates at a density of 2000 cells/well, cultured overnight, and treated with various inhibitors. Then, cells were pulse labeled with 10 μM BrdU for 24 h. After fixation, BrdU incorporation was determined by the BrdU Cell
Proliferation Assay kit (Merck Millipore) according to the manufacturer’s instructions. For MTT assay, cells seeded on 96-well plate at a density of $1 \times 10^4$ cells per well were treated with various agents, followed by the addition of MTT solution (5 mg/ml). Next, the medium was removed and cells were lysed by DMSO. Cell viability was determined by absorbance measurement at 570 nm.

**Sample preparation for liquid chromatography-tandem mass spectrometry (LC–MS/MS)**

$1 \times 10^7$ HeLa cells were lysed in denaturing buffer (8 M urea and 20 mM HEPES pH 8.0), sonicated, and centrifuged at 13,000 rpm for 15 min at 25°C. An aliquot of 5 μg protein was reduced with 10 mM dithioerythritol at 37 °C for 1 h, and then alkylated with 25 mM iodoacetamide in the dark for 1 h. The protein sample was digested with Lys-C/trypsin for 19 h and terminated with formic acid in the dark for 1 h. The protein sample was desalted by Zip-Tip and lyophilized prior to LC–MS/MS analysis.

**Shotgun proteomic identifications**

NanoLC–nanoESi-MS/MS analysis was performed on a Thermo UltiMate 3000 RSLCnano system connected to a Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanospray interface (New Objective, Woburn, MA, USA) and followed procedures as described previously [28]. Peptide mixtures were loaded onto a 75 μm ID, 25 cm length PepMap C18 column (Thermo Fisher Scientific) packed with 2 μm particles with a pore width of 100 Å and were separated using a segmented gradient in 120 min from 5 to 35% solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nl/min. Solvent A was 0.1% formic acid in water. The mass spectrometer was operated in the data-dependent mode. Briefly, survey scans of peptide precursors from 350 to 1600 m/z were performed at 240 K resolution with a $2 \times 10^5$ ion count target. Tandem MS was performed by isolation window at 1.6 Da with the quadrupole, higher-energy collisional dissociation fragmentation with normal-ized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS$^2$ ion count target was set to $1 \times 10^4$ and the max injection time was 50 ms. Only those precursors with charge state 2–6 were sampled for MS$^2$. The instrument was run in top speed mode with 3 s cycles; the dynamic exclusion duration was set to 15 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on.

Peptide identification was performed using the percolator node within Proteome Discoverer (v 2.4.1.15; Thermo Scientific, Waltham, MA, USA) against the Swiss-Prot Human database (561,911 entries total). Search criteria used were trypsin digestion, variable modifications set as carbamidomethyl (C), oxidation (M), ubiquitinylation (K) allowing up to two missed cleavages, mass accuracy of 10 ppm for the parent ion and 0.02 Da for the fragment ions. The false discovery rate (FDR) was set to 1% for peptide identifications. For label-free quantification, precursor ions intensities were extracted using Minora Feature Detector node in Proteome Discoverer with a 2 ppm mass precision and 2 min retention time shift.

**Bioinformatics**

For in silico analysis of *BCRP3* expression, RNA-seq data of different types of normal and cancer tissues were downloaded from The Cancer Genome Atlas (TCGA) or Genotype-Tissue expression (GTEx) database via the UCSC Xena platform (http://xena.ucsc.edu) [29]. Gene Ontology (GO) analysis was carried out by DAVID (https://david.ncifcrf.gov/), and filtered by $P$ value<0.05.

**Statistics**

The unpaired two sided Student’s t-test was used to compare between two groups and one-way or two-way ANOVA with Tukey’s post hoc test was used for multi-group comparisons.

**Results**

*BCRP3* promotes autophagy

By analyzing TCGA data sets, we identified lncRNA *BCRP3* based on its lower expression in tumor than normal tissues in many cancer types, including colon, esophagus, brain, skin, stomach, testis, kidney, lung, ovary, prostate, and liver (Fig. 1A). The *BCRP3* gene is located on the chromosome 22q11.23 and is overlapping in an antisense orientation with the gene of lncRNA *POM121L10P* (Fig. 1B). RNA-seq data retrieved from the GTEx database revealed the expression of *BCRP3* in many cell types and tissues (Fig. 1C and Additional file 1: Fig. S1). However, Ribo-seq data indicated the devoid of ribosome binding to *BCRP3*, which was in a sharp contrast to the RNA of its neighboring coding gene *GGT1* (Fig. 1D). Accordingly,
Fig. 1 (See legend on previous page.)
BCRP3 is denoted by National Center for Biotechnology Information (NCBI) GenBank as a pseudogene which gives rise to a lncRNA of 1.4 kb. The down-regulation of BCRP3 in many cancer types suggested its role in tumor suppression. We therefore tested the functions of BCRP3 in cancer. While overexpression of BCRP3 in HCT116 human colorectal cancer cell line did not affect cell viability at the basal state, it significantly increased cell viability upon treatment with a chemotherapeutic drug 5-FU (Additional file 1: Fig. S2A). Monitoring DNA damage by γH2A.X showed no difference between control and BCRP3-overexpressed cells at each time point after 5-FU treatment, suggesting that BCRP3 does not affect DNA damage sensing/repair (Additional file 1: Fig. S2B). Notably, autophagy represents one mechanism that leads to the resistance of cancer cells to chemotherapy, but in contrast plays a suppressive role for tumor initiation [30, 31]. The latter is in line with the lower expression of BCRP3 in tumor than normal tissues. Furthermore, we found that autophagy blockage by 3-MA or bafilomycin A1 diminished the chemoresistance effect of BCRP3 (Additional file 1: Fig. S2C). We thus explored the role of BCRP3 in autophagy. Remarkably, overexpression of BCRP3 in HCT116 cells increased the number of autophagosomes (detected by LC3 puncta) in cells cultured in MEM (for measuring basal autophagy activity) or EBSS (for monitoring starvation-induced autophagy activity) (Additional file 1: Fig. S3A). A similar finding was observed in HeLa cells overexpressing BCRP3 (Fig. 2A).

Importantly, the increased autophagosome number by BCRP3 overexpression was also evident in cells treated with bafilomycin A1 to block autophagic turnover (Fig. 2A), indicating that BCRP3 promotes autophagosome formation rather than inhibiting autophagosome fusion with lysosome for degradation. Western blot analysis showed that BCRP3 overexpression increased LC3 lipidation (designated as LC3-II) and decreased the abundance of p62, an autophagic cargo (Fig. 2B), further supporting an enhancement of autophagy activity. In the reciprocal set of experiments, we depleted BCRP3 expression in HeLa or 293T cells by shRNAs. Importantly, BCRP3 knockdown decreased autophagosome numbers and LC3 lipidation in cells cultured in MEM, EBSS, MEM with bafilomycin A1, or EBSS with bafilomycin A1 (Fig. 2C, D and Additional file 1: Fig. S3B, C). These findings collectively identify a role of BCRP3 in promoting autophagosome biogenesis.

**BCRP3 acts downstream of ULK1 and upstream of PI3P production**

Next, we investigated the functional position of BCRP3 in the autophagosome biogenesis process. ATG16L1 and WIPI2 are two proteins recruited to the PI3P-containing phagophore, a nascent autophagosomal structure, to facilitate LC3 lipidation [32, 33]. Notably, BCRP3 knockdown decreased the formation of ATG16L1 and WIPI2 puncta in EBSS-cultured cells (Fig. 3A and Additional file 1: Fig. S4). Furthermore, BCRP3 knockdown in EBSS-cultured cells diminished GFP-DFCP1 puncta (also known as omegasomes, PI3P-enriched compartments serving as the precursors of autophagosomes) [34] (Fig. 3B). In the reciprocal set of experiments, BCRP3 overexpression in EBSS-cultured cells increased ATG16L1 puncta and GFP-DFCP1 puncta (Fig. 3C, D). However, BCRP3 knockdown did not affect the kinase activities of TORC1, AMPK, and ULK1 in EBSS-cultured cells, as monitored by ULK1 S757 phosphorylation, ULK1 S317 phosphorylation, and ATG13 S318 phosphorylation, respectively [35, 36] (Fig. 3E). Likewise, BCRP3 overexpression did not alter AMPK and ULK1 activities in cells cultured in EBSS (Fig. 3F). Since TORC1, AMPK, and ULK1 act upstream of VPS34 complex in the autophagosome biogenesis process, our data suggest an impact of BCRP3 on the VPS34 complex.

**BCRP3 binds VPS34 complex to increase its enzymatic activity**

To determine the mechanism by which BCRP3 regulates the function of VPS34 complex, we first interrogated the subcellular localization of BCRP3. Cell fractionation followed by qRT-PCR analysis found that BCRP3 is mainly present in the cytoplasm (Fig. 4A). This finding was further confirmed by RNA FISH analysis with a BCRP3 antisense probe (Fig. 4B). Thus, we examined whether BCRP3 acts directly on the cytoplasmic-residing VPS34 complex. Western blot analysis showed that BCRP3 knockdown did not alter the abundance of VPS34 complex subunits, including VPS34, Beclin 1, VPS15, ATG14, and AMBRA1 (Additional file 1: Fig. S5A). Furthermore,
Fig. 2 (See legend on previous page.)
immunoprecipitation analyses revealed that BCRP3 knockdown did not compromise the integrity of VPS34 complex as well as the oligomerization of Beclin 1 (Additional file 1: Fig. S5A, B), the latter of which is crucial for the assembly of VPS34 complex [37]. We isolated VPS34 complex from cells transfected with GFP-ATG14 using GFP-Trap. Remarkably, this complex was readily associated with in vitro transcribed BCRP3 (Fig. 4C), suggesting a direct binding between BCRP3 and VPS34 complex. Furthermore, RNA immunoprecipitation analysis demonstrated the association of BCRP3 with each of the VPS34 complex component in vivo (Fig. 4D–H). Thus, these data identify BCRP3 as a binding partner of the VPS34 complex.

We next determined whether BCRP3 could modulate the catalytic activity of VPS34 complex. Using the GFP-2xFYVE probe [38], we found that BCRP3 knockdown in EBSS-cultured cells decreased the cellular level of PI3P, the product of VPS34 complex (Fig. 5A). Furthermore, VPS34 complex isolated from control cells showed a higher enzymatic activity in the in vitro kinase assay, compared to that isolated from BCRP3 knockdown cells (Fig. 5B). To demonstrate a direct role of BCRP3 in stimulating the enzymatic activity of VPS34 complex, we isolated VPS34 complex from FLAG-VPS34 transfected cells and incubated this complex with in vitro transcribed BCRP3. Addition of BCRP3 significantly enhanced the enzymatic activity of VPS34 complex, whereas a control RNA failed to do so (Fig. 5C). These data provide compelling evidence for the stimulation of VPS34 enzymatic activity by BCRP3.

**BCRP3 is upregulated by certain proteotoxic insults to contribute to aggrephagy-induction**

Having identified the function and mechanism of BCRP3 in promoting autophagy, we next investigated whether BCRP3 expression could be induced under certain autophagy-stimulating conditions. Our analysis found that BCRP3 expression was upregulated in a time-dependent manner by treatment of cells with proteasome inhibitor MG132 or oxidative stressor As2O3 (Fig. 6A, B). As2O3 is known to induce the formation of cytoplasmic protein aggregate called aggresome and aggresome-like induced structure (ALIS), respectively, and aggrephagy represent one mechanism for removing these aggregates [21, 39]. Thus, we investigated the effect of BCRP3 on aggrephagy. Remarkably, BCRP3 knockdown decreased aggrephagy in MG132 and As2O3-treated cells, as monitored by p62/ LC3 double positive puncta (Fig. 6C, D). This was accompanied by increased accumulations of aggresome or ALIS (marked by Ub/p62 double positive puncta) and the total cellular ubiquitinated proteins (Fig. 6E–H). BCRP3 knockdown in basal conditions, however, did not obviously increased the total cellular ubiquitinated proteins (Additional file 1, Fig. S6B). Thus, our study reveals the induction of BCRP3 by proteotoxic stress to stimulate aggrephagy activity for protein quality control.

**Accumulation of growth/survival inhibitors and induction of TGF-β pathway by BCRP3 deficiency in proteotoxicity**

Next, we sought to understand the impact of BCRP3-mediated aggrephagy on proteome landscape. We performed label-free quantitative LC–MS/MS analysis to detect the protein expression profiles in control and BCRP3 knockdown cells treated with or without MG132. Proteins preferentially accumulated in MG132-treated BCRP3 knockdown cells compared with MG132-treated control cells were defined with the following criteria: protein level [BCRP3 knockdown/control] > 1.5, P < 0.05, and a total of 189 proteins were recovered. Among them, 55 were also accumulated in BCRP3 knockdown cells under unstressed conditions and therefore were excluded (Fig. 7A). GO analysis of the remaining 134 proteins (listed in Additional file 1: Table S3) revealed “positive regulation of apoptotic process”, “negative regulation of cell proliferation”, “cell death”, “co-Smad protein phosphorylation” and “release of cytochrome c from mitochondria” as the enriched GO terms (Fig. 7B). Among these GO terms, the enrichment of co-Smad protein phosphorylation is intriguing and both TGF-β1 and Smad2,
Fig. 3 (See legend on previous page.)
**Fig. 4** BCRP3 is mainly distributed to the cytoplasm and binds VPS34 complex. A Subcellular localization of BCRP3 in HeLa cells detected by cell fractionation followed by qRT-PCR analysis. GAPDH and Neat1 were used as cytoplasmic and nuclear RNA controls, respectively. B RNA FISH analysis of BCRP3 (red) in HeLa cells. Nuclei were stained with DAPI (blue). Bar, 10 μm. C In vitro RNA protein binding assay. GFP-ATG14 transiently expressed in 293T cells was immunoprecipitated with GFP-Trap beads and the immunocomplexes were incubated with in vitro transcribed BCRP3. The bound RNAs were extracted and analyzed by qRT-PCR, whereas the presence of VPS34 complex components in the immunocomplexes was analyzed by Western blot (left). Data are means ± SD from three independent experiments. P values are determined by unpaired t-test, ***P < 0.001. D-H RNA immunoprecipitation assay using indicated antibodies followed by qRT-PCR analysis. Data were normalized to the IgG control and expressed as means ± SD, n = 3. P values are determined by unpaired t-test, *P < 0.05, **P < 0.01, ***P < 0.001

(See figure on next page.)

**Fig. 5** BCRP3 stimulates the activity of VPS34 complex. A Confocal microscopy analysis of control or BCRP3-deficient HeLa cells, transiently transfected with GFP-2xFYVE and starved in EBSS for 1 h. Representative images are shown on the left and quantitative data are on the right. Bar, 10 μm. Data are means ± SD from three independent experiments and 7 cells per group per experiment were counted. P values are determined by one-way ANOVA with Tukey's post hoc test, *P < 0.05. B FLAG-VPS34 was immunoprecipitated from control or BCRP3-deficient HeLa cells. The immunocomplexes were analyzed by Western blot (middle) or incubated with phosphatidylinositol (PI) substrate and ATP (left). PI3P production was measured by ELISA assay and normalized to FLAG-VPS34 protein levels (right). C FLAG-VPS34 immunoprecipitated from transfected HeLa cells was incubated with PI substrate and ATP, together with in vitro transcribed BCRP3 or lambda RNA (left). PI3P production was measured by ELISA and normalized to FLAG-VPS34 protein levels (right). Data in (B) and (C) are means ± SD from three independent experiments. P values are determined by one-way ANOVA with Tukey's post hoc test, *P < 0.05, **P < 0.01, ***P < 0.001
Fig. 5 (See legend on previous page.)
a downstream effector of TGF-β signaling, appeared in this category (Fig. 7B). Of note, TGF-β pathway elicits potent cytostatic effects including growth arrest and apoptosis under certain cellular contexts [40]. We therefore evaluated the impact of BCRP3 on TGF-β signaling in more details. Consistent with the data from proteomics analysis, BCRP3 knockdown in MG132-treated cells increased Smad2 and p-Smad2 levels and these effects were abrogated by blocking the lysosome degradation with bafilomycin A1 (Fig. 7C), thus supporting a role of BCRP3-mediated aggrephagy in the selective degradation of Smad2. Furthermore, BCRP3 knockdown in MG132-treated cells, but not control cells, increased the activity of a Smad-responsive reporter 4xSBE-Luc and the mRNA levels of DAPK1, p15INK4B (also known as CDKN2B) and p21Cip1 (also known as CDKN1A) (Fig. 7D, E), which are the cytostatic effectors of TGF-β/Smad2 pathway [41–43]. These findings identify the function of BCRP3 in selective downregulation of TGF-β signaling, cell death-promoting factors and proliferation inhibitors during proteotoxicity-induced aggrephagy.

**BCRP3 prevents proteotoxicity-induced growth arrest and apoptosis partly through downregulating TGF-β signaling**

The identification of BCRP3 functions in aggrephagy and proteome landscape alteration prompted us to investigate its impact on cell proliferation and survival under proteotoxicity. Through BrdU incorporation and MTT assays, we found that BCRP3 knockdown decreased proliferation and viability, respectively, in MG132-treated cells but not control cells (Fig. 8A, B). BCRP3 knockdown also enhanced apoptosis, PARP cleavage and caspase 3 cleavage in MG132-treated cells but not untreated cells (Fig. 8C, D). Remarkably, the effects of BCRP3 knockdown on cell proliferation, viability and apoptosis were all reversed partially by blocking TGF-β signaling with the TβRI inhibitor SB431542 (Fig. 8A-C). Furthermore, enforced activation of TGF-β pathway in MG132-treated cells enhanced apoptosis (Fig. 8E). Together, our study uncovers a role of BCRP3 in maintaining cell proliferation and viability and preventing apoptosis during proteotoxicity, which are mediated in part through downregulating TGF-β signaling.

**Discussion**

Transcriptional, post-transcriptional and posttranslational regulations are the three major mechanisms governing many cellular processes, including autophagy. Besides these commonly employed regulatory mechanisms, a novel “riboregulation” concept has recently emerged, which is referred to as a regulatory mechanism with the following features [44]. First, the direct interaction between a regulatory RNA and its target protein can lead to a functional change of the target protein. Furthermore, the level or activity of the regulatory RNA and/or target protein can be altered by a biological cue. In this study, we identify IncRNA *BCRP3* as a positive regulator of autophagy by binding and activating the VPS34 complex, a core component in both bulk and selective autophagy processes. Furthermore, *BCRP3* is upregulated under certain proteotoxic conditions to enhance aggrephagy activity for the clearance of ubiquitinated protein aggregates. These properties of *BCRP3* indicate its role as a novel “riboregulator” of autophagy. To date, only couple riboregulatory mechanisms of autophagy have been identified, including IncRNA *NBR2*-mediated AMPK activation in controlling energy stress-induced autophagy [26] and small ncRNA *Vault RNA1-1*-modulated p62 oligomerization in suppressing aggrephagy [45]. Notably, a recent high-throughput screen identified 63 IncRNAs that affect autophagosome numbers in basal or Torin 1-treated conditions [46], supporting a profound role of IncRNAs in autophagy regulation. However, whether any of these IncRNAs fulfills the riboregulation characters remains to be studied.

We show that BCRP3 functions as a RNA activator of the VPS34 complex. Importantly, BCRP3 affects neither the cellular abundance nor the integrity of VPS34 complex. Furthermore, BCRP3 is able to stimulate the
Fig. 6 (See legend on previous page.)
**Fig. 7** BCRP3 deficiency in proteotoxicity leads to the accumulation of proteins involving in growth inhibition, cell death, and TGF-β/Smad2 signaling. **A** Venn diagram showing the numbers of enriched proteins after BCRP3 knockdown together with or without 10 µM MG132 treatment for 12 h. **B** GO enrichment analysis of the 134 proteins shown in (A). Selective enriched GO terms are shown by the order of fold enrichment (bottom to top). **C** Western blot analysis of indicated proteins in control or BCRP3-deficient HeLa cells treated with 10 µM MG132 together for 12 h together with or without 200 nM bafilomycin A1 for 2 h. **D** Control or BCRP3-deficient HeLa cells were transfected with 4 x SBE-Luc reporter construct, treated with 10 µM MG132 for 12 h and analyzed for luciferase activity. **E** qRT-PCR analysis of relative DAPK1, p15, and p21 levels in control or BCRP3-deficient HeLa cells treated with 10 µM MG132 for 12 h. Data in (D), (E) are means ± SD from three independent experiments. *P values are determined by one-way ANOVA with Tukey's post hoc test, *P < 0.05, ***P < 0.001.
activity of purified VPS34 complex in vitro, without the need of a membrane environment. These findings suggest that BCRP3 binding to the VPS34 complex results in a conformational change to elevate its enzymatic activity, even though we cannot rule out the possibility for an additional role of BCRP3 in promoting the membrane targeting of VPS34 complex. Of note, numerous post-translational mechanisms have been identified to regulate the activity of VPS34 complex [7, 47], highlighting its high susceptibility to conformational change-induced regulation. In addition, the activity of VPS34 can also be modulated by various binding partners. For instance, recent structural analyses revealed that the tight association of VPS15 with VPS34 restrains the activation loop of VPS34 to inhibit its catalytic activity [48], and the recruitment of NRBF2 to VPS34 complex facilitates an allosteric unleashing of the VPS34 kinase domain to drive VPS34 activation.

**Fig. 8** BCRP3 promotes proliferation and survival in proteotoxic cells. A BrdU incorporation assay for control or BCRP3-deficient HeLa cells treated with 10 µM MG132, 4 µM SB431542 and incubated with BrdU for 24 h. B, C MTT (B), and apoptosis (C) assays for control or BCRP3-deficient HeLa cells treated with 10 µM MG132 for 12 h followed by 4 µM SB431542 for 2 h. Data in (A), (B), and (C) are means ± SD from three independent experiments. P values are determined by two-way ANOVA with Tukey’s post hoc test, **P < 0.01, ***P < 0.001. D, E Western blot analysis of indicated proteins in HeLa cells treated with or without 10 µM MG132 for 16 h, followed by 5 ng/ml TGF-β for 2 h.
mediated aggrephagy in the selective removal of a set of proteins, including proteins with anti-proliferation/cell death functions or toxic protein aggregates, likely contribute to the cell growth and survival defects.

BCRP3 was identified based on its downregulation in many types of tumor tissues, compared with the normal tissues. However, whether BCRP3 plays a suppressive role in tumor initiation through its autophagy-enhancing function remains unclear. Studying the tumor-initiation function often requires the utilization of genetically modified mouse models, but the lack of murine homologue of BCRP3 precludes such study. Although the function of BCRP3 in cancer remains elusive, our finding for the upregulation of BCRP3 in response to certain proteotoxic stresses and its effect on aggrephagy induction for maintaining proteostasis and cell viability imply an impact of BCRP3 on preventing pathological states associated with the accumulation of protein aggregates. These proteinopathies represent a large group of disease states, including various neurodegenerative diseases, and evidence has emerged that autophagy plays protective roles against these disease states [56, 57]. Future study will aim to interrogate the function and expression of BCRP3 in these human pathological states.

**Conclusions**

We identified IncRNA BCRP3 as a riboactivator of autophagy through its binding and stimulating the catalytic activity of VPS34 complex. In response to certain proteotoxic stresses, BCRP3 is upregulated to enhance aggrephagy activity. BCRP3 deficiency under proteotoxicity not only compromises protein quality control but leads to the accumulation of anti-proliferation and cell death proteins/signaling molecules to suppress cell proliferation and survival.

**Abbreviations**

3-MA: 3-Methyladenine; 5-FU: 5-Fluorouracil; ALIS: Aggresome-like induced structure; BCRP3: Break point region pseudogene 3; EBSS: Earle’s Balanced Salt Solution; FBS: Fetal bovine serum; FDR: False discovery rate; FISH: Fluorescence in situ hybridization; GO: Gene ontology; LC–MS/MS: Liquid chromatography-tandem mass spectrometry; LIR: LC3-interacting region; IncRNAs: Long non-coding RNAs; MEM: Minimum essential medium; NCBI: National Center for Biotechnology Information; PI3K: Phosphatidylinositol-3-kinase; PI3P: Phosphatidylinositol-3-phosphate; PS: Penicillin/streptomycin; qRT-PCR: Quantitative real-time PCR; RPMI: Roswell Park Memorial Institute; TβRI: Type I TGF-β receptor; TCGA: The Cancer Genome Atlas.

**Supplementary Information**

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Additional file 1: Fig. S1. BCRP3 expression in various human tissues. DNA-seq data from indicated tissues were retrieved from GTEx database. Fig. S2. BCRP3 desensitizes cancer cells to chemotherapeutic agent without affecting DNA damage sensing/repair. A HCT116 cells stably expressing vector or BCRP were treated with or without 5 μM 5-FU for 48 h, and cell viability was determined by MTT assay. The expression levels of BCRP3 were analyzed by qRT-PCR and shown on the right. Data are means.
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Author contributions
R.L.Y. conceived the study, designed and performed experiments and analyzed data. C.L.L., C.C.L. and L.H.L. performed the experiments and analyzed data. F.Y.C. helped mass spectrometry analysis. H.Y.C. initiated the study. H.Y.C. and C.L.L. performed the experiments and analyzed data. C.L.L., C.C.L. and L.H.L. performed bioinformatics analyses. R.H.C. directed and coordinated the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The original mass spectrometry data for proteome analysis are deposited to the ProteomeXchange Consortium via PRIDE partner repository with the project accession number PXD030756. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Content for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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