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Master Thesis

一種新型長鏈非編碼 RNA *BCRP3* 通過激活自噬促進蛋白質穩態和細胞存活

A novel long noncoding RNA *BCRP3* promotes protein homeostasis and cell survival by activating autophagy

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一種新型長鏈非編碼 RNA BCRP3 通過激活自噬促進蛋白質穩態和細胞存活

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本論文係欒玖霖君（R09B48005）在國立臺灣大學基因體與系統生物學學位學程完成之碩（博）士學位論文，於民國 111 年 7 月 21 日承下列考試委員審查通過及口試及格，特此證明

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摘要

長鏈非編碼 RNA(LncRNA) 能調節許多生物反應，最近研究發現 lncRNAs 在自噬調節中扮演重要作用。然而目前對 lncRNAs 調控自噬的機制尚不清楚。本研究中，我們發現一種新型 lncRNA *BCRP3* 在多種腫瘤中的表達異常低，並做為自噬的正調節因子。進一步我們確認 *BCRP3* 主要位於細胞質中並能提高 VPS34 活性來啟動自噬。當細胞處於蛋白毒性刺激下會促進 *BCRP3* 的基因表現，進而促進泛素化蛋白質聚集體的清除。當蛋白質毒性刺激下， *BCRP3* 缺乏不僅影響蛋白質動態平衡的調節，還導致 TGF-β/Smad2 訊號被啟動，最後造成生長抑制、細胞凋亡。總上所述，我們研究揭示了 *BCRP3* 通過增強 Vps34 複合物的活性進而促進自噬作用來維持蛋白質動態平衡和細胞存活。

關鍵詞：LncRNA、自噬、VPS34複合物、TGF-β信號、細胞死亡、癌症

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Abstract

Long noncoding RNAs (LncRNAs) have been implicated in regulating many biological processes. Recent studies have also shown that lncRNAs play important roles in autophagy regulation. However, the underlying mechanism of lncRNAs in autophagy has not been unveiled. In this study, we identified a novel lncRNA *BCRP3* as a positive modulator of autophagy and with abnormally low expression in a variety of tumors. *BCRP3* is mainly located in the cytoplasm and binds to the VPS34 complex, which in turn initiates autophagy under basal and starvation conditions. In response to proteasome inhibition or oxidative stress-induced proteotoxicity, *BCRP3* is upregulated to promote autophagy, thus facilitating the clearance of ubiquitinated protein aggregates. *BCRP3* deficiency under proteotoxic stress not only comprises the protein quality control, but also leads to growth inhibition, cell death, and apoptosis. These cell survival defects are mediated in part through the upregulation of the TGF-β/Smad2 pathway. In conclusion, our study revealed the role of *BCRP3* as a positive regulator of autophagy by binding to VPS34 complex and *BCRP3* is important for the maintenance of protein homeostasis and cell survival.

Keywords: autophagy, cancer, cell death, LncRNA, TGF-β signaling, VPS34 complex

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I. Introduction

Autophagy

1. Autophagy in cancer

Autophagy is a cellular self-protective mechanism that can be used to eliminate damaged organelles and misfolded proteins. Dysregulation of autophagy frequently contributes to a variety of diseases, including metabolic diseases, neurodegenerative diseases, cancer, and more. Autophagy plays dual and dynamic roles in cancer progression and suppression, depending on cell contexts and stages of cancer development[1]. In early tumorigenesis, autophagy serves as a control mechanism to prevent tumorigenesis and inhibit cancer progression. Autophagy maintains homeostasis in normal cells, and this homeostatic activity constitutes a powerful barrier against malignant transformation. However, many oncoproteins escape this surveillance of homeostasis by inhibiting autophagy. Once tumors are advanced and under environmental stress, autophagy in tumor cells constitutes a means of coping with intracellular and environmental stress, thereby favoring tumor survival and growth, and promoting the ability of cancer to metastasize and invade[2]. This suggests that the regulation of autophagy can serve as an effective intervention strategy for cancer therapy. Many current cancer treatments affect

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autophagy. Understanding the underlying mechanisms of autophagy in cancer could lead to more effective treatment of patients[3].

2. Mechanism of autophagy

Autophagy induction

Autophagy recycling enables cellular renewal and energy balance, allowing cells to adapt to stressful situations. When cells are stimulated by external stress such as hypoxia or nutrient deprivation, cells initiate autophagy through complex protein networks and signaling pathways to maintain cellular stability[4]. When the cells are in a low energy state, the reduction of intracellular ATP simultaneously activates AMP-activated protein kinase (AMPK). AMPK can directly activate Ulk1 and further promote autophagy by phosphorylating Ulk1 at Ser 317 and Ser 777. When cells are in a nutrient-rich condition, mTOR has a higher activity, which in turn inhibits the interaction between Ulk1 and AMPK to prevent Ulk1 activation, thereby inhibiting autophagy.[5, 6].

Nucleation

Activation of autophagy requires upstream recruitment of the ULK1 complex. The ULK1 complex is responsible for integrating signals of cellular nutrient status and transmitting autophagy-initiated signals to downstream ATG

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proteins. The ULK1 complex contains other protein components, including FIP200, ATG 13, and ATG101[7-9]. Activated ULK1 complex recruits the PI3K lipid kinase complex to phagopore for the formation of omegasomes [10]. The PI3K (Phosphatidylinositol 3-kinase/ VPS34) complex are tetrameric and share core subunits Vps15, Vps34 and Beclin1. As the first step in autophagosome formation, autophagosome nucleation requires the interaction of Beclin1 with ATG14 and promotes the catalytic activity of VPS34 to further increase PI(3)P levels[11]. The Beclin1-VPS34 complex is a pleiotropic regulator of complex autophagy. Beclin1 can regulate VPS34 kinase activity, which is essential for autophagy and other membrane trafficking processes. It is currently known that the Vps34 complex can be divided into two different complexes: complex I and complex II. The fourth subunit Atg14 in complex I or UVRAG in complex II define the separate role of these two complexes. Complex I mainly regulates autophagosome biogenesis, while complex II is involved in late autophagosome maturation and endosomal trafficking[12]. Vps34 complex I is one of the core ATG proteins that initiate autophagosome production, so it must have high membrane curvature. This curvature-sensing ability mainly comes from Atg14 binding Vps34 and Beclin1. However, the main function of VPS34 complex II is within the endocytic compartment. The

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study also found that UVRAG is present on Rab9-positive endocytic compartments and may regulate autophagy through the ATG9 cycle[13, 14]. The ULK1 complex binds to the ER and vesicles of ATG9 with the help of Vps34 complexes, and these elements aggregate gradually to form spherical structures unique to early autophagy[15].

Expansion and closure

The ATG12-ATG5-ATG16L1 complex is thought to mediate LC3 lipidation by recruiting LC3 to the membrane and facilitating the transfer reaction to phosphatidylethanolamine(PE)[16]. The autophagy factor ATG12-ATG5 conjugate has E3 ligase-like activity that promotes lipidation of LC3 family members. The N-terminal region of ATG16L1 can bind to ATG12-ATG5 conjugate and recruit it to the autophagosome membrane[17]. The core of autophagy is mediated by a ubiquitin-like protein (UBL) cascade in which homodimeric E1 enzymes direct UBL LC3 and Atg12 to their respective E2 enzymes [18]. Finally, LC3 is transferred from ATG3's catalytic Cys to phosphatidylethanolamine (PE) in a reaction stimulated by the Atg12-Atg5 complex[19]. The membrane-bound form of LC3 (LC3-II) is a classic marker of the autophagosome. In addition, ATG4 was also found to participate in the sealing of autophagosomes by processing the LC3 family[20, 21].

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Maturation and fusion

After autophagosome expansion and closure, it must undergo maturation to shed most of the ATG proteins from the autophagosome membrane. The final critical step in autophagy is the acquisition of degradative enzymes by autophagosomes through fusion with lysosomes. The autophagosome-lysosome fusion step consists of two SNARE complexes, STX17-SNAP29-VAMP7/VAMP8. In addition, the multi-level regulation of Rab GTPase, tethering factors, SNARE recruitment to form complexes and lipid components ensures that autoCes can precisely bridge and fuse with lysosomes, further preventing the risk of lysosomal enzymes disrupting and infiltrating the cell membrane.[22, 23].

3. The roles of autophagy in aggrephagy

The proteins and organelles within the cell need to coordinate at all levels to control cell quality to ensure cellular health and appropriate response to stress and injury. Two major control systems responsible for protein degradation in eukaryotic cells: the ubiquitin-proteasome system (UPS) and autophagy. UPS is dependent on Ub as a degradation signal as well as as a size-limiting substrate, so it is primarily limited in size by proteasome narrow channels and is primarily used to degrade single unfolded polypeptides. On the other hand, autophagy mainly

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degrades larger structures such as protein complexes, cell aggregates, organelles [24, 25]. The most studied Ub receptor is p62/SQSTM1, which binds to UB through its Ub-binding domain (UBD). And the PB domain in p62 can further induce the formation of p62 body, and finally lead to liquid-liquid phase separation [26, 27]. Finally, it binds to the autophagic membrane through its LC3 interaction region (LIR) domain. Then use autophagy to degrade larger structures [28, 29].

When misfolded and aggregated proteins are present, these proteins lose their physiological functions and become cytotoxic. Selective autophagy is a quality control system that can be induced by a variety of stressors, including oxidative stress, protein aggregation [24]. Removal of cytotoxicity is very important in cells, and when cells cannot properly remove cytotoxicity, it can further lead to disease. For instance, neurodegenerative disorders of ageing include Alzheimer disease, Parkinson disease, Huntington disease, and related tauopathies. Their common characteristics: accumulation of abnormally misfolded proteins, such as tau, TAR DNA-binding protein 43 (TDP43), and mutant forms of huntingtin (Htt) [30]. In summary, understanding the mechanism of clearance of cytotoxic proteins and strategies to enhance their therapeutic effects are the primary issues.

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Long non-coding RNAs (lncRNAs)

1. Functions of lncRNAs

RNA represents the genetic information in the genome and plays important roles in the decoding, regulation and expression of genes. However, less than 2% of RNAs are transcribed into proteins, and the majority of transcripts are noncoding RNAs (ncRNA) [31]. Long non-coding RNAs (lncRNAs) are defined as ncRNAs longer than 200 nucleotides, and lncRNAs have a greater diversity in sequence, shape, and function than other classes of ncRNAs. According to Human GENCODE, the human genome contains more than 16,000 lncRNA genes[32]. The multifaceted roles of lncRNAs depend on their specific cellular localization and the adoption of specific structural modules with interacting partners[33]. In the nucleus, lncRNAs can interact with nuclear proteins, chromatin, and other nuclear RNAs, and recruit specific cofactors into the nucleus to further form phase-separated liquid-liquid domains[34]. In the cytoplasm, lncRNAs can control mRNA stability and translation, regulate protein stability, modulate signaling pathways, and retain the structural and functional integrity of subcellular organelles[35].

LncRNAs can function as decoys, guides, and scaffolds in the nucleus. For instance, lncRNAs can act as guides to assist ribonucleoprotein complexes to

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locate to specific target sites, and they can also act as decoys to tether RNA-binding proteins and prevent them from accessing their targets. The structural features of lncRNAs allow them to act as scaffolds to assemble different molecules to perform certain functions at a single locus. In addition, lncRNAs can act as chromatin organizers to organize chromatin structure through direct interaction with DNA for transcriptional regulation. In many biological signals, such lncRNAs can simultaneously bind multiple effector partners, thereby temporally and spatially combining activated or inhibited effectors. LncRNAs are critical for precise control of intermolecular interactions and signal-specific dynamics[36]. More and more studies have shown that lncRNAs are extremely important for regulating the cellular physiological environment and signal transmission. It has also been found that many diseases are caused by deregulation of lncRNAs. Therefore, understanding lncRNAs can provide many common diseases management and treatment.

2. lncRNA in cancer

Recent studies have found that lncRNAs can regulate gene expression, and thus affect cell proliferation, survival, migration, and genomic stability[37]. LncRNAs are platforms for complex interactions with miRNAs, mRNAs, proteins, and act as important regulators. LncRNAs play important roles in chromatin

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organization, transcriptional and post-transcriptional regulation. Their abnormal expression confers the ability of cancer cells to develop tumors, grow and metastasize[38]. LncRNAs have also been found to be involved in cancer immunity, cancer metabolism, and cancer metastasis[39]. LncRNAs play key roles in regulating the activity of proteins as well as other RNAs. They can participate in cell differentiation, cell cycle, proliferation and apoptosis by regulating gene expression[40]. More and more studies have found that lncRNAs can act as tumor suppressor genes or oncogenes by regulating gene expression or regulating protein function[41].

A number of lncRNAs elicit pro-tumor functions. For instance, *HOTAIR* expression is associated with poor prognosis in breast, colon and lung tumors. *NORAD* contributes to the development of various cancers and its expression is upregulated and associated with poor prognosis in various cancers. LncRNA *DANCR* promotes tumor progression by targeting *miR-216a-5p* in breast cancer[42]. When *DANCR* is overexpressed, it is often associated with poor tumor prognosis in patients with advanced colorectal cancer [43]. Furthermore, multiple lncRNAs, such as *PVT1, CCAT1, CCAT, PCAT1,* and *MINCR,* regulate the expression of proto-oncogene *MYC* [44].

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Numerous lncRNAs have been found to inhibit tumor progression. For example, *GAS5* expression is inversely correlated with the size, stage, and metastasis of many types of tumors, and various lncRNAs have been found to inhibit tumor pathways by regulating *p53*[44]. *loc285194* can directly regulate *p53* or suppress tumors in part through the negative regulation of *miR-211*. This result suggests the existence of a competing endogenous RNA regulatory network, in which *loc285194* and *miR-211* are mutually negatively regulated[45]. *PDCD4-AS1* has also been found in breast cancer to promote the stability of *PDCD4* mRNA and control the interaction between *PDCD4* and RNA decay-promoting factors to control breast cancer progression[46]. LncRNA *TUG1* can promote tumor suppression by activating caspase-3 and -9-mediated intrinsic pathways and by promoting apoptosis and inhibiting Bcl-2-mediated anti-apoptotic pathways[47].

In summary, numerous studies have shown that lncRNAs play critical roles in tumorigenesis and tumor progression. Aberrant lncRNA expression may accompany DNA damage, immune escape, and cellular metabolic disturbances in cancer cells[39]. Therefore, lncRNAs have broad application prospects in cancer diagnosis, prognosis, and treatment. For instance, PCA3 has been permitted for the diagnosis of prostate cancer [48].

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3. lncRNA in autophagy

Recent studies have shown that various ncRNAs can affect autophagy by regulating autophagy-related (ATG) proteins[49]. It is also found that LncRNAs regulate the expression of ATG genes by directly binding to DNA, RNA or protein [50]. LncRNAs regulate autophagy in the context of cancer initiation, progression, and resistance to chemo- or radiotherapy[51]. Activation of mTOR signaling is known to inhibit autophagy signaling, and there are many lncRNAs that affect mTOR activity by directly binding to the mTOR complex or indirectly regulate proteins acting upstream or downstream of mTOR[52]. In addition, it was also found that lncRNA *CASC19* can promote autophagy through the AMPK-mTOR pathway, thereby causing the resistance of nasopharyngeal carcinoma to radiotherapy[53]. The LncRNA *SNHG6* has been reported to promote chemoresistance of miR-26a-5p via ULK1-induces autophagy[54]. Aberrant overexpression of *H19* inhibits mTOR phosphorylation and promotes ULK1 phosphorylation, which in turn promotes the proliferation and autophagy of various cancers including glioma cells[55]. Apart from ULK1, the PI3K complex is regulated by multiple lncRNAs. Multiple lncRNAs can sub-regulate the PI3K complex. Meanwhile, lncRNA may stimulate or inhibit autophagy nucleation by interacting with Beclin1 or activate autophagy initiation by increasing the

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expression of ATG13. There are also lncRNAs that promote autophagosome elongation/closure by binding to ATG genes involved in the ubiquitin-like binding system[50].

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TGF-beta pathway

1. Mechanism of TGF-β pathway

The transforming growth factor-beta (TGF-beta) pathway has been known to orchestrate multiple cellular processes, including growth, survival, and cell fate specification. Deregulation of TGF-beta family signaling results in disorders such as developmental abnormalities, fibrotic diseases, tumorigenesis, and immune disorders[56]. TGF-β signaling begins with TβRII, a receptor to which TGF-β binds directly. TβRII forms a heteromeric complex with TβRI, and ligand-induced activation of TβRII leads to activation of TβRI serine-threonine kinase activity through phosphorylation. After phosphorylation of TβRI, key components of the downstream TGF-β signaling pathway including canonical and non-canonical downstream signaling pathways are activated[57]. The canonical pathway refers to TGF-β signaling through SMAD proteins. Once receptor-regulated SMADs (SMAD2, SMAD3) are phosphorylated, they form a trimeric complex with common mediator SMADs (SMAD4). The complex is then translocated to the nucleus to regulate target gene transcription[56]. In addition to TGF-β signaling through SMADs, TGF-β also utilizes a variety of intracellular signaling pathways to regulate various cellular functions such as MAPK (including ERK, JNK, and p38 MAPK) and PI3K-Akt pathway. These signaling pathways are called TGF-β

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non-canonical pathways (SMAD-independent pathways). TGF-β non-canonical pathways activate through receptors directly occupied by ligands to enhance, attenuate, or otherwise regulate downstream cellular responses [58].

2. The functions and mechanisms of TGF-β pathway in regulating cell cycle and cell survival

TGF-β plays an important role in tumor cells by regulating proliferation, differentiation, and death of various cell types[59]. TGF-β can exhibit tumor suppressive effects by inhibiting cell cycle progression and promoting apoptosis. TGF-β can block cell cycle progression and inhibit growth through the canonical SMAD pathway by increasing the expression of CDK inhibitors p15, p21, p27 and/or p57. Tumor cells often reduce tumor suppressive effects by inactivating or disrupting the TGF-β pathway. In most cases, tumors mutate and lead to loss of function of the core pathways of TGF-β (TGFBR2, SMAD2, SMAD4), further inhibiting the tumor suppressive ability of TGF-β[60]. Besides, TGF-β induces cell apoptosis by enhancing the activation of the death receptors, and pro-apoptotic factors, such as BCL-2, BIM, and DAPK. TGF-beta prevents the phosphorylation and degradation of the pro-apoptotic protein BIM through its rapid inhibition of ERK, resulting in the accumulation of BIM protein and the initiation of apoptosis in cells[61]. In addition, TGF-β can induce apoptosis through the action of Smad2,

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Smad3 and Smad4 Induces expression of death-associated protein kinase (DAPK).[62] In summary, TGF-β-mediated growth inhibition and apoptosis lead to decreased cell survival.

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II. Materials and Methods

Oligonucleotides

shRNA	target sequences
shLuc	5’-TTACGCTGAGTACTTCGA-3’
sh*BCRP3*#1	5’-ATATTGGACGCTGGACCCGC-3’
sh*BCRP3* #2	5’-GACGCTGGACCCGCAGGCCC-3

Plasmids

GFP-DFCP1	From Dr. Wei Yuan Yang’s lab
4 × SBE-Luc construct	from Rik Derynck (University of California at San Francisco)

Antibodies

Protein	Company	Cat. number	Source	Titer
LC3	Cell Signaling	2775	Rabbit	WB (1:1000)
GAPDH	GeneTex	GTX100118	Rabbit	WB (1:5000)
WIPI2	Abcam	ab105459	Rabbit	IF (1:200)
ULK1	Santa Cruz	sc-33182	Rabbit	WB (1:1000)
ULK1-pS757	Cell Signaling	14202	Rabbit	WB (1:1000)
ULK1-pS317	Cell Signaling	6887	Rabbit	WB (1:1000)
Atg13	Sigma-Aldrich	SAB4200100	Rabbit	WB (1:1000)
Atg13-pS318	Rockland	600-401-C49	Rabbit	WB (1:1000)
VPS34	Cell Signaling	4263	Rabbit	WB (1:1000)
VPS34	Echelon Biosciences	Z-R015	Rabbit	IP (1:150)
HA	Cell Signaling	3724	Rabbit	WB (1:5000)
FLAG	GeneTex	GTX115043	Rabbit	WB (1:5000)
H2A.X	Sigma-Aldrich	05-636	Mouse	WB (1:1000)
H3	Abcam	ab1791	Rabbit	WB (1:1000)
Caspase 3	Abcam	ab32351	Rabbit	WB (1:1000)
PARP	Santa Cruz	sc-8007	Mouse	WB (1:1000)
Ubiquitin	Abcam	Ab7254	Mouse	WB (1:1000)
Ubiquitin (FK2)	Enzo	BML-PW8810-0100	Mouse	IF (1:300)
p-Smad2	Cell Signaling	8828	Rabbit	WB (1:1000)
Smad2	Cell Signaling	8685	Rabbit	WB (1:1000)

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Primers

Primers	Forward (5'→3')	Reverse (5'→3')
BCRP3	CACAACCTCGCCACTGTCTT	CACTGGCATCTAGGCCTTCG
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG
Neat1	CAGTTAGTTTATCAGTTCTCC CATCCA	GTTGTTGTCGTCACCTTTCAA CTCT
DAPK1	CGAGGTGATGGTGTATGGTG	CTGTGCTTTGCTGGTGGA
P15	CACCCCCACCCACCTAATTC	TGAGTGTCGAGGGCCAGATA
P21	CCTCATCCCGTGTTCTCCTTT	GTACCACCCAGCGGACAAGT

Chemicals

TβRI (type I TGF-β receptor) inhibitor SB431542, 5-fluorouracil (5-FU), Tunicamycin, Puromycin, Bafilomycin A1, 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).

Cell culture and transfection

HCT116 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640, 293FT cells were cultured in Dulbecco's Modified Eagle medium (DMEM), and HeLa cells were cultured in Minimum Essential Medium (MEM). All mediums were containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (PS), and 1 mM sodium pyruvate. *BCRP3*-deficient HeLa cells were generated by lentiviral transduction of *BCRP3* shRNAs. For starvation, cells were incubated with Earle's Balanced Salt Solution (EBSS) for the indicated times. Plasmid transfection was

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performed using Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Lentiviral package and transduction

Lentiviruses were produced in 293FT cells co-transfected with packaging plasmid (pCMV8.91), envelope VSV-G plasmid (pMD.G), and *BCRP3* cDNA or shRNA expression plasmids at a ratio of 4:1:4. After 48 hours of incubation, the lentivirus-containing supernatant was collected and filtered. In *BCRP3* knockdown, 70% confluent HeLa and HEK293T cells were transduced with lentivirus carrying *BCRP3*-specific shRNA in medium containing polybrene (8 μg/ml) and treated with 1 μg/ml puromycin (Sigma- Aldrich) selection of transduced cells (St. Louis, MO, USA). For *BCRP3* overexpression, HeLa and HCT116 cells were transduced with lentiviruses carrying pLAS5w.Pneo-*BCRP3* and screened with 0.5 mg/ml G418 Geneticin (Invitrogen, Thermo Fisher Scientific).

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 PMSF and protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The lysate was clarified by centrifugation at 15,490 × g for 15 min. Protein concentration was determined with Bradford reagent. Lysates containing equal amounts of protein were boiled with sample buffer for 10 min and separated by

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SDS-PAGE and transferred to PVDF membranes. Membranes were then blocked with 5% nonfat milk and probed with primary and subsequent secondary antibodies and signal detected using 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 PMSF, and protease inhibitor cocktail). After centrifugation at 15,490 × g, the supernatant was pre-cleared with Protein A beads for 1 hr and then incubated with the desired antibody for 1.5 hr. The beads were washed 3 times, denatured with sample buffer, and subjected to western blot 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.

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 reverse transcribed using the iScript cDNA synthesis kit (Bio-Red, Richmond, CA, USA). qPCR was performed on a LightCycler® 480 Instrument II System (Roche) using FastStart Universal SYBR Green Master Reagent (Roche). GAPDH was used as reference gene.

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Subcellular fractionation assay

1×10^7 cells were lysed in 10 ml cytoplasmic lysis buffer (0.256 M sucrose, 8 mM Tris–HCl pH 7.5, 4 mM $MgCl_2$, 0.8% Triton X-100, and 0.25% PBS) with gentle pipetting and inverting, followed by rotation at 4℃ for 10 min and incubation on ice for another 10 min. After centrifugation at 2,500 × g for 15 minutes, transfer 250 µl of the supernatant to a new tube and save (cytoplasmic fraction). The pellet was washed 3 times with 10 ml ice-cold PBS, followed by 1 ml RIP buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 0.5 mM dithiothreitol, 1 : 100 SuperRNaseIn (Invitrogen, Thermo Fisher Scientific) and protease inhibitor cocktail). Lysates were sonicated using Qsonica (30 s ON, 30 s OFF 15 min) and centrifuged at 13,000 rpm for 10 min. Finally the supernatant (nuclear fraction) was collected. Equal amounts of cytoplasm and nucleus were then subjected to RNA extraction with 1 ml of Trizol reagent.

PI3K activity assay

FLAG-VPS34 complexes were immunoprecipitated from HeLa cells and assayed for activity using a class III PI3K ELISA kit (K-3000, Echelon Biosciences, Salt Lake City, UT, USA). 30 µl of kinase reaction buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM $MnCl_2$ and 50 µM ATP), 9 µl of 500 µM PI substrate and 20 µl of ddH_2O were first added to the immune complexes, together with in vitro

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transcribed BCRP3 or control RNA. After 30 min incubation at 30°C, the reaction was stopped by adding an additional 12 μl of 100 mM EDTA. The amount of PI3P produced was further tested by a competitive ELISA assay. Finally, detect the amount of PI3P by reading the colorimetric change in absorbance at 450 nm. and normalized to the amount of immunoprecipitated FLAG-VPS34.

BrdU incorporation and MTT assays

To measure BrdU incorporation, cells were seeded at 2000 cells/well in 96-well plates and incubated overnight before treatment with various drugs. Cells were finally pulsed with 10 μM BrdU for 24 hr. After fixation, cell proliferation was performed by BrdU to determine BrdU incorporation by an assay kit (Merck Millipore). For MTT assay, cells seeded on 96-well plate at a density of 1×10^4 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.

Bioinformatics

RNA-seq data of different types of normal and cancer tissues were downloaded from the UCSC Xena platform (http://xena.ucsc.edu). Using python to classify different tissues, the extracted *BCRP3* expression was filtered out at the end of the normal and tumor gene expression, and the tissues with normal and tumor differences were filtered

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by P-value < 0.05. The protein obtained by label-free quantitative LC-MS/MS analysis was compared knockdown/control was > 1.5, P-value < 0.05, and the basal control group was filtered out, and the obtained protein was sent to DAVID (https://david.ncifcrf.gov/) for Gene Ontology (GO) analysis, filtered by P value < 0.05, and finally plotted in R language. For in silico analysis of *BCRP3* expression, RNA-seq data of different types of normal and cancer tissues were downloaded from via the UCSC Xena platform (http://xena.ucsc.edu). 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 two groups and one-way or two-way ANOVA with Tukey's post hoc test was used for multi-group comparisons.

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III. Results

BCRP3 promotes autophagy

By analyzing the UCSC Xena platform dataset, we found that the tumor expression of lncRNA *BCRP3* in 11 cancer types, including colon, esophagus, brain, skin, stomach, testis, kidney, lung, ovary, prostate and liver, was lower than in normal tissues (Figure 1). The *BCRP3* gene is located on chromosome 22q11.23 as a pseudogene based on the annotation by Biotechnology Information (NCBI) GenBank and produces a 1.4 kb lncRNA. The low expression of *BCRP3* in many cancer types suggests that the gene may play a repressive role in tumors. Therefore, we further tested the function of *BCRP3* in cancer cells. We found that overexpression of *BCRP3* did not affect the cell viability of HCT116 human colorectal cancer cell line in the basal state, but significantly improved cell viability after treatment with the chemotherapeutic drug 5-FU (Figure 2A). However, monitoring DNA damage with γH2A.X showed that we found no difference between control cells and *BCRP3*-overexpressing cells after 5-FU treatment (Figure 2B). This suggests that *BCRP3* does not affect DNA damage and repair.

Autophagy is known to play an inhibitory role in tumorigenesis, but it is also a mechanism by which cancer cells often develop resistance to chemotherapy. The chemoresistance effect of *BCRP3* was further reduced by blocking autophagy with 3-

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MA or bafilomycin A1 (Figure 3). Therefore, we examined the role of *BCRP3* in autophagy using LC3 as a marker. Overexpression of *BCRP3* increased LC3 lipidation (referred to as LC3-II) in HCT116 cells cultured in both MEM (basal) or EBSS (starvation-induced) (Figure 4A). On the other hand, knockdown of *BCRP3* reduced LC3 lipidation in cells cultured in MEM, and specifically *BCRP3* promoted autophagosome formation by blocking anaphase of autophagy with bafilomycin A1 (Figure 4B). In conclusion, our study found that *BCRP3* can promote autophagosome production and thus promote autophagy.

BCRP3 promotes autophagy by enhancing VPS34 activity

To gain insight into how *BCRP3* promotes autophagy, we prioritized the subcellular localization of *BCRP3*. *BCRP3* was found mainly in the cytoplasm by cell fractionation and qRT-PCR analysis (Figure 5). We next investigated the function of *BCRP3* during autophagosome biogenesis. *BCRP3* knockdown reduces GFP-DFCP1 puncta in starvation-stimulated cells, so we know that *BCRP3* knockdown reduces the amount of omegasomes expressed (Appendix 1 by Ruei-Liang Yen). This means that *BCRP3* can initiate autophagy by promoting omegasomes. Next, we wanted to determine if *BCRP3* affects upstream regulators such as mTOR, AMPK and ULK1. However, *BCRP3* knockdown did not affect the kinase activities of mTOR, AMPK and

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ULK1 in starvation-stimulated cells as monitored by ULK1 S757 phosphorylation, ULK1 S317 phosphorylation and ATG13 S318 phosphorylation, respectively (Appendix 2 by Ruei-Laing Yen). We next wanted to determine whether *BCRP3* could affect the activity of the VPS34 complex. We isolated VPS34 complex from control and *BCRP3* knockdown cells and used an in vitro kinase assay to determine the phosphorylation of PI to produce PI3P. W We found that *BCRP3* knockdown had higher VPS34 complex activity compared to control cells (Figure 6). In summary, our data suggested that *BCRP3* promotes the activity of VPS34 complex.

BCRP3 is upregulated under proteotoxic stresses to promote the accumulation of ubiquitinated proteins

After we determined that *BCRP3* promotes autophagy, we next wanted to investigate which autophagy-stimulating condition could induce *BCRP3* expression. The analysis showed that *BCRP3* expression was up-regulated by stimulating cells with the proteasome inhibitor MG132 or the oxidative stressor As2O3 (Figure 7A, B). However, *BCRP3* expression levels were not affected by starvation and other proteotoxic stressors such as HSP90 inhibitors (17-AAG and 17-DMAG), puromycin (Figure 7C). Since both MG132 and As2O3 trigger the accumulation of ubiquitinated protein, we wanted to understand whether *BCRP3* could promote the clearance of these

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ubiquitinated proteins through autophagy. Knockdown of *BCRP3* under basal conditions had no effect on total cellular ubiquitinated proteins (Figure 8A). However, under stimulation with proteasome inhibitor MG132 and the oxidative stressor As2O3, total cellular ubiquitinated protein accumulation was significantly increased in *BCRP3* knockdown cells, comparing to control cells (Figure 8B, C). In summary, our study revealed that induction of *BCRP3* by proteotoxic stresses further stimulates autophagy activity for protein quality control.

BCRP3 deficiency under proteotoxicity leads to the accumulation of growth/survival inhibitors and TGF-β pathway upregulation

Next, we wanted to further understand the impact of *BCRP3* deficiency upon proteotoxic stimuli on the overall proteome. We performed label-free quantitative LC-MS/MS analysis to examine the protein expression profile of *BCRP3* knockdown cells under MG132 treatment relative to controls. Protein accumulation in *BCRP3* knockdown cells under MG132 treatment (proteotoxicity) was defined using criteria: *BCRP3* knockdown/control protein expression level > 1.5, P value < 0.05, 189 proteins were obtained. We further excluded 55 proteins that also accumulate under basal conditions (Figure 9 A). Finally, the remaining 134 proteins were analyzed by GO and found "positive regulation of apoptosis process", "negative regulation of cell

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proliferation", "cell death", "mitochondrial release of cytochrome c" and "common partner SMAD protein phosphate" 'ization' paths have higher GO richness. Special white, phosphorylation of common partner SMAD proteins including TGF-β1 and Smad2 (downstream effectors of TGF-β signaling) (Figure 9B). The TGF-β pathway is known to trigger growth arrest and apoptosis, so we further evaluated the effect of *BCRP3* on TGF-β signaling. *BCRP3* knockdown upon MG132 treatment resulted in increased levels of Smad2 and p-Smad2, and blocking lysosomal degradation with bafilomycin A1 abolished these effects. These results support a role for *BCRP3*-mediated aggregated phagocytosis in the selective degradation of Smad2 and are consistent with data from proteomic analyses (Figure 10A). Furthermore, *BCRP3* knockdown under MG132 treatment increased the Smad response reporter 4xSBE-Luc (Figure 10B). We further demonstrated that TGF-β downstream genes including DAPK1, p15 and p21, which play important roles in apoptosis and antiproliferative effectors, were up-regulated in *BCRP3* knockdown cells treated with MG132 (Figure 10C). Our study found that *BCRP3* induces autophagic degradation under proteotoxic stimuli to downregulate TGF-β signaling, a cell death promoter, and a proliferation inhibitor.

BCRP3 protects against proteotoxicity causing growth arrest and apoptosis by downregulating TGF-β signaling

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Based on the functional identification of *BCRP3* in autophagy and the altered proteomic landscape, we wanted to further investigate its effects on cell proliferation and survival under proteotoxic stimuli. Using BrdU incorporation and MTT assays, we found that *BCRP3* knockdown reduced cell proliferation and viability under MG132 treatment, but not control cells. However, blocking TGF-β signaling with the TβRI inhibitor SB431542 partially reversed the effects of *BCRP3* knockdown on cell proliferation, viability (Figure 11A, B). In MG132-treated cells, *BCRP3* knockdown enhanced markers of apoptosis including cleaved PARP and active caspase 3, but did not affect MG132-untreated cells (Figure 12). In addition, MG132-treated cells under TGF-β pathway enforcement enhanced apoptosis (Figure 13). In conclusion, our study found that *BCRP3* maintains cell proliferation and viability and prevents apoptosis in response to proteotoxic stimuli, which is mediated in part by downregulating TGF-β signaling.

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V. Discussion

BCRP3 promotes protein homeostasis by activating autophagy

In this study, we identified the lncRNA *BCRP3* as an important factor in the positive regulation of autophagy. *BCRP3* is upregulated in the presence of proteasome inhibition (MG132) or oxidative stress-induced (AS2O3) proteotoxicity, further *BCRP3* is mainly localized to the cytoplasm and increases the enzymatic activity of the VPS34 complex to promote aggregated autophagy. *BCRP3* deficiency under proteotoxicity results in preferential accumulation of proteins in growth inhibition, cell death, apoptosis, and TGF-β/Smad2 pathways (Figure 14). In conclusion, our studies will identify As an activator of the VPS34 complex, *BCRP3* maintains cellular fitness by regulating protein quality through selective degradation and plays a key role in cell growth and survival.

BCRP3 upregulates Vps34 enzymatic activity

Our study shows that *BCRP3* increases lipid kinase activity of the Vps34 complex. Importantly, Previous studies in our laboratory found that *BCRP3* does not affect the integrity of the VPS34 complex. Furthermore, *BCRP3* can stimulate the activity of purified VPS34 complexes in an in vitro environment, suggesting that *BCRP3* can stimulate the activity of VPS34 complexes without a membrane environment. These results suggest that the binding of *BCRP3* to the VPS34 complex may enhance its

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enzymatic activity by changing the conformation of the VPS34 complex, but we cannot exclude other possibilities that *BCRP3* promotes membrane targeting of the VPS34 complex. In addition, many studies have also known that the activity of VPS34 complex can be regulated by post-translational modification mechanisms[63]. It is possible that *BCRP3* can induce the activity of the VPS34 complex through post-translational modification. In conclusion, the mechanism by which *BCRP3* regulates the VPS34 complex in detail is unclear, and its mechanism of stimulating VPS34 activity remains to be further investigated.

BCRP3 is induced under proteasome inhibitor and oxidative stressor

Our results show that the proteasome inhibitor MG132 and the oxidative stressor arsenic trioxide can promote *BCRP3* gene expression, both of which are known to cause protein misfolding and ubiquitinated protein aggregates. However, we found that several other drugs that trigger proteotoxic stress, such as puromycin and HSP90 inhibitors, did not alter *BCRP3* gene expression levels. Although the mechanism of promoting *BCRP3* is still unclear, it is possible that proteasome inhibitor MG132 can cause some transcription factors to not be degraded normally, and then these transcription factors promote *BCRP3* gene expression level[64]. On the other hand, oxidative stressor arsenic trioxide can cause oxidative damage and lead to the destruction of protein structure and cause abnormal accumulation of protein, which may

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promote the accumulation of *BCRP3* in the cytoplasm and induce aggregated phagocytosis to compensate for the degradation ability [65]. The exact molecular mechanism of *BCRP3* induction is not fully understood, but *BCRP3* is identified as a new player mediating the control of protein quality between the ubiquitin-proteasome system and the autophagy process.

BCRP3 affects growth inhibition, apoptosis and TGF-β/Smad2 pathway under proteotoxicity

Our study found that depletion of *BCRP3* under proteotoxicity leads to accumulation of the protein, with major effects including growth inhibition, cell death, apoptosis, and the TGF-β/Smad2 pathway. Consistent with the accumulation of these proteins, *BCRP3* deficiency in proteotoxic cells impairs cell viability and proliferation and increases apoptosis mediated from activation of the TGF-β/Smad2 pathway. However, we found that TGF-β pathway blockade does not completely reverse the deleterious effects of *BCRP3* deficiency in proteotoxic cells, indicating the involvement of other proteins which act together with TGF-β pathway to impair cell proliferation and viability.

Currently, We do not know the underlying mechanism of how *BCRP3*-deficient cells preferentially accumulate specific proteins in response to proteotoxicity. These proteins may be direct cargos of autophagy mediated by *BCRP3* or indirectly

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accumulated/induced through autophagy deficiency. However, we know that some of these proteins are degraded in a *BCRP3*-dependent and lysosome-dependent manner during proteotoxicity, suggesting the possibility as direct cargos of autophagy. Future study for identifying direct autophagic cargos mediated by *BCRP3* in proteotoxicity could be conducted by LC3-based proximity labeling followed by LC-MS/MS analysis [66].

The importance of *BCRP3* in cancer

BCRP3 is significantly down-regulated in tumor tissues of many cancer types. Although this suggests a tumor suppressive function of *BCRP3*, we found that *BCRP3* overexpression does not affect cell proliferation and survival. Noted that autophagy plays dual roles in cancer, that is, suppressive role during tumor initiation and promoting role once tumors are formed. Our findings that *BCRP3* overexpression promotes resistance of tumor cells to chemotherapeutic agent supports the tumor-promoting role. However, whether *BCRP3* suppresses tumor formation during tumor initiating stage has not been explored. The tumor-initiating function is usually evaluated by genetic mouse model, but because mice lack the *BCRP3* gene counterpart, they cannot be studied in this way. Although the function of *BCRP3* in tumor initiation remains unclear, the impact of *BCRP3* on preventing pathological conditions associated with proteotoxicity is a direction worthy of further investigation, such as various

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neurodegenerative diseases, since autophagy is now known to play a protective role in these disease states [67]. Future studies will focus on understanding the function and expression of *BCRP3* for these pathologies.

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VI. Figure

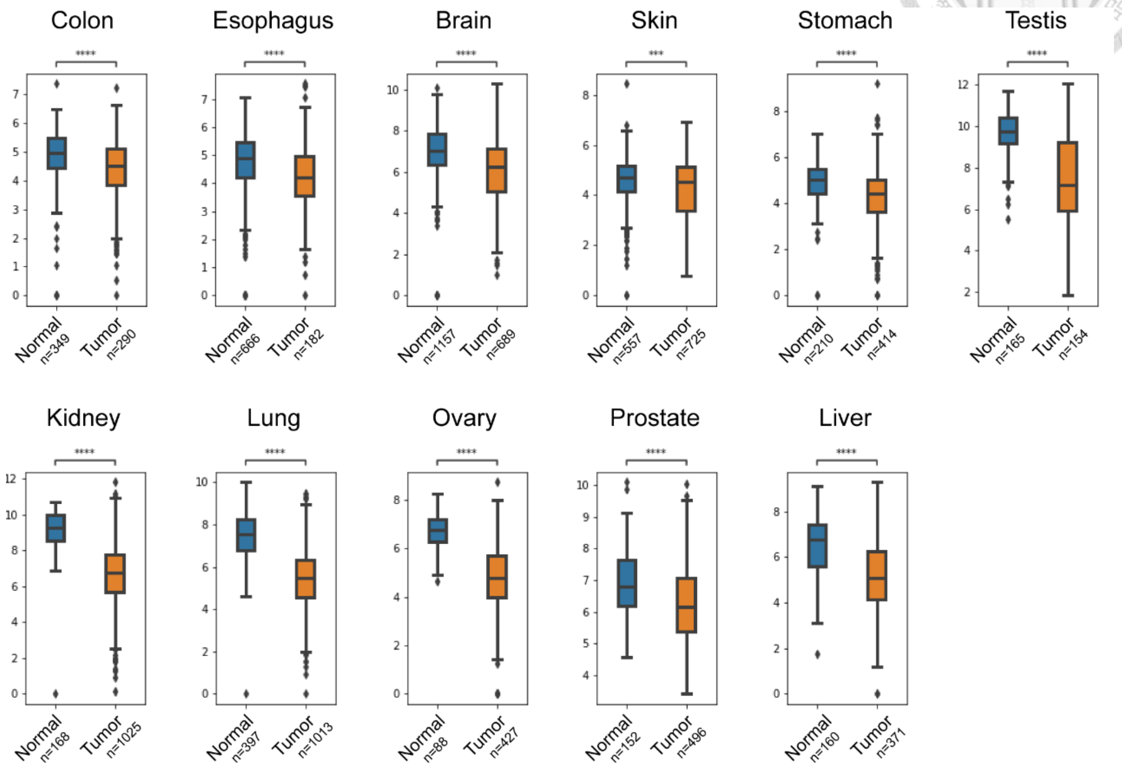

Figure 1. *BCRP3* gene expression level in various cancers and normal tissues.

BCRP3 gene expression level in tumors and normal tissues, including colon, esophagus, brain, skin, stomach, testis, kidney, lung, ovary, prostate, and liver cancers by analyzing Xena data sets. *P* values are determined by unpaired t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

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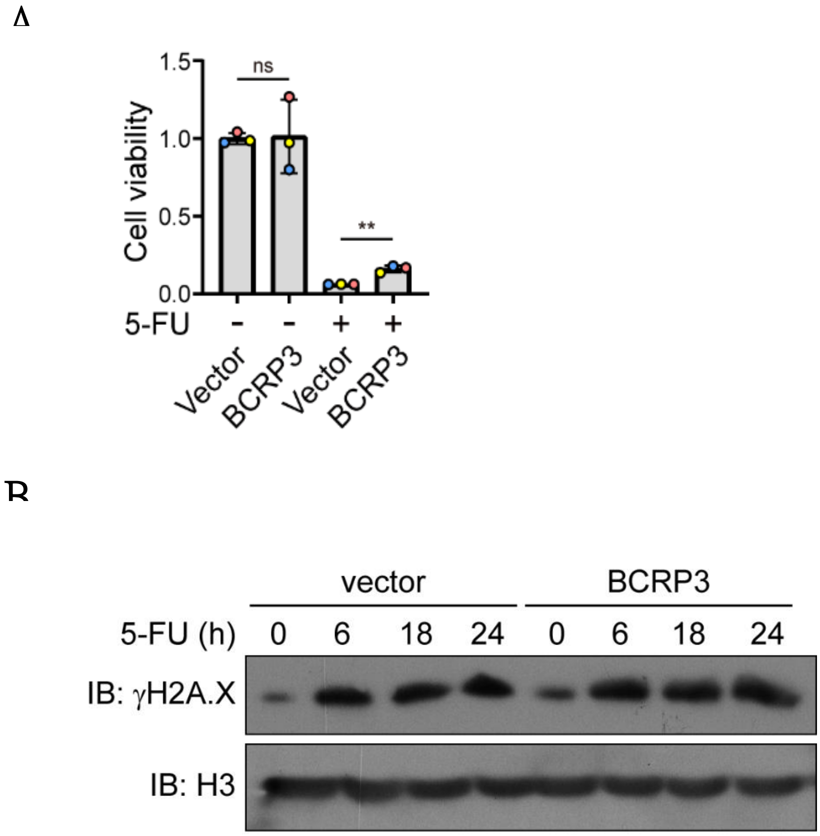

Figure 2. Overexpression of *BCRP3* increases the resistance of tumor cells to chemotherapy but does not affect DNA damage repair.

(A) Control or *BCRP3* overexpressed HCT116 human colorectal cancer cells were treated with or without 5 μM 5-FU for 48 hours. *P* values are determined by mean ± SD., $^{**}P < 0.01$. (B) Western blot analysis of γH2AX in control or *BCRP3* overexpressed HCT116 human colorectal cancer cells treated with 5 μM 5-FU for indicated time periods.

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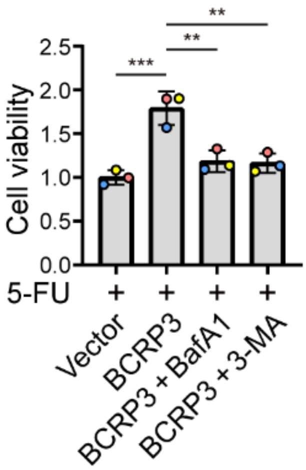

Figure 3. Autophagy inhibition reverses *BCRP3*-induced chemoresistance.

HCT116 stably cells were treated with 5 μM 5-FU for 48 hours, followed by 200 nM bafilomycin A1 or 10 mM 3-MA for 2 hours. Cell viability was determined by MTT assay. *P* values are determined by one-way ANOVA, ***P* < 0.01, ****P* < 0.001.

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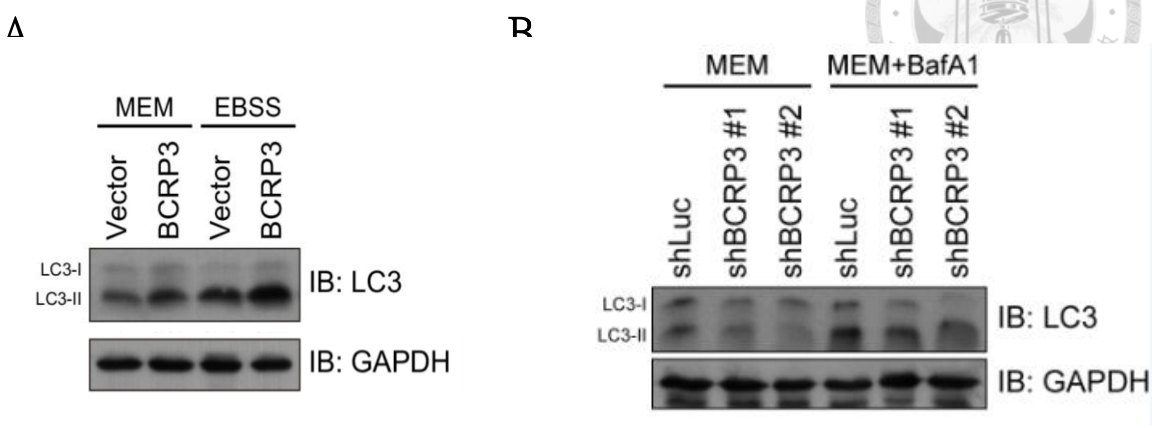

Figure 4. *BCRP3* increases LC3 lipidation.

(A) Control or expressing *BCRP3* in HCT116 stably cells were cultured in MEM and EBSS starved for 2 hours (B) Control or *BCRP3*-depleted 293T cells were cultured in MEM and treated with 200 nM Bafilomycin A1 for 2 hours. Western blot analysis of LC3 proteins is normalized with that of GAPDH and shown on the bottom.

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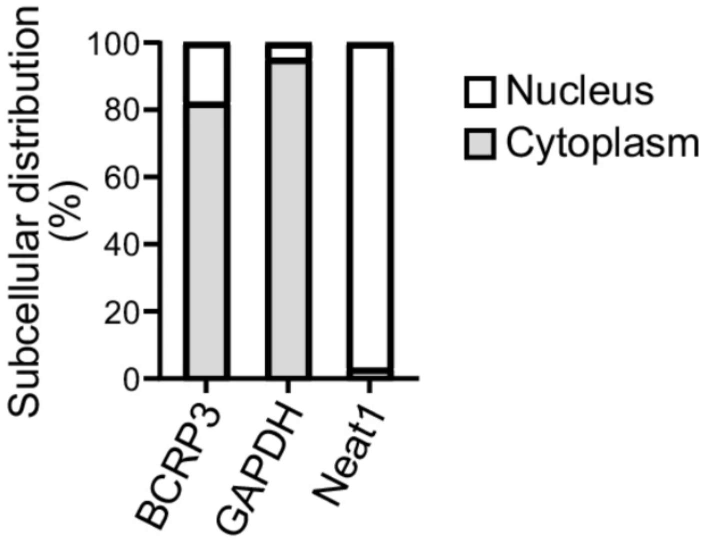

Figure 5. *BCRP3* is mainly localized in cytoplasm.

Subcellular localization of *BCRP3* in HeLa cells revealed by cell fractionation followed by qRT-PCR analysis. *GAPDH* or *Neat1* were used as a nuclear or cytoplasmic lncRNA control, respectively.

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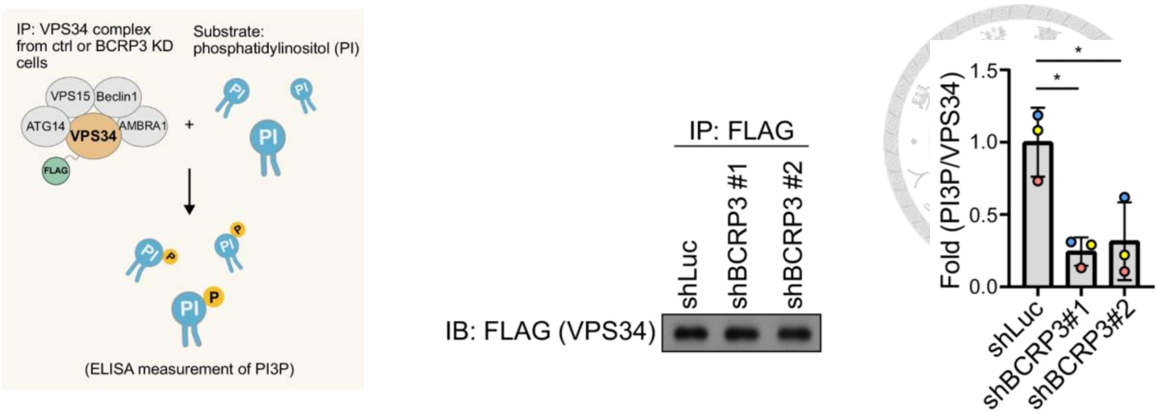

Figure 6. *BCRP3* enhances Vps34 activity.

FLAG-VPS34 immunoprecipitated from transfected HeLa cells were incubated with PI substrate and ATP, together with in vitro transcribed *BCRP3* or lambda RNA. PI3P production was measured by ELISA and FLAG-VPS34 protein levels were normalized. P values are determined by one-way ANOVA, *$P < 0.05$.

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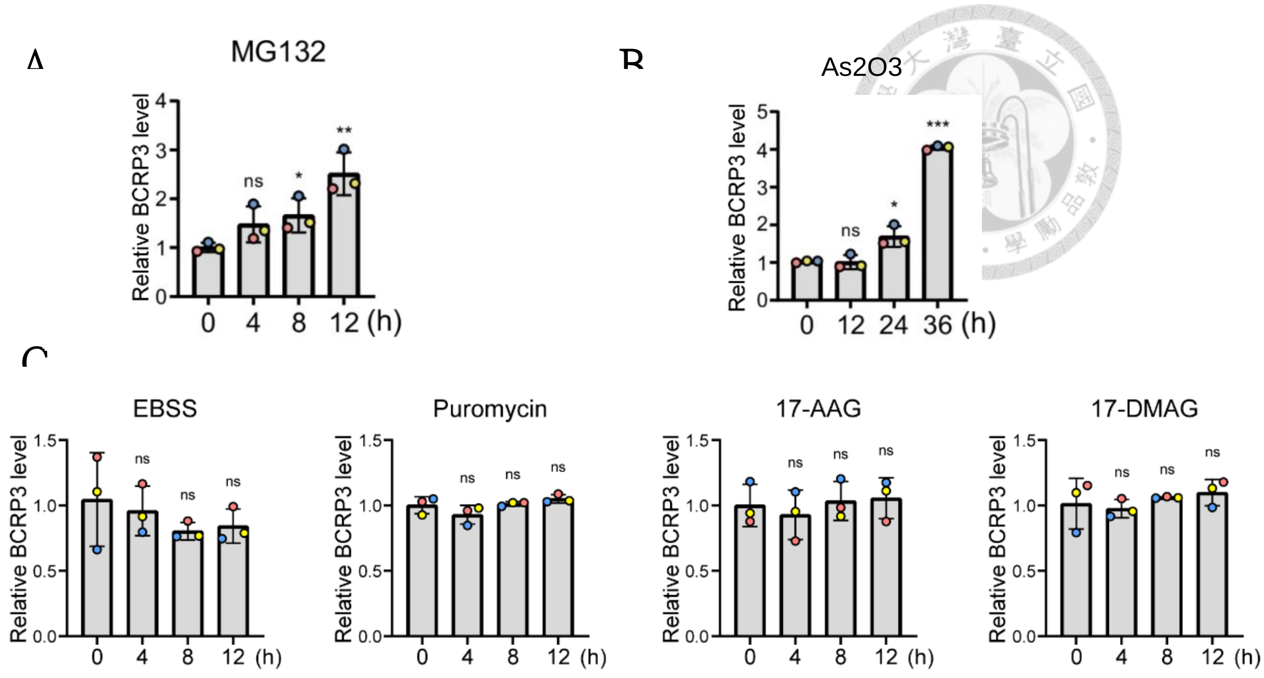

Figure 7. ***BCRP3*** **is upregulated by certain proteotoxic stresses.**

(A) qRT-PCR analysis of *BCRP3* levels in HeLa cells under 10 μM MG132 treatment for 12 hours. (B) qRT-PCR analysis of relative *BCRP3* levels in HeLa cells under 4 μM As2O3 treatment for 36 hours. (C) qRT-PCR analysis of relative *BCRP3* levels in HeLa cells under EBSS for 12 hours, 10 mg/ml puromycin, HSP90 inhibitors (10 mM 17-AAG, 10 mM 17-DMAG) for 12 hours. P values are determined by one-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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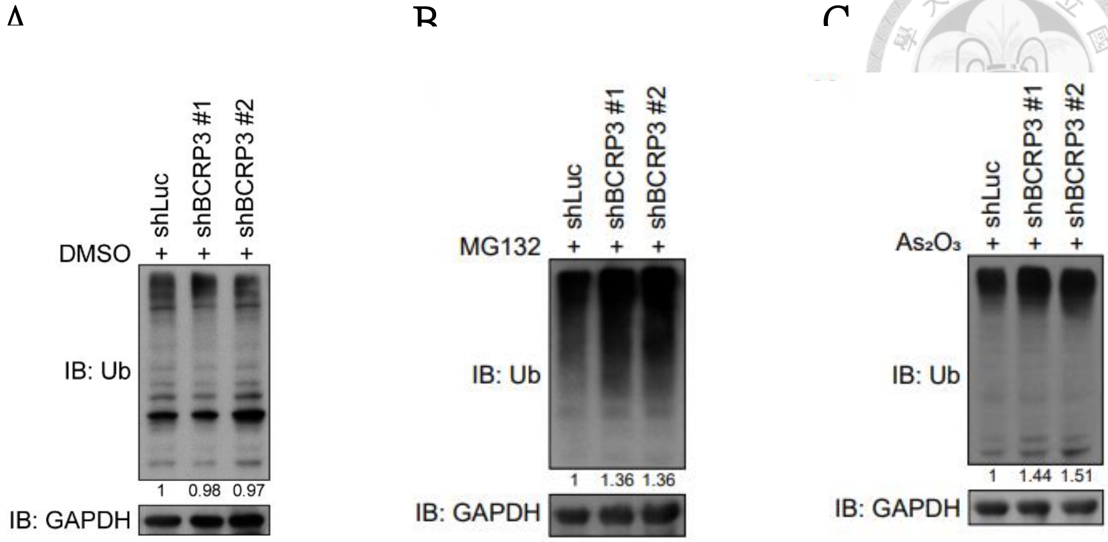

Figure 8. *BCRP3* deficiency increases the accumulations of ubiquitinated proteins under proteotoxic stresses.

(A) Western blot analysis using control or *BCRP3*-depleted HeLa cells cultured in normal conditions. Ubiquitinated protein levels were quantified, normalized with that of GAPDH, and shown on the bottom. (B-C) Western blot analysis using control or *BCRP3*-depleted HeLa cells cultured in 10 μM MG132 for 12 hours or 4 μM As2O3 for 24 hours. Ubiquitinated protein levels were quantified, normalized with that of GAPDH, and shown on the bottom.

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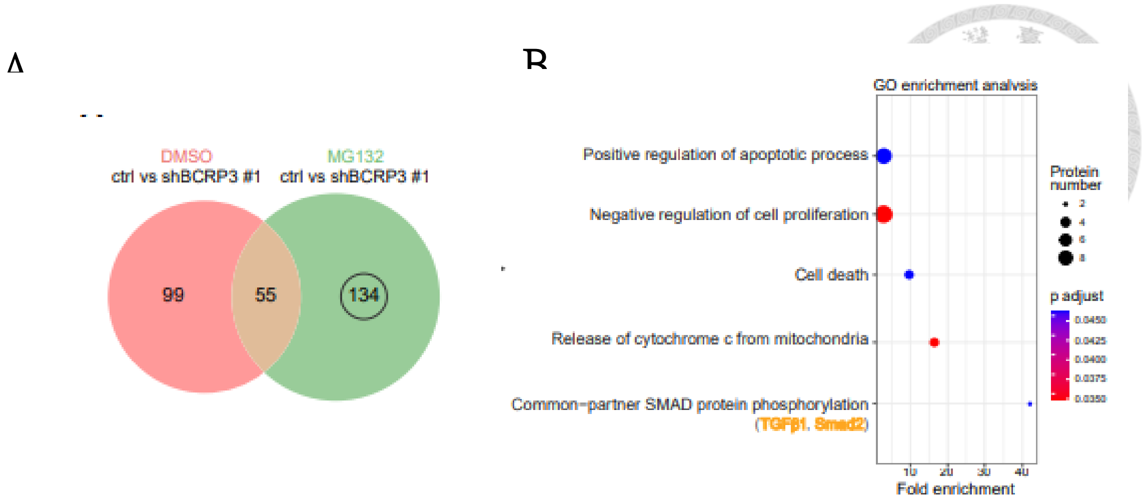

Figure 9. *BCRP3* deficiency under proteotoxic stress leads to the accumulation of proteins in growth arrest, apoptosis, and TGF-β pathway.

(A) Venn diagram showing the numbers of enriched proteins after control or *BCRP3* deficiency together with or without 10 μM MG132 treatment for 12 hours. (B) Gene ontology (GO) term analysis of the 134 proteins are shown by the order of fold enrichment.

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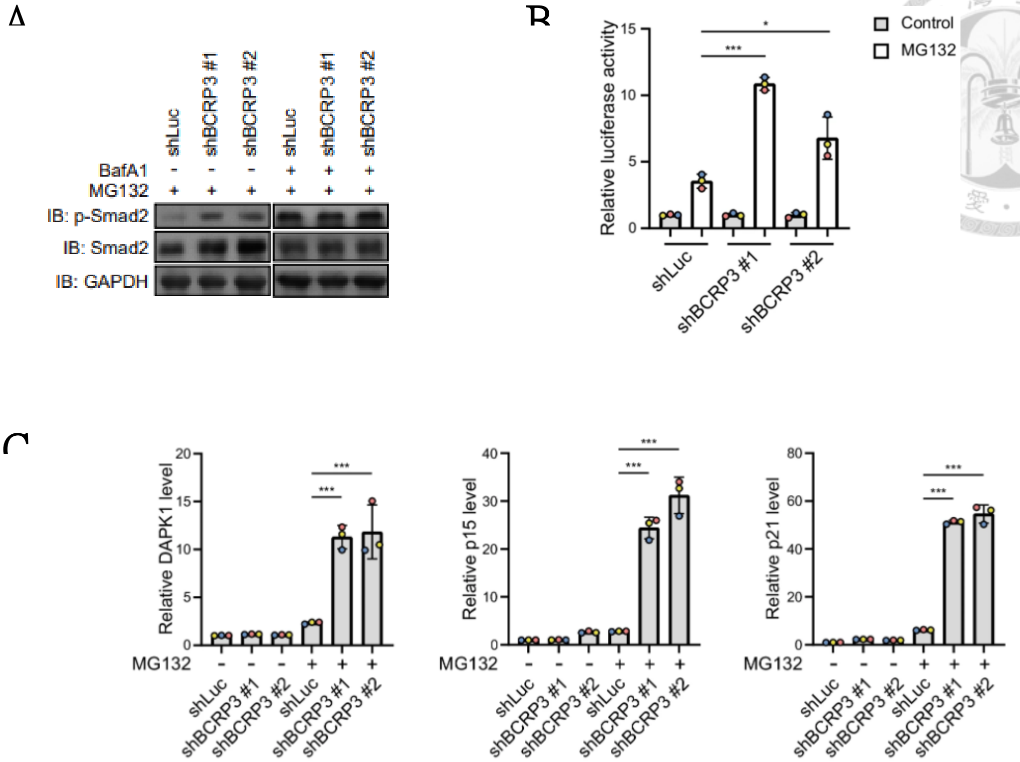

Figure 10. *BCRP3* deficiency under proteotoxicity enhances TGF-β signaling towards growth inhibition and apoptosis.

(A) Western blot analysis of p-SMAD2 and total SAMD2 in *BCRP3*-dpeleted HeLa cells treated with 10 μM MG132 for 12 hours and 200 nM Bafilomycin A1 was added in the final 2 hours. (B) Smad-responsive reporter activity (4xSBE-Luc) analyzed in control or *BCRP3*-depleted HeLa cells under 10 μM MG132 treatment for 12 hours. (C) qRT-PCR analysis of *DAPK1*, *p15*, and *p21* expression levels in control or *BCRP3*-depleted HeLa cells treated with 10 μM MG132 for 12 hours. P values are determined by one-way ANOVA, $*P < 0.05$, $***P < 0.001$

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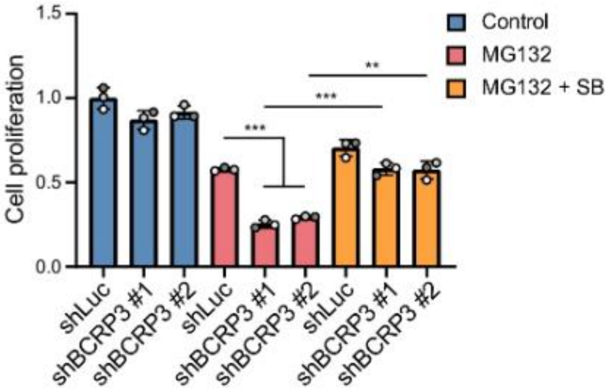
A



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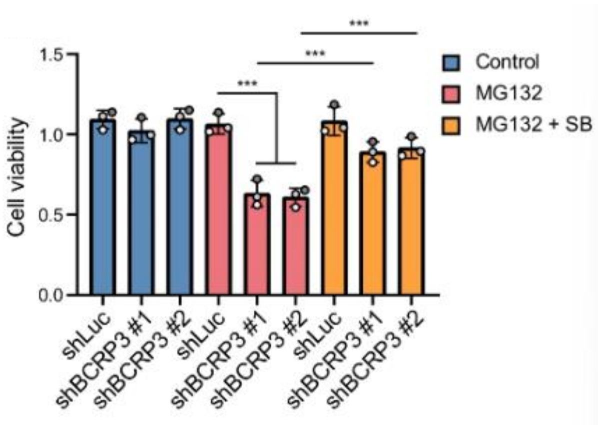

Figure 11. *BCRP3* deficiency under proteotoxicity decreases proliferation and cell viability.

(A) Control or *BCRP3*-depleted HeLa cells were treated with 10 µM MG132 for 12 hours or in combination with 4 µM SB431542 (the inhibitor of TGF-beta receptor) for 2 hours, and cell proliferation was measured by BrdU incorporation analysis. (B) Control or *BCRP3*-depleted HeLa cells were treated with 10 µM MG132 for 12 hours or in combination with 4 µM SB431542 (the inhibitor of TGF-beta receptor) for 2 hours, and cell viability was measured by MTT assays. P values are determined by one-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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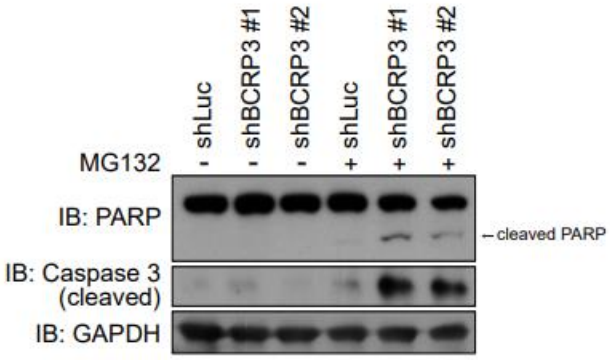

Figure 12. ***BCRP3*** **deficiency under proteotoxicity enhances apoptosis.**

Western blot analysis of PARP, Caspase3 in control or *BCRP3*-deficient HeLa cells were treated with 10 µM MG132 for 12 hours with or without 200 nM Bafilomycin A1 treatment 2 hours.

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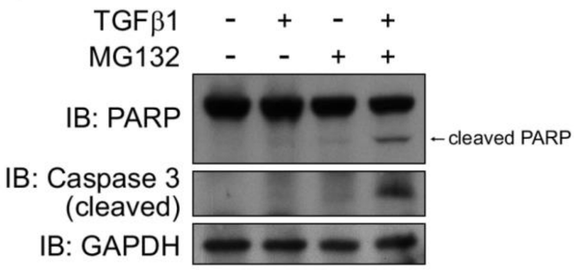

Figure 13. TGF-β pathway enhances apoptosis in MG132-treated cells.

Western blot analysis of PARP, Caspase3 in HeLa cells treated with or without 10 μM MG132 for 16 hours, followed by 5 ng/ml TGF-β for 2 hours.

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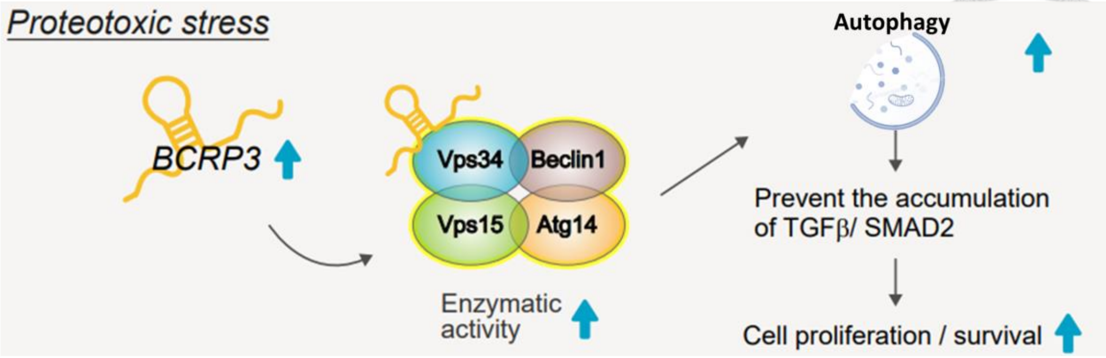

Figure 14. The model showing the mechanism for *BCRP3* in protein homeostasis and cell survival by activating autophagy.

In proteotoxic stress, LncRNA *BCRP3* induces autophagy by enhancing the activity of Vps34 complex for the maintenance of protein homeostasis and cell survival.

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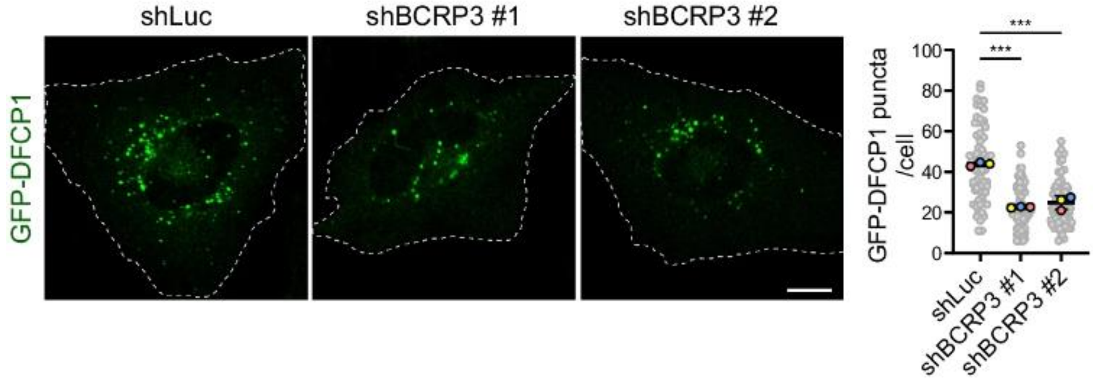
VII Appendix



Appendix 1. *BCRP3* deficiency impairs omegasome formation.

Control or *BCRP3*-depleted HeLa cells transfected with GFP-DFCP1 were EBSS starved for 2 hours, fixed and visualized by confocal microscopy. Measure GFP-DFCP1 puncta using ImageJ software. Quantification is shown on the right (mean ± SD; n > 70 cells, ***P < 0.001). Scale bar, 10 μm.

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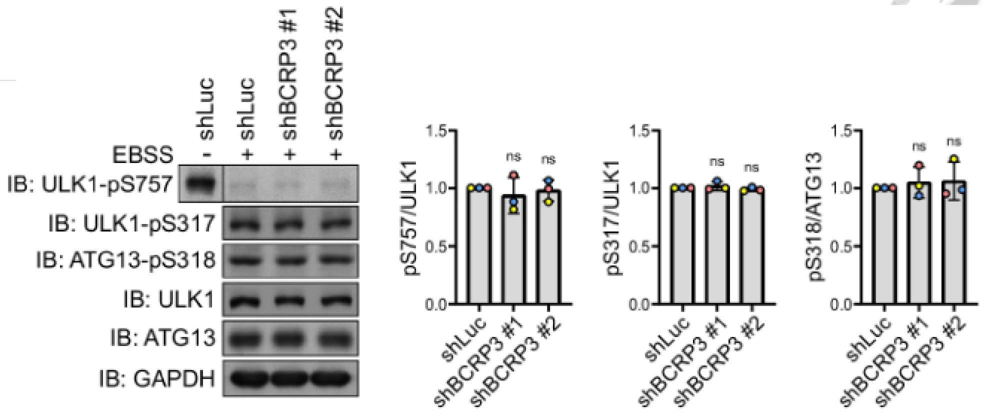

Appendix 2. *BCRP3* deficient did not affect ULK1, mTOR, and AMPK.

Control or *BCRP3*-depleted HeLa cells were EBSS starved for 1 hour before lysis. Whole cell lysates were immunoblotted with antibodies against total proteins or ULK1-pS757, ULK1-pS317 and Atg13-pS318, corresponding to the activities of ULK1, mTOR, and AMPK, respectively.

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Reference

1. Li, X., S. He, and B. Ma, *Autophagy and autophagy-related proteins in cancer.* Mol Cancer, 2020. **19**(1): p. 12.
2. Galluzzi, L., et al., *Autophagy in malignant transformation and cancer progression.* EMBO J, 2015. **34**(7): p. 856-80.
3. Levy, J.M.M., C.G. Towers, and A. Thorburn, *Targeting autophagy in cancer.* Nat Rev Cancer, 2017. **17**(9): p. 528-542.
4. Wong, S.Q., et al., *Autophagy in aging and longevity.* Hum Genet, 2020. **139**(3): p. 277-290.
5. Mihaylova, M.M. and R.J. Shaw, *The AMPK signalling pathway coordinates cell growth, autophagy and metabolism.* Nat Cell Biol, 2011. **13**(9): p. 1016-23.
6. Kim, J., et al., *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1.* Nat Cell Biol, 2011. **13**(2): p. 132-41.
7. Mizushima, N., *The role of the Atg1/ULK1 complex in autophagy regulation.* Curr Opin Cell Biol, 2010. **22**(2): p. 132-9.
8. Turco, E., D. Fracchiolla, and S. Martens, *Recruitment and Activation of the ULK1/Atg1 Kinase Complex in Selective Autophagy.* J Mol Biol, 2020. **432**(1): p. 123-134.
9. Zachari, M. and I.G. Ganley, *The mammalian ULK1 complex and autophagy initiation.* Essays Biochem, 2017. **61**(6): p. 585-596.
10. Karanasios, E., et al., *Dynamic association of the ULK1 complex with omegasomes during autophagy induction.* J Cell Sci, 2013. **126**(Pt 22): p. 5224-38.
11. Pyo, J.O., J. Nah, and Y.K. Jung, *Molecules and their functions in autophagy.* Experimental and Molecular Medicine, 2012. **44**(2): p. 73-80.
12. Funderburk, S.F., Q.J. Wang, and Z. Yue, *The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond.* Trends Cell Biol, 2010. **20**(6): p. 355-62.
13. Itakura, E., et al., *Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG.* Mol Biol Cell, 2008. **19**(12): p. 5360-72.
14. Thoresen, S.B., et al., *A phosphatidylinositol 3-kinase class III sub-complex containing VPS15, VPS34, Beclin 1, UVRAG and BIF-1 regulates cytokinesis and degradative endocytic traffic.* Exp Cell Res, 2010. **316**(20): p. 3368-78.
15. Karanasios, E., et al., *Autophagy initiation by ULK complex assembly on ER tubulovesicular regions marked by ATG9 vesicles.* Nat Commun, 2016. **7**: p. 12420.
16. Lystad, A.H., S.R. Carlsson, and A. Simonsen, *Toward the function of mammalian ATG12-ATG5-ATG16L1 complex in autophagy and related processes.* Autophagy, 2019. **15**(8): p. 1485-1486.

doi:10.6342/NTU202201725

17. Otomo, C., et al., *Structure of the human ATG12~ATG5 conjugate required for LC3 lipidation in autophagy.* Nat Struct Mol Biol, 2013. **20**(1): p. 59-66.
18. Kaiser, S.E., et al., *Noncanonical E2 recruitment by the autophagy E1 revealed by Atg7-Atg3 and Atg7-Atg10 structures.* Nat Struct Mol Biol, 2012. **19**(12): p. 1242-9.
19. Taherbhoy, A.M., et al., *Atg8 transfer from Atg7 to Atg3: a distinctive E1-E2 architecture and mechanism in the autophagy pathway.* Mol Cell, 2011. **44**(3): p. 451-61.
20. Satoo, K., et al., *The structure of Atg4B-LC3 complex reveals the mechanism of LC3 processing and delipidation during autophagy.* EMBO J, 2009. **28**(9): p. 1341-50.
21. Agrotis, A., et al., *Human ATG4 autophagy proteases counteract attachment of ubiquitin-like LC3/GABARAP proteins to other cellular proteins.* J Biol Chem, 2019. **294**(34): p. 12610-12621.
22. Yim, W.W. and N. Mizushima, *Lysosome biology in autophagy.* Cell Discov, 2020. **6**: p. 6.
23. Nakamura, S. and T. Yoshimori, *New insights into autophagosome-lysosome fusion.* Journal of Cell Science, 2017. **130**(7): p. 1209-1216.
24. Lamark, T. and T. Johansen, *Aggrephagy: selective disposal of protein aggregates by macroautophagy.* Int J Cell Biol, 2012. **2012**: p. 736905.
25. Park, C. and A.M. Cuervo, *Selective Autophagy: Talking with the UPS.* Cell Biochemistry and Biophysics, 2013. **67**(1): p. 3-13.
26. Rogov, V., et al., *Interactions between Autophagy Receptors and Ubiquitin-like Proteins Form the Molecular Basis for Selective Autophagy.* Molecular Cell, 2014. **53**(2): p. 167-178.
27. Pohl, C. and I. Dikic, *Cellular quality control by the ubiquitin-proteasome system and autophagy.* Science, 2019. **366**(6467): p. 818-822.
28. Lamark, T., et al., *NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets.* Cell Cycle, 2009. **8**(13): p. 1986-1990.
29. Sun, D., et al., *Phase Separation in Regulation of Aggrephagy.* J Mol Biol, 2020. **432**(1): p. 160-169.
30. Boland, B., et al., *Promoting the clearance of neurotoxic proteins in neurodegenerative disorders of ageing.* Nat Rev Drug Discov, 2018. **17**(9): p. 660-688.
31. Djebali, S., et al., *Landscape of transcription in human cells.* Nature, 2012. **489**(7414): p. 101-8.
32. Statello, L., et al., *Gene regulation by long non-coding RNAs and its biological functions.* Nat Rev Mol Cell Biol, 2021. **22**(2): p. 96-118.

doi:10.6342/NTU202201725

33. Yao, R.W., Y. Wang, and L.L. Chen, *Cellular functions of long noncoding RNAs.* Nat Cell Biol, 2019. **21**(5): p. 542-551.
34. Akkipeddi, S.M.K., A.J. Velleca, and D.M. Carone, *Probing the function of long noncoding RNAs in the nucleus.* Chromosome Res, 2020. **28**(1): p. 87-110.
35. Noh, J.H., et al., *Cytoplasmic functions of long noncoding RNAs.* Wiley Interdiscip Rev RNA, 2018. **9**(3): p. e1471.
36. Wang, K.C. and H.Y. Chang, *Molecular mechanisms of long noncoding RNAs.* Mol Cell, 2011. **43**(6): p. 904-14.
37. Huarte, M., *The emerging role of lncRNAs in cancer.* Nat Med, 2015. **21**(11): p. 1253-61.
38. Yang, G., X. Lu, and L. Yuan, *LncRNA: a link between RNA and cancer.* Biochim Biophys Acta, 2014. **1839**(11): p. 1097-109.
39. Jiang, M.C., et al., *Emerging roles of lncRNA in cancer and therapeutic opportunities.* Am J Cancer Res, 2019. **9**(7): p. 1354-1366.
40. Liu, Y. and M. Zhao, *lnCaNet: pan-cancer co-expression network for human lncRNA and cancer genes.* Bioinformatics, 2016. **32**(10): p. 1595-7.
41. Guzel, E., et al., *Tumor suppressor and oncogenic role of long non-coding RNAs in cancer.* North Clin Istanb, 2020. **7**(1): p. 81-86.
42. Tao, W., et al., *LncRNA DANCR contributes to tumor progression via targetting miR-216a-5p in breast cancer: lncRNA DANCR contributes to tumor progression.* Biosci Rep, 2019. **39**(4).
43. Liu, Y., et al., *Over-expression of lncRNA DANCR is associated with advanced tumor progression and poor prognosis in patients with colorectal cancer.* Int J Clin Exp Pathol, 2015. **8**(9): p. 11480-4.
44. Taniue, K. and N. Akimitsu, *The Functions and Unique Features of LncRNAs in Cancer Development and Tumorigenesis.* Int J Mol Sci, 2021. **22**(2).
45. Liu, Q., et al., *LncRNA loc285194 is a p53-regulated tumor suppressor.* Nucleic Acids Res, 2013. **41**(9): p. 4976-87.
46. Jadaliha, M., et al., *A natural antisense lncRNA controls breast cancer progression by promoting tumor suppressor gene mRNA stability.* PLoS Genet, 2018. **14**(11): p. e1007802.
47. Li, J., et al., *LncRNA TUG1 acts as a tumor suppressor in human glioma by promoting cell apoptosis.* Exp Biol Med (Maywood), 2016. **241**(6): p. 644-9.
48. Qian, Y., L. Shi, and Z. Luo, *Long Non-coding RNAs in Cancer: Implications for Diagnosis, Prognosis, and Therapy.* Front Med (Lausanne), 2020. **7**: p. 612393.
49. Liu, P.F., et al., *Regulatory effects of noncoding RNAs on the interplay of oxidative stress and autophagy in cancer malignancy and therapy.* Semin Cancer Biol, 2020.

doi:10.6342/NTU202201725

50. Yang, L., et al., *Long non-coding RNAs involved in autophagy regulation.* Cell Death Dis, 2017. **8**(10): p. e3073.
51. Liang, J., L. Zhang, and W. Cheng, *Non-coding RNA-mediated autophagy in cancer: A protumor or antitumor factor?* Biochim Biophys Acta Rev Cancer, 2021. **1876**(2): p. 188642.
52. Aboudehen, K., *Regulation of mTOR signaling by long non-coding RNA.* Biochim Biophys Acta Gene Regul Mech, 2020. **1863**(4): p. 194449.
53. Liu, H., et al., *lncRNA CASC19 Contributes to Radioresistance of Nasopharyngeal Carcinoma by Promoting Autophagy via AMPK-mTOR Pathway.* Int J Mol Sci, 2021. **22**(3).
54. Wang, X., et al., *LncRNA SNHG6 promotes chemoresistance through ULK1-induced autophagy by sponging miR-26a-5p in colorectal cancer cells.* Cancer Cell Int, 2019. **19**: p. 234.
55. Zhao, W., et al., *Long noncoding RNA H19 contributes to the proliferation and autophagy of glioma cells through mTOR/ULK1 pathway.* Neuroreport, 2021. **32**(5): p. 352-358.
56. Yu, Y. and X.H. Feng, *TGF-beta signaling in cell fate control and cancer.* Curr Opin Cell Biol, 2019. **61**: p. 56-63.
57. Vander Ark, A., J. Cao, and X. Li, *TGF-beta receptors: In and beyond TGF-beta signaling.* Cell Signal, 2018. **52**: p. 112-120.
58. Zhang, Y.E., *Non-Smad pathways in TGF-beta signaling.* Cell Res, 2009. **19**(1): p. 128-39.
59. Zhang, Y., P.B. Alexander, and X.F. Wang, *TGF-beta Family Signaling in the Control of Cell Proliferation and Survival.* Cold Spring Harb Perspect Biol, 2017. **9**(4).
60. Katz, L.H., et al., *Targeting TGF-beta signaling in cancer.* Expert Opin Ther Targets, 2013. **17**(7): p. 743-60.
61. Ramesh, S., et al., *TGF beta-mediated BIM expression and apoptosis are regulated through SMAD3-dependent expression of the MAPK phosphatase MKP2.* Embo Reports, 2008. **9**(10): p. 990-997.
62. Jang, C.W., et al., *TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase.* Nature Cell Biology, 2002. **4**(1): p. 51-58.
63. Rostislavleva, K., et al., *Structure and flexibility of the endosomal Vps34 complex reveals the basis of its function on membranes.* Science, 2015. **350**(6257): p. aac7365.
64. Szeto, J., et al., *ALIS are stress-induced protein storage compartments for substrates of the proteasome and autophagy.* Autophagy, 2006. **2**(3): p. 189-99.

doi:10.6342/NTU202201725

65. Dikic, I., *Proteasomal and Autophagic Degradation Systems.* Annu Rev Biochem, 2017. **86**: p. 193-224.
66. Zellner, S., M. Schifferer, and C. Behrends, *Systematically defining selective autophagy receptor-specific cargo using autophagosome content profiling.* Mol Cell, 2021. **81**(6): p. 1337-1354 e8.
67. Lim, J. and Z. Yue, *Neuronal aggregates: formation, clearance, and spreading.* Dev Cell, 2015. **32**(4): p. 491-501.

doi:10.6342/NTU202201725