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Overexpression of the Ubiquilin-4 (UBQLN4) is Associated with Cell Cycle Arrest and Apoptosis in Human Normal Gastric Epithelial Cell Lines GES-1 Cells by Activation of the ERK Signaling Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

AEF 1,2,3,4 Shengkai Huang*
AEF 1,2,3 Xin Dong*
BD 5 Jia Wang
BDE 6 Jie Ding
BD 2,3,4 Yan Li
BD 2,3,4 Dongdong Li
BD 2,3,4 Hong Lin
BD 2,3,4 Wenjie Wang
E 2,3,4 Mei Zhao
AF 7 Qing Chang
AF 8 Ning Zhou
AE 1 Wei Cui
AEFG 2,3,4 Changzhi Huang

1 Department of Clinical Laboratory, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P.R. China
2 State Key Laboratory of Molecular Oncology, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P.R. China
3 Department of Etiology and Carcinogenesis, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P.R. China
4 Beijing Key Laboratory for Carcinogenesis and Cancer Prevention, Beijing, P.R. China
5 Department of Clinical Laboratory, Meitan General Hospital, Beijing, P.R. China
6 State Key Laboratory of Cardiovascular Disease, Anesthesia Center, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P.R. China
7 Department of Ultrasound, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P.R. China
8 The Central People's Hospital of Zhanjiang, Zhanjiang, Guangdong, P.R. China

* These authors contributed equally to this work

Corresponding Authors: Changzhi Huang, e-mail: huangpumc@163.com, Wei Cui, e-mail: wendycuiwei@sina.cn, Ning Zhou, e-mail: zjzhou121@163.com, Qing Chang, e-mail: chq.qing@163.com

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Background: Ubiquilin-4 (UBQLN4) is a component of the ubiquitin-proteasome system and regulates the degradation of many proteins implicated in pathological conditions. The aim of this study was to determine the role of UBQLN4 in regulating the proliferation and survival of the normal gastric epithelial cell line GES-1.

Material/Methods: We constructed GES-1 lines stably overexpressing UBQLN4 by lentiviral infection. Cell proliferation, apoptosis, and the cell cycle were analyzed using the MTT assay and flow cytometric assays. Phosphorylation of ERK, JNK, p38, and expression of cyclin D1 were detected by western blot analysis.

Results: Overexpression of UBQLN4 significantly reduced proliferation and induced G2/M phase arrest and apoptosis in GES-1 cells. Moreover, upregulation of UBQLN4 increased the expression of cyclin D1 and phosphorylated ERK, but not JNK or p38.

Conclusions: These data suggest that UBQLN4 may induce cell cycle arrest and apoptosis via activation of the ERK pathway and upregulation of cyclin D1 in GES-1 cells.

MeSH Keywords: Apoptosis • Cell Cycle • MAP Kinase Signaling System • Ubiquitins

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Background

Ubiquilin-4 (UBQLN4) is a member of the ubiquilin protein family, each of which contains an N-terminal ubiquitin-like domain and a C-terminal ubiquitin-associated domain (UBA). The UBA domain of UBQLN4 binds polyubiquitin chains and has a role in the stability of UBQLN4 and in the subcellular localization of UBQLN4 [1]. UBQLN4 functions primarily to facilitate protein degradation via the ubiquitin-proteasome system, this places it within the ubiquitin-like protein family that is involved in proteasome-mediated activities [2]. Research has identified the UBQLN4 as a cellular target of the small hydrophobic (SH) protein of the mumps virus, the ectopic expression of UBQLN4 leads to a re-distribution of SH to punctate structures in transfected or infected mammalian cells [3]. In addition to its classic role in protein degradation, UBQLN4 has been implicated in a number of pathological and physiological processes, including ubiquitin-independent proteasomal degradation [4], autophagy, and receptor trafficking [5]. UBQLN4 is a BCL2 associated athanogene 6 (BAG6) binding factor that also eliminates newly synthesized defective polypeptides [6]. A recent study indicated that UBQLN4 is associated with amyotrophic lateral sclerosis and its expression compromises motor axon morphogenesis in mouse motor neurons and in zebrafish [7]. UBQLN4 is thought to regulate the abundance of proteins implicated in these events.

Ubiquilin also influences the fate of proteins involved in cell proliferation and apoptosis. For example, UBQLN1 stabilizes BCLb, a member of the BCL2 family of apoptosis-regulating proteins. UBQLN1 promotes the mono-ubiquitination of BCLb on multiple lysine residues, inducing its relocation to the cytosol, and it has been suggested that the anti-apoptotic activity of BCLb regulated by UBQLN1 may be an important factor in lung cancer [8,9].

Notably, many key apoptosis-related molecules, including BCL2 and caspase family proteins, are regulated by 2 major signaling pathways: the RAS/RAF/MEK/ERK pathway and the RAS/phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathways [8]. Upregulation of ERK and AKT contributes to abnormal cell proliferation and tumorigenesis [10] and prevents apoptosis by phosphorylating and inhibiting caspase-9. ERK activation also induces cell cycle arrest and apoptosis of cells exposed to destructive agents. For example, irradiation- and chemical-induced DNA damage promote G2/M accumulation and cell apoptosis via enhanced ERK expression [11]. However, in some cells, aberrant activation of MEK–ERK signaling promotes cell cycle progression but results in apoptosis [12,13].

Alterations in the level or activity of cell cycle regulatory proteins such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors can cause exit and re-entry into the cell cycle, but the mechanisms that regulate the expression of these proteins are poorly understood [14,15]. Cyclin E and CDK2 are thought to drive progression through G1/S, and CDC25-regulated cyclin B, and CDK2 has been reported to control the G2/M transition [16,17]. Previous studies have shown that activation of mitogen-activated protein kinase (MAPK) family proteins, including ERK, JNK, and p38, regulate apoptosis and cell cycle progression of cancer cells [18–20].

In this study, we investigated the function of UBQLN4 in the human gastric epithelial cell line GES-1, including its relationship to ERK signaling, cell cycle progression, and apoptosis. Our goal was to determine whether UBQLN4 plays a role in these processes via activation of the ERK pathway in GES-1 cells.

Material and Methods

Cell culture

The gastric cancer cell lines MKN45 and BGC-823 and the immortalized normal human gastric epithelial cell line GES-1 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone), 100 IU/mL penicillin, and 100 µg/mL streptomycin and were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Lentivirus production and generation of stable cell lines

Human *UBQLN4* cDNA was kindly provided by Dr. Jiahuai Han and was cloned into the pLVX-Puro vector (designated pLVX) with an N-terminal Flag or EGFP tag. Lentiviruses were produced by co-transfection of 293T cells with empty pLVX or pLVX-UBQLN4 together with the packaging vectors psPAX2 and pMD2.G using X-tremeGENE HP DNA Transfection Reagent (Roche, USA) according to the manufacturer's instructions. At 48 hours post-transfection, the supernatants were collected, filtered, and added to GES-1, MKN45, or BGC-823 cells. After 24 hours, the cells were transferred to fresh complete medium containing 2 µg/mL puromycin and cultured for 2 weeks to generate stably transfected cell lines.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay

Cells were plated into 96-well plates at a density of 1.5×10^3 cells/well (n=8 wells per condition), and cell viability/proliferation was examined every 24 hours for 72 hours. Briefly, at the appropriate time, 20 µL of MTT solution (5 mg/mL;

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Sigma-Aldrich, USA) and 90 µL DMEM were added to the cells, and the plates were incubated at 37°C for 4 hours. The medium was aspirated and 150 µL of dimethyl sulfoxide was added to each well. Absorbance at 492 nm was measured on a microplate spectrophotometer (Thermo Fisher Scientific, MA, USA). All assays were repeated at least 3 times.

Protein extraction and western blotting

Cells were lysed in lysis buffer (150 mM NaCl, 1.5% NP-40, 50 mM Tris-HCl, pH 7.4, 0.1% sodium dodecyl sulfate [SDS], 50 µg/mL phenylmethylsulfonyl fluoride, and fresh proteinase inhibitor cocktail [Roche]) for 30 min on ice, and then centrifuged at 13 000 rpm for 15 min at 4°C. The supernatant was collected, and total protein concentration was measured with a BCA assay (Sigma-Aldrich). Proteins were separated on 6–12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline (TBS) for 1 hour at room temperature (RT), and then incubated with primary antibodies overnight at 4°C. The membranes were then washed in TBS containing 1% Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour at RT. Membranes were washed again with 1% Tween 20 in TBS and treated with enhanced chemiluminescence detection reagents (Applygen Technologies, China). Finally, protein bands were detected with a Fujifilm LAS-4000 imager (Fujifilm Life Science, USA). The primary antibody dilutions and sources were as follows: UBQLN4 (1: 1000; Santa Cruz Biotechnology, USA); cyclin D1 (1: 1000), p38 (1: 1000), phosphorylated (p)-p38 (Thr180/Tyr182,1: 1000), ERK (1: 1000), p-ERK (Thr202/Tyr204, 1: 1000), JNK (1: 1000), p-JNK (Thr183/Tyr185, 1: 1000), AKT (1: 1000), p-AKT (Ser473, 1: 1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1: 1000), all from Cell Signaling Technology (MA, USA).

Flow cytometric analysis

For cell cycle analysis, cells were harvested, washed with phosphate buffered saline (PBS), and fixed in 75% ethanol at –20°C overnight. RNA was removed by incubating the cells with RNase A (100 µg/mL; Sigma-Aldrich) and 0.25% (v/v) Triton X-100 at 37°C for 30 min. Cells were then stained with propidium iodide (PI) solution (50 µg/mL; Sigma-Aldrich) for 30 min at RT and analyzed on a BD LSR II flow cytometer (BD Biosciences, CA, USA). For analysis of apoptosis, an Annexin V-PE Apoptosis Detection Kit (BD Biosciences, CA, USA) was used according to the manufacturer's instructions. In brief, cells were washed twice with cold PBS and then resuspended in 1× Binding Buffer at a concentration of 1×10^6 cells/mL. Aliquots of 100 µL were transferred to a 5 mL culture tube and mixed with 5 µL each of Annexin V-PE and 7-aminoactinomycin D (7-AAD). The cells were gently vortexed and incubated for 15 min at RT in the dark. Then 1× Binding Buffer (400 µL/tube) was added and the cells were analyzed by flow cytometry within 1 hour.

Fluorescence microscopy

EGFP-expressing GES-1 cells were cultured to 60–80% confluence, washed 3 times with PBS, fixed with 4% paraformaldehyde, and then permeabilized with 0.5% Triton X-100. Cells were incubated with 4',6-diamidino-2-phenylindole (DAPI), mounted, and visualized on a fluorescence microscope.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics V20.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). Differences between groups were analyzed with Student's *t*-test. *P* values <0.05 were considered statistically significant. Prism 5.0 software (GraphPad, La Jolla, CA, USA) was used to generate the graphs.

Results

Overexpression of UBQLN4 suppresses proliferation and promotes apoptosis in human gastric epithelial cells

To assess the influence of UBQLN4 on proliferation and apoptosis in human gastric epithelial cells, we stably overexpressed UBQLN4 in GES-1 cells. Western blot analysis confirmed robust expression of UBQLN4 in pLVX-UBQLN4-infected cells compared with the control uninfected or pLVX (empty vector)-infected cells (Figure 1A). Bright field microscopy showed that UBQLN4-overexpressing cells were smaller and shriveled compared with control cells, suggesting they were undergoing apoptosis (Figure 1B). Furthermore, fluorescence microscopy showed that the nuclei of EGFP-UBQLN4-overexpressing cells were abnormal compared with the control cells (Figure 1C). Consistent with these observations, evaluation of cell proliferation using the MTT assay indicated that UBQLN4 overexpression significantly inhibited the proliferation of GES-1 cells and the gastric cancer cell lines MKN45 and BGC-823 (Figure 1D). Taken together, these data suggest that UBQLN4 overexpression might reduce proliferation and induce apoptosis in GES-1 cells.

UBQLN4 induces cell cycle arrest and apoptosis in GES-1 cells

To evaluate whether the morphological changes in UBQLN4-overexpressing cells were consistent with cell death, we investigated the cell cycle and apoptosis using flow cytometric assays. When labeled with the fluorescent markers Annexin V-PE and 7-AAD, early apoptotic cells appear as Annexin V-PE+/7-AAD$^-$ (lower right quadrant in Figure 2A) and late apoptotic cells as

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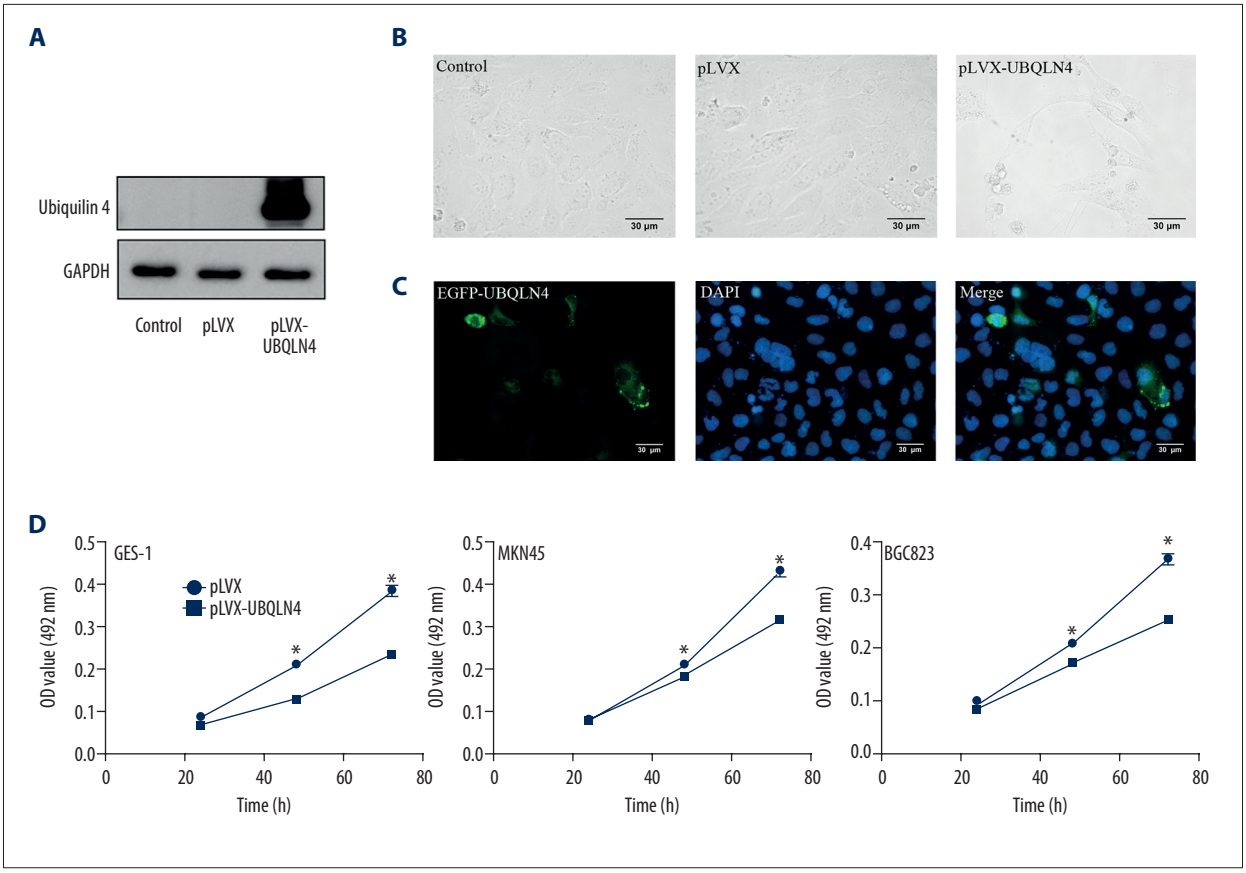

Figure 1. Overexpression of UBQLN4 induced apoptosis in GES-1 cells. (**A**) Western blot analysis of UBQLN4 protein expression in uninfected (control) GES-1 cells or cells infected with empty (pLVX) or UBQLN4-encoding (pLVX-UBQLN4) lentiviruses. GAPDH was probed as a loading control. (**B**) Light microscopic images showing the morphology of GES-1 cells as described in (**A**). Scale bar, 30 μm. (**C**) Fluorescence microscopy of GES-1 cells expressing an EGFP–UBQLN4 fusion protein. Nuclei were stained with DAPI. Scale bar, 30 μm. (**D**) MTT proliferation assay of pLVX- or pLVX-UBQLN4-infected GES-1, MKN45, and BGC-823 cells. Data are shown as the mean ±SD of 8 experiments. * $P<0.05$ for comparison with the control.

Annexin V-PE+/7-AAD+ (upper right quadrant). Overexpression of UBQLN4 caused a marked increase in the proportion of early apoptotic cells compared with uninfected or pLVX-infected cells (Figure 2A). Moreover, cell cycle analysis of PI-stained cells indicated that UBQLN4 expression induced arrest of the cells at the G2/M transition. At 48 hours post-infection, 38.3±0.2% of control cells and 28.0±1.3% of UBQLN4-overexpressing cells were in G0/G1, compared with 24.6±0.6% and 31.6±0.9% in G2/M. At 72 hours, these percentages were 39.8±1.2% and 26.7±1.1% in G0/G1 and 24.4±0.4% and 32.1±0.9% in G2/M (Figure 2B). These results demonstrated that overexpression of UBQLN4 induces cell cycle arrest in the G2/M phase and increases apoptosis in GES-1 cells.

UBQLN4 activates ERK and increases cyclin D1 levels

ERK signaling is crucial for numerous cell responses, including proliferation, migration, differentiation, and apoptosis, indicating that ERK has a dual role in regulating cell growth and survival [21,22]. ERK signaling promotes entry into the cell cycle and induces apoptosis by elevating cyclin D1 levels [15,23]. To determine whether a similar pathway may explain the effects of UBQLN4 overexpression in gastric cells, we examined expression of cyclin D1 and components of the ERK signaling pathway. Indeed, western blot analysis showed that GES-1-pLVX-UBQLN4 cells expressed higher levels of cyclin D1 (Figure 3A) and phosphorylated (activated) ERK (Thr202/Tyr204) compared with control cells (Figure 3B). Moreover, this effect was not restricted to normal cells since UBQLN4 overexpression in MKN45 and BGC-823 also increased phosphorylated ERK levels (Figure 3B). Expression of phosphorylated ERK (Thr202/Tyr204) and AKT (Ser473) were both increased in a time-dependent manner after serum starvation of UBQLN4- overexpressing GES-1 cells compared with control cells (Figure 3C). These observations suggest that UBQLN4 overexpression may induce apoptosis via activation of the ERK signaling pathway. Importantly, phosphorylation of JNK (Thr183/Tyr185) and p38 (Thr180/Tyr182) were not affected by UBQLN4 expression

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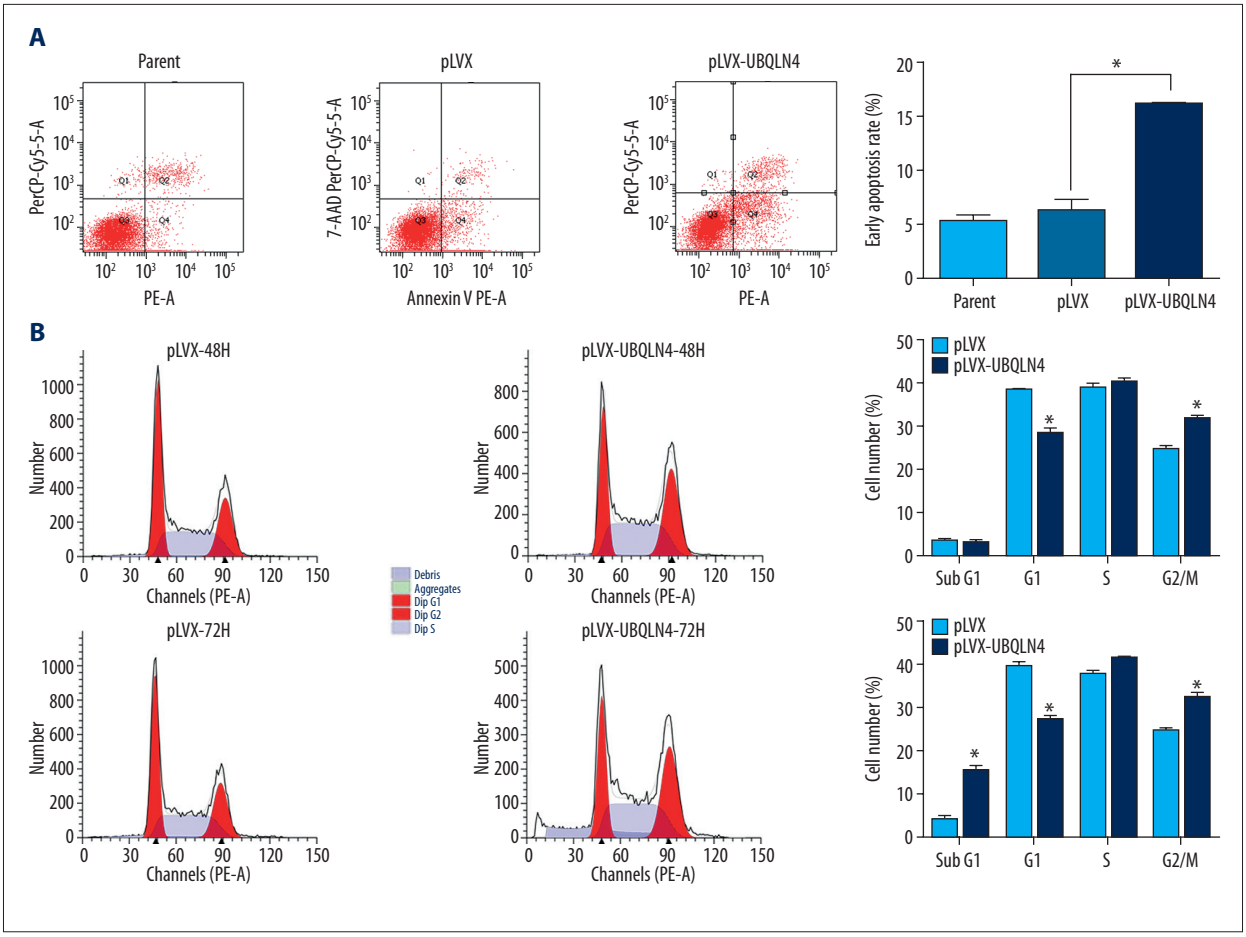

Figure 2. UNQLN4 induced cell cycle arrest and apoptosis in GES-1 cells. (**A**) Flow cytometry dot plots and quantification of early apoptosis of Annexin V-PE/7-AAD-labeled control or UBQLN4-expressing GES-1 cells. (**B**) Flow cytometry histograms and quantification of PI-labeled GES-1 cells in each phase of the cell cycle at 48 hours (upper) and 72 hours (lower) after infection with pLVX or pLVX-UBQLN4. Data are shown as the mean ±SD of 3 experiments. * $P<0.05$ for comparison with the control.

(Figure 3D), suggesting that UBQLN4 selectively activates ERK, but not JNK or p38. Taken together, these data indicate that UBQLN4 may induce cell cycle arrest and promote apoptosis in gastric cells through the ERK signaling pathway and cyclin D1.

Discussion

Ubiquitin-mediated proteolysis constitutes an important component of the core oscillator which drives the cell cycle in all eukaryotes [24]. Here, we sought to understand the function of UBQLN4 in regulating life and death in GES-1 cells. Using a lentivirus-mediated overexpression system, we demonstrated that UBQLN4 reduces proliferation and induces cell cycle arrest and apoptosis in GES-1 cells. Many studies have sought to understand the cell cycle machinery and signaling pathways that control cell cycle arrest and apoptosis [25]. Cell cycle checkpoints exist to ensure that DNA damage is effectively repaired; accordingly, irreparable damage can activate cell cycle arrest and apoptotic signaling pathways [26]. Arrest in G2/M phase of the cell cycle can reduce proliferation and induce apoptosis by inhibiting the symmetrical segregation of damaged chromosomes during mitosis [27]. In the present study, we found that UBQLN4 overexpression increased the proportion of GES-1 cells in G2/M compared with control cells, suggesting that a block in G2/M transition may have triggered the apoptotic program.

Apoptosis is a biochemically and morphologically distinct mode of cell death elicited by exposure of cells to cancer chemotherapeutic agents and other toxic compounds [28]. The molecular mechanisms that control aberrant expression of cell cycle proteins, such as cyclin D1, and lead to cell cycle arrest and/or reentry are poorly understood [29]. Cyclin D1 is a key regulator of the G1/S transition via activation of CDK4, which phosphorylates Rb protein [30,31]. Our data show that cyclin D1

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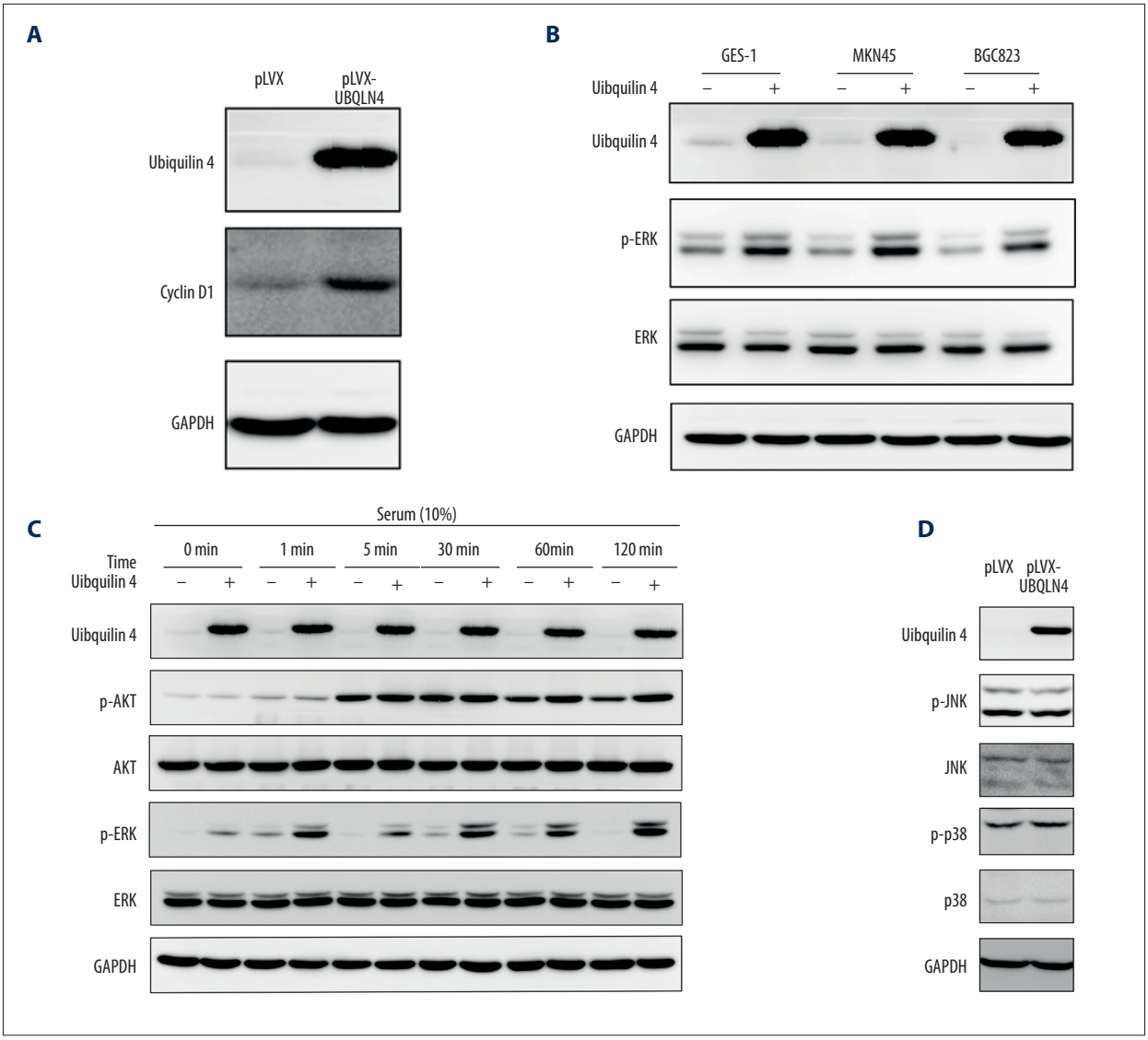

Figure 3. UBQLN4 overexpression activated ERK signaling and increased cyclin D1 expression. (**A**) Western blot analysis of cyclin D1 in GES-1-pLVX-UBQLN4 and GES-1-pLVX cells. (**B**) Western blot analysis of total and phosphorylated ERK in GES-1, MKN45, and BGC-823 cells infected with pLVX-UBQLN4 or pLVX. (**C**) Western blot analysis of total and phosphorylated ERK and AKT in GES-1-pLVX and GES-1-pLVX-UBQLN4 cells after serum starvation for 12 hours followed by the addition of 10% FBS for the indicated times. (**D**) Western blot analysis of total and phosphorylated JNK and p38 in GES-1-pLVX and GES-1-pLVX-UBQLN4 cells. GAPDH served as a loading control for all blots.

expression is increased by UBQLN4 overexpression in GES-1 cells. A recent study identified a positive feedback pathway between cyclin D1 and the MEK-ERK pathway [32].

The proliferation and survival of cancer cells is controlled by a multitude of intracellular signaling cascades, and previous studies have identified key roles for MAPKs, including ERK and JNK, in apoptosis and cell cycle regulation [33,34]. Other studies, however, have shown that ERK activation can lead to cell cycle arrest and apoptosis when cells are exposed to noxious agents [35].

Conclusions

In our study, we observed that phosphorylation of ERK, but not JNK or p38, was significantly increased by UBQLN4 expression in GES-1 cells as well as MKN45 and BGC-823 gastric cancer cells. Moreover, this appeared to occur concomitantly with arrest of GES-1 cells in G2/M. Collectively, our data suggest a role for UBQLN4 in regulating the cell cycle and apoptosis of GES-1 cells via the ERK signaling pathway. Further work in this area may contribute to our understanding of gastric cancer and suggest potential therapeutic targets.

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Conflict of interest

None.

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