Cdc20 protects cells from Ara-c-induced apoptosis in AML via targeting autophagy

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Research

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Abstract

Objective

This study aims to explore the role and mechanism of Cdc20 on Ara-c chemosensitivity of AML cells.

Methods

Evaluation experiments of effects of Cdc20 on Ara-c chemosensitivity were performed with AML cell transfected with constructs overexpressing Cdc20 or AML cell transfected with Cdc20 shRNA through observing cell viability, apoptosis rate,expression of apoptosis protein.The level of autophagy was assessed by transmission electron microscopy and western blotting.

Results

After exposure to Ara-c, Cdc20 expression is down-regulated. Intracellular Cdc20 expression inhibited Ara-c-induced apoptosis as shown by increasing cell viability and decreasing expression of cleaved caspase3.The expression of LC3B was mediated by Cdc20 expression,which further inhibits autophagy. Moreover, Cdc20 mediated LC3B-decreasing promotes the expression of P-Akt and P-ERK and inhibits ROS generation.

Conclusion

It was determined that Cdc20 promoted the degradation of LC3B, thereby inhibiting autophagy and decreasing Ara-c-induced apoptosis in AML cells.

Background

Acute myeloid leukemia is an aggressive hematological tumor with rapid development. In recent years, research on the pathogenesis and prognosis of AML has made revolutionary progress. However, consolidation or intensive treatment after induction chemotherapy is still the conventional treatment for AML, three points One is that adult AML can be cured, and increasing the sensitivity of chemotherapy has become an urgent problem.

Autophagy refers to the process in which cells form autophagosomes through double-layer membranes to wrap the substances to be degraded, and then combine with lysosomes to form autophagolysosomes, which degrade the contents of the packages. While helping cells avoid nutritional stress, it also affects many cell functions, such as apoptosis [1], cell differentiation [2], and cell cycle [3]. The formation and development of leukemia, and the activation of autophagy may promote resistance to standard chemotherapy [4].

As an activator of APC, Cdc20 can act on the substrate containing the degradation cassette domain to regulate the process of mitosis and regulate the cell cycle [5-7]; regulate cell apoptosis [8-10] and

participate in the regulation of tumor drug resistance [11-12], involved in the occurrence, development and migration of tumors [13-16]. The role of Cdc20 in autophagy and chemosensitivity of leukemia cells remains unclear. Therefore, it is important to determine the role of Cdc20 in leukemia cells, paying particular attention to autophagy and its potential impact on chemotherapy sensitivity.

Materials And Methods

Cell culture and reagents

The human leukemia cell lines THP-1, Kasumi were from the Blood Research Laboratory of Qilu Hospital of Shandong University, and fetal bovine serum (LONSERA) and ampicillin and streptomycin were cultured in 1640 medium (Gibco, Los Angeles, California) at 37 ℃. 95% air and 5% CO2 in a humid atmosphere. All cell lines were used within 20 passages. Antibodies against Cdc20 were obtained from Abcam. Antibodies against LC3, p62, cleaved PARP, Akt, p-Akt, P-Erk, Erk and GAPDH were obtained from Cell Signaling Technology. Horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (Nakasugi Golden Bridge).

Cell transfection

Control/Cdc20 shRNAs,pHBLV Control/Cdc20 were obtained from GK gene, transfected with leukemia cells according to the instructions, 24 hours later, the complete medium was used instead of the lentivirus-containing medium, and the appropriate concentration of purine After the mycin selection, PCR and Western blot analysis were performed to verify infection efficiency.

Si-LC3B was obtained from Boshang Bio, according to the manufacturer's instructions, and transfected into leukemia cells using Micropoly Cell transfection reagent at a concentration of 20μM for 48h. The transfection efficiency was verified by PCR.

Cell viability

Cell viability was assessed by the Cell Counting Kit 8 (CCK-8) test. THP-1 and Kasumi cells were seeded into 96-well plates. The indicated concentrations and time points were then treated with cytarabine. 10 μl of CCK-8 reagent was then added to each well and cultured for 4 hours. The relative number of viable cells was determined by measuring the optical density (O.D.) of the cell lysate at 450 nm.

Apoptosis determination

After drug treatment at the specified concentration and time, it was washed twice with PBS. The Annexin V-APC / 7AAD apoptosis detection kit was used to assess the degree of cell apoptosis by flow cytometry. Western blot analysis of c-caspase3, Bcl-2was also performed.

Real-time quantitative PCR

Total RNA was extracted from the cells using TRIzol (Invitrogen). Real-time quantitative PCR (qRT-PCR) experiments were performed using SYBR-Green reagent (Takara Bio Inc., Shiga, Japan) with gene-specific primers for Cdc20 (5'- ACGGTTTTGATGTAGAGGAAGC-3 'and reverse primers, 5'-GATACGGTCTGGCAGGGAAG -3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH forward primer,5' -GAAGGTGAAGGTCGGAGTC-3' and reverse primer, 5'-GAAGATGGTGATGGGATTTC-3'). Each sample was performed in triplicate, and the control group was set to 1.

Western blot analysis

Collect the cells and lyse them in the protein extraction kit (Beyotime), then use the BCA kit (Beyotime) to determine the protein concentration. An equal amount of cell lysate was separated by SDS-PAGE and transferred to a PVDF membrane(Millipore). The membrane was blocked with 5% skim milk, and then incubated with primary antibodies (1: 1000) overnight, and then incubated with a polyclonal HRP-conjugated secondary antibody (1: 2000) 1 h at room temperature. The film is visualized by enhanced chemiluminescence. GAPDH was used as a loading control.

Transmission electron microscopy

Cells were washed with 0.1 cacodylate buffer (pH 7.4) and fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in PBS. Subsequently, the rest of the procedure was conducted using the standard protocol. The thin sections were stained with uranyl acetate and lead citrate for observation under hitachi Transmission Electron Microscope .

Reactive oxygen species (ROS)

ROS produced in cells were detected by using ethidium dihydrogen (DHE). DHE can dye superoxide anions (O_2 -). After the cells were treated, they were washed twice with ice-cold phosphate buffered saline (PBS), treated with DHE (10^{-5} M), and incubated at 37℃ for 30 minutes. After probe treatment, cells were washed at least twice with ice-cold PBS. In the vicinity of the excitation wavelength of 535 nm and the emission wavelength of 610 nm, the intracellular ROS level was measured using a flow cytometer.

Pulse tracking analysis

Lentivirus-infected leukemia cells were prepared with cycloheximide (CHX, 20 μM) or CHX (20 μM) plus MG132 (10 μM), and protein lysates were prepared at specified time points. The level of endogenous LC3B protein was detected by Western blot analysis of anti-LC3B Ab, and the results were quantified and normalized to GAPDH.

Statistical analysis

All results were presented as mean ±SD.Paired Sample T tests were used to assess statistically significant differences.Values of $p<0.05$ were considered as statistically significant.

Results

Cdc20 expression is down-regulated in AML during apoptosis

After exposure to cytarabine, AML cell lines undergo apoptosis, and the expression of Cdc20 is significantly reduced. These results indicate that the level of Cdc20 is down-regulated during apoptosis.

Cdc20 enhances chemotherapy resistance of leukemia cells

To explore the role of Cdc20 in chemotherapy-induced apoptosis,we transfected Cdc20 shRNA into THP-1 cell lines. PCR and Western blot showed that Cdc20 mRNA and protein expression were significantly reduced (Fig. 2a and2b); knockdown of Cdc20 inhibited cell proliferation with or without Ara-c.(Fig.2c and 2d).High levels of apoptotic cell death were found, as shown by an increase of Annexin V-PE positive cells (Fig. 2e) and an increase level of cleaved caspase3 in Cdc20 knockdown cells compared with control shRNA treated groups.In contrast, Bcl2 expression is down-regulated (Fig.2f).

Western blot .

In order to further clarify the role of Cdc20 in AML cell apoptosis during chemotherapy, lentivirus overexpressing Cdc20 was transfected into Kasumi cell line, a decrease in the number of Annexin V-APC positive cells (Fig.3c)and a decrease in the level of cleaved caspase3 compared with control shRNA treated cells(Fig.3d), confirming an antiapoptotic role of Cdc20 in AML cells. The experimental results show that down-regulation of Cdc20 increases apoptosis, making AML cells sensitive to Ara-C treatment;There is a close relationship between autophagy and apoptosis, which can antagonize each other and promote each other.To explore whether autophagy influences the effects of Cdc20-mediated resistance to apoptosis after exposure to Ara-c.we treated pHBLV Cdc20 and Cdc20 shRNA transfected cells with an autophagy inducer Rapamycin,which can efficiently induce activation of autophagy ,causes higer apoptosis level.(Fig2e and 3c)

Cdc20 inhibits autophagy

We assess whether Cdc20 has an effect on autophagy ,and autophagic flux were analyzed by transmission electron microscopy(Fig.4a and 4b).Cdc20 shRNA brought THP-1 cells decreased autophagy activity during chemotherapy compared with control shRNA treated cells as measured by ultra-structural analysis of autophagosomes using transmission electron microscopy. It shows that cells with knockdown of Cdc20 has a higher level of autophagy. In contrast, the autophagy activity of the Cdc20-overexpressing group decreased .Rapamycin increased autophagic flux of AML cells with or without Ara-c.Then we examined the expression of autophagy protein under basic and chemotherapy conditions(Fig4c and Fig3d). Down-regulation of Cdc20 leads to increased expression of LC3B protein.Only the change in LC3B matches the change in autophagy flux.We speculate that Cdc20 may affect autophagy flux and thus apoptosis by regulating the expression of LC3B.

Cdc20 affects autophagy through degradation of LC3B

To explore how Cdc20 affects the expression of LC3B,we used cycloheximide (CHX, a protein synthesis inhibitor) to determine the half-life of LC3B. As shown in Fig.6a,compared with the pHBLV control, Cdc20 pHBLV infection resulted in overexpression of Cdc20 leading to a shortened half-life of LC3B. In contrast, compared with Control shRNA, Cdc20 shRNA knockout Cdc20 can prolong the half-life of LC3B, which shows that Cdc20 can shorten the half-life of LC3B. The half-life of a protein may be shortened through various means. On the one hand, it can be achieved through the down-regulation of the mRNA level, that is, the transcription level, or it may be achieved by the increased degradation of this protein after translation. In order to prove that the overexpression of Cdc20 degrades LC3B through the ubiquitin protease system, rather than regulating Cdc20 through mRNA levels. Therefore, in this topic, we used qPCR to detect the level of LC3B mRNA in Cdc20 knockdown cells and control cells. The result is shown in Fig.5b.Compared with the control group, there is no significant difference in LC3B mRNA compared to the control group, indicating that Cdc20 regulates LC3B from the post-translational modification level. We speculate that as previously studied, it can degrade LC3B through the proteasome pathway, so Cdc20 is up-down and applied on the basis of CHX We added MG132 (a proteasome inhibitor) treatment and found that MG132 can reverse the shortened LC3B half-life caused by Cdc20 overexpression. These results indicate that Cdc20 regulates LC3B protein levels in a proteasome-dependent manner.Heart disease studies have shown that Cdc20 interacts with LC3B and promotes ubiquitination and degradation of LC3B through the proteasome[17],so we speculate that this is also true in acute myeloid leukemia.

Cdc20 affects chemotherapy resistance through ERK, Akt pathway and ROS

Akt and Erk signaling pathways, ROS are closely related to autophagy and apoptosis. In order to further explore how autophagy exerts anti-drug resistance, we used Ara-C to treat THP-1 cells transfected with Cdc20 shRNA. The expression of P-Akt and P-Erk was reduced under the condition of chemotherapy . At the same time, the Cdc20 shRNA group showed a higher level of ROS. The expression of P-Akt and P-Erk in Kasumi cells overexpressing Cdc20 is higher under chemotherapy, and the level of ROS in the overexpression Cdc20 group is lower than that in the control group.These results indicate that Cdc20 promotes Akt and Erk pathways and inhibits ROS production by inhibiting autophagy.

Discussion

Cytarabine and daunorubicin are standard chemotherapeutic drugs for AML. However, inherent and acquired resistance often lead to treatment failure, leading to refractory and recurrence failure outcomes. The mechanisms of drug resistance include abnormal DNA repair, ABC transporter-mediated multidrug resistance, and apoptosis tolerance. Autophagy can play a cancer-promoting effect by promoting cell survival and tumor cell resistance to chemotherapy; on the other hand, it can also play a tumor suppressor effect as one of the mechanisms of chemotherapy-induced cell death. Studies have shown that bortezomib can induce the MDS/AML autophagy pathway to trigger cell death, TRAF6 is necessary to maintain the survival of MDS/AML progenitor cells, and the autophagy degradation induced by bortezomib can lead to cell death[18].Bortezomib induces autophagic death of endothelial cells, which is

manifested by intense cytoplasmic vacuolization with evidence of autophagosomes at electron microscopy[19].In the treatment of AML, it is unclear whether autophagy plays a role in drug sensitivity. In this study, we demonstrated that the down-regulation of Cdc20 or the activation of autophagy increases the drug sensitivity of AML cells.

Cdc20 is a ubiquitin ligase which contains C-box, KEN-box, MIM theme (the theme interacting with Mad2), CRY-box and 7 WD40 repeats can specifically recognize proteins containing D-BOX motifs. After ubiquitinating to modify the substrate, it is degraded by the ubiquitin-proteasome (UPS) pathway. Previous studies have shown that Cdc20 passes through the substrate The ubiquitination and degradation of cyclins, protein-dependent kinases and pro-apoptotic proteins play an important role in the regulation of cell cycle and apoptosis[20][21]. Other studies have shown that the inhibition of Cdc20 can reduce the degradation of the pro-apoptotic protein Bim, thereby inhibiting the docetaxel resistance of CRPC[22]. Aneuploid acute myeloid leukemia was characterized by increased genomic complexity based on exonic variants,which was associated with Cdc20 upregulation[23].In human adult T-cell leukemia cells, the elevated APC (Cdc20) activity obtained by expressing the Tax virus oncoprotein can be resistant to anticancer drugs[24].

As the adaptor protein of the E3 ubiquitinated ligase complex, Cdc20 can target LC3 to proteasome-mediated degradation in a D-box-dependent manner [17]. Consistent with previous findings, our data indicate that the expression of LC3B can be regulated by Cdc20 and is related to Ara-c resistance in AML cell lines. We evaluated the autophagy activity of AML cells before and after chemotherapy and found that knocking down Cdc20 increased the autophagy-related protein LC3B,which enhanced the autophagy flux. The overexpression of Cdc20 led to the opposite finding. Enhancing autophagy promotes the death of AML cells caused by chemotherapy. When autophagy inducers are used, the protective effect of chemotherapy induced by Cdc20 overexpression is reversed. These results demonstrate that Cdc20 protects cells from Ara-c-induced apoptosis in AML via targeting autophagy.

Inhibition of Cdc20 can increase autophagy activity, and the protein level of LC3B increases. Overexpression of Cdc20 is contrary to the result, indicating that Cdc20 can regulate autophagy activity by regulating the expression of LC3B. The knockdown of Cdc20 does not affect the expression of LC3B at the transcriptional level. MG132 and CHX were used to analyze the expression of LC3B in AML cells. The results showed that down-regulation of Cdc20 can prolong the half-life of LC3B, and overexpression of Cdc20 can shorten the half-life of LC3B, and MG132 can Reversing this shortening, these results indicate that Cdc20 regulates the activation of autophagy by reducing the stability of LC3B, and it is regulated at the proteasome level.

In cancer treatment, the induction of ROS can trigger the death signal pathway [8] and induce tumor cell apoptosis [25]. Studies have shown that ZnO NPs increase ROS production and induce cell apoptosis/necrosis in human liver cancer HepG2 cells and human MM cells [26–27]. The generation of ROS is critical for camalexin exerting anti-tumor effect on leukemia cells [28] AraC treatment of leukemia

induces apoptosis through the generation of reactive oxygen species (ROS) [29]. Our study found that AML cells down-regulated by Cdc20 showed higher ROS levels after chemotherapy.

The expression and phosphorylation of Bcl2 family proteins are controlled by the survival signaling pathway mainly regulated by Akt and MAPK/Erk1/2 pathways, and reactive oxygen species (ROS) can activate these two pathways [30]. Studies have shown that the Rac activator Tiam1 can control the intracellular redox balance through the generation of ROS, thereby regulating ERK phosphorylation and the sensitivity of keratinocytes to apoptosis signals [31]. Inhibition of ROS in human pancreatic tumor cells Panc-1 can reduce the level of phosphorylated (active) Akt and induce apoptosis [32]. Our research shows that AML cells down-regulated by Cdc20 have higher death levels after chemotherapy, and lower levels of P-ERK, P-AKT, and Bcl-2.

Conclusion

In conclusion, here we showed chemotherapy agents decreased Cdc20 expression in AML cells.Cdc20

acts as an important regulator of autophagy that leads to inhibited apoptosis. Cdc20 can reduce the stability of LC3B through post-translational level reduction, thereby inhibiting autophagy; the reduction of autophagy level leads to the increase of Ara-c-induced apoptosis level;In addition, AML cells with Cdc20 shRNA have lower levels of P-ERK, P-AKT, and Bcl-2 and higher level of ROS after chemotherapy, thus caused incresased apoptosis .These results support an investigation of Cdc20 as a potential target for AML therapy.

Abbreviations

Cdc20: Cell division cycle 20 homologue ;

Ara-C: Cytarabine;

AML**:**Acute myeloid leukemia;

CHX:cycloheximide.

Declarations

Acknowledgements

Not applicable

Authors' contributions

DSM and XRR designed the study. DSM and KJY performed the in vitro experiments. WY ,WJY and MDX prepared the Figs. DSM , WT, CSY and LZH collected and analyzed the data. DSM and KJY wrote the

manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Ethics approval and consent to participate

All procedures of this study were in accordance with the ethical standards of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

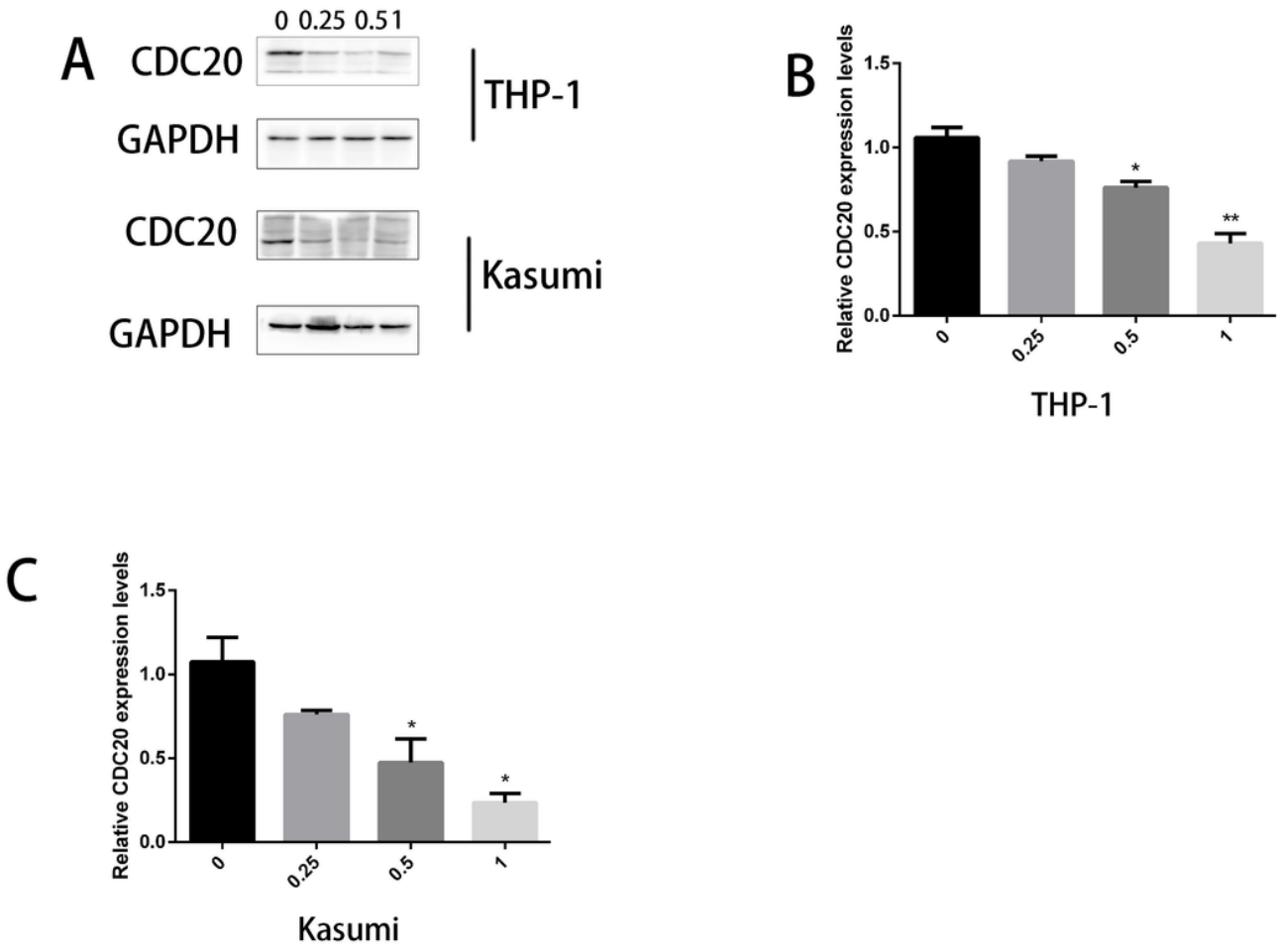

Figure 1

Ara-c changes Cdc20 expression in AML cells. THP-1and Kasumi cells were treated with Ara-c (0,0.25,0.5,1μmol/L)for 24 h. a Whole cell lysates were subjected to western blot analysis for Cdc20 expression. b and c. Cdc20 mRNA level was assessed by quantitative real-time PCR (b; n=3; *p<0.05 ,**p<0.01vs .untreated group).

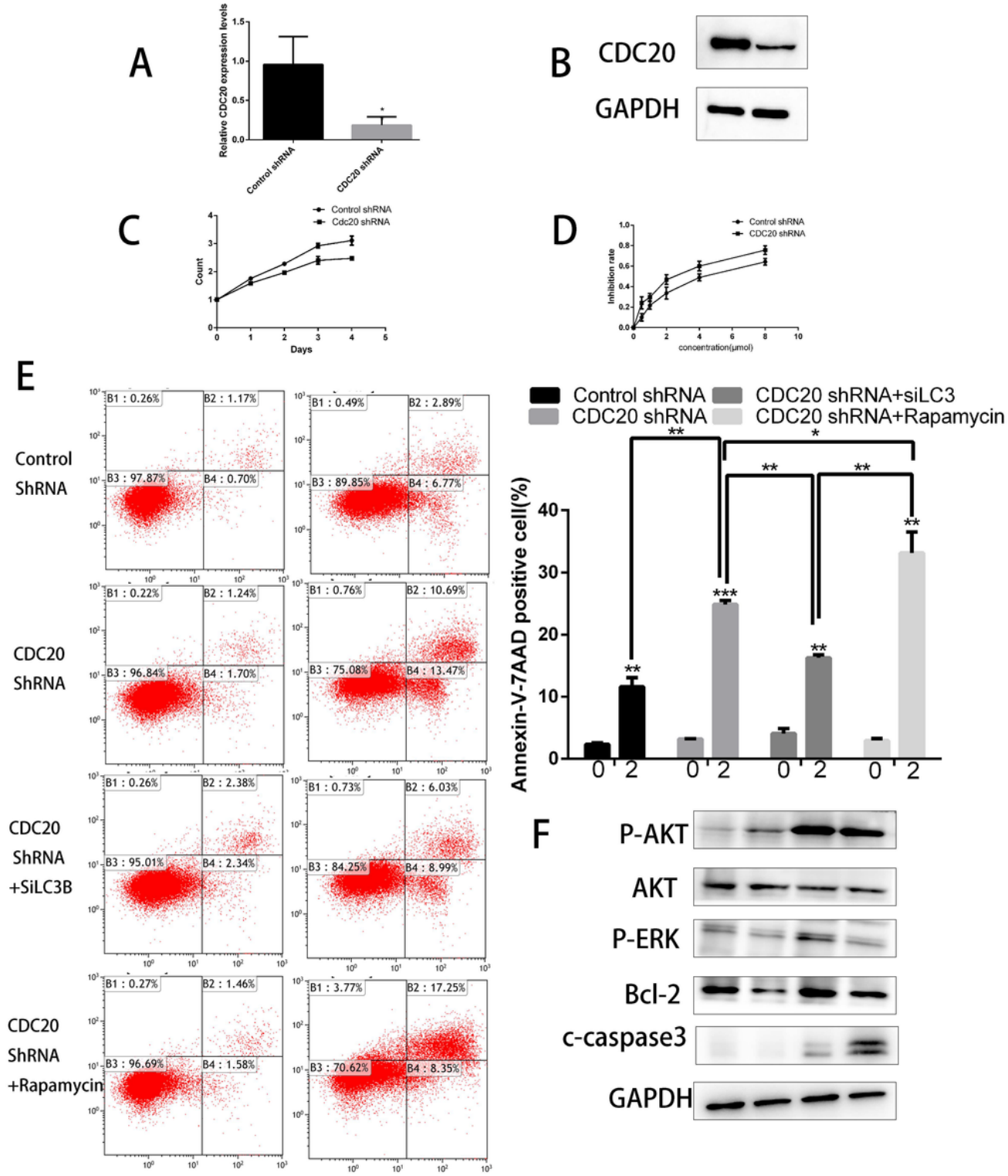

Figure 2

Knockdown of Cdc20 promotes AML cell sensitivity to Ara-c by increasing apoptosis. a THP-1 cell was transfected with control shRNA and Cdc20 shRNA for 48 h. Then the expression levels of Cdc20 were analyzed by Western blot(a) and quantitative real-time PCR(b) (n=3;* p<0.05 vs. control shRNA group).c and d after transfection with control shRNA and Cdc20 shRNA,cell viability was determined by CCK-8. c the cell viability was determined at 0, 24 ,48 ,72and 96h. d the cells were treated with Ara-

c(0,0.5,1,2,4,8μmol/L)for 24h.e Cdc20 shRNA transfected THP-1 cell was pretreated with rapamycin (100 nmol/L) for 6 h and then were exposed to Ara-c(2μmol/L) for an additional 24 h,then apoptosis was analyzed by flow cytometric analysis of Annexin V-APC/7AAD staining (c; n=3; **p<0.01 ,***p<0.001vs, control shRNA group)f cleaved caspase3 in THP-1 cells were analyzed by Western blot .

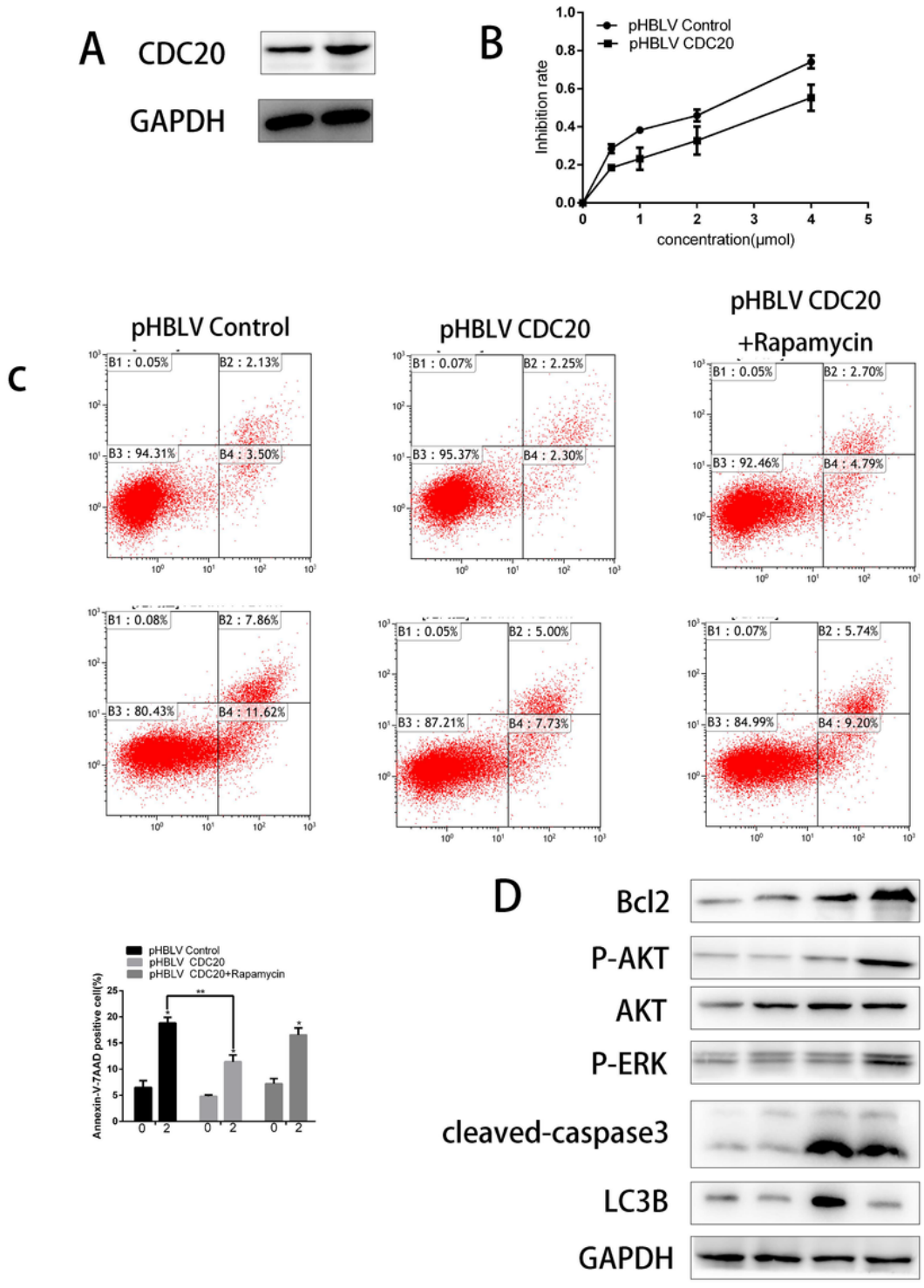

Figure 3

Cdc20 reduces AML cells sensitivity to Ara-c by decreasing apoptosis.Kasumi cell was infected with control (pHBLV control) and Cdc20-expressing lentiviruses (pHBLV Cdc20). a.The protein level of Cdc20 was assayed by Western blot.b. after transfection with pHBLV control and pHBLV Cdc20,cell viability was determined by CCK-8. c the cell viability was determined at 0, 24 ,48 ,72and 96h. d the cells were treated with Ara-c(0,0.5,1,2,4,8μmol/L)for 24h.c. After transfection with pHBLV control and pHBLV Cdc20, the cells were treated with Ara-c(2μmol/L) for 24h,then apoptosis was analyzed by flow cytometric analysis of Annexin V-APC/7AAD staining (c; n=3; **p<0.01 ,***p<0.001vs, control shRNA group).d. cleaved caspase3 in Kasumi cells were analyzed by Western blot.

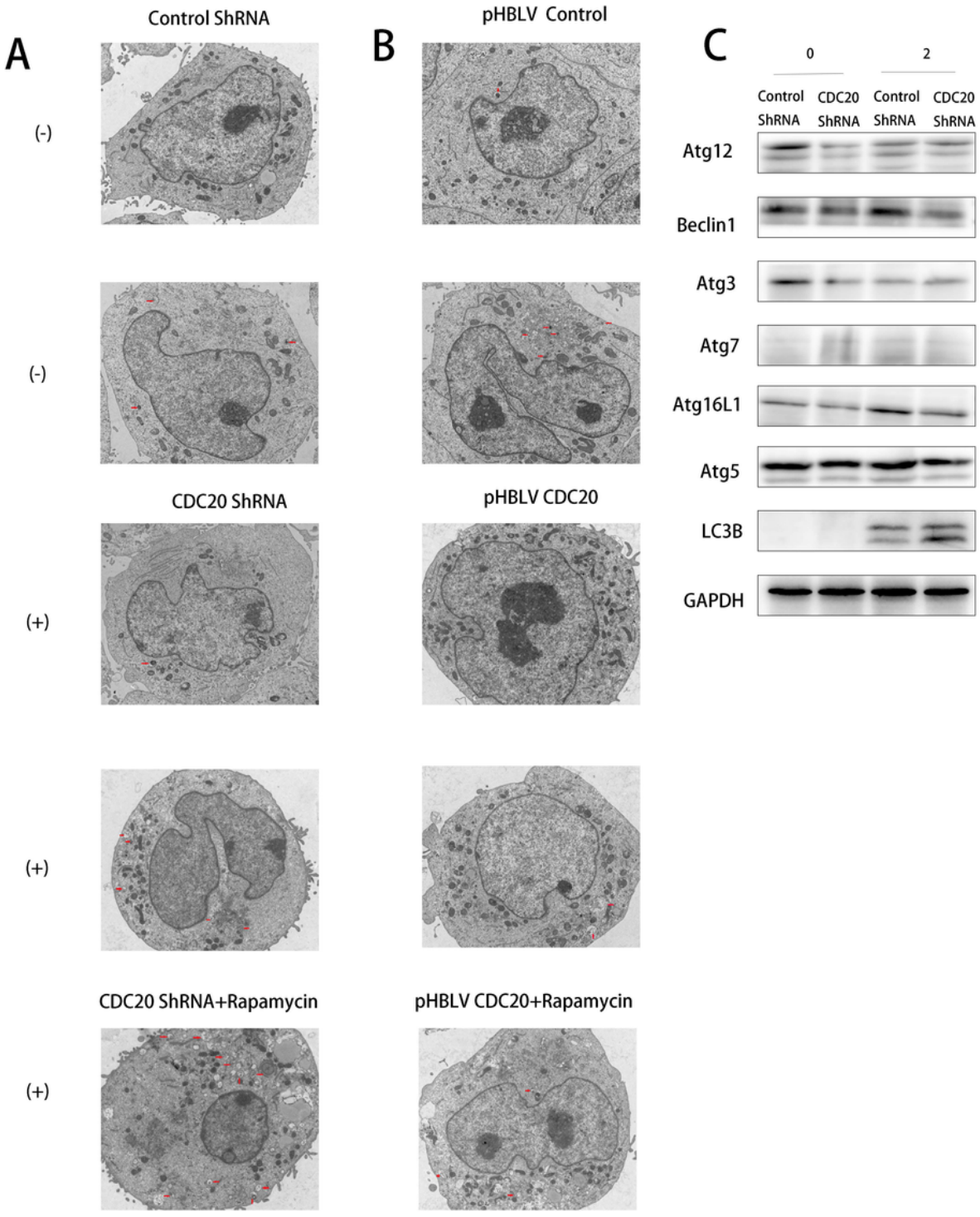

Figure 4

Autophagy mediates Cdc20-induced sensitivity of AML cells to Ara-c..Regulation of autophagy level can affect apoptosisa.Control/Cdc20 shRNA transfected THP-1 cells were treated with Ara-c(2μmol/L)in the presence or absence of Rapamycin .b.Cdc20-overexpressing Kasumi cells were treated with Ara-c(2μmol/L) for 24h in the presence or absence of Rapamycin(100 nmol/L for 6 h). a and b .Autophagic flux were analyzed by transmission electron microscopy ,autophagosome-like structures were indicated

(red arrows).c Autophagy activity indicated by LC3B and Atg12,Atg3,Atg5,Atg7,Atg12,Atg16L1 were assessed by Western blot.

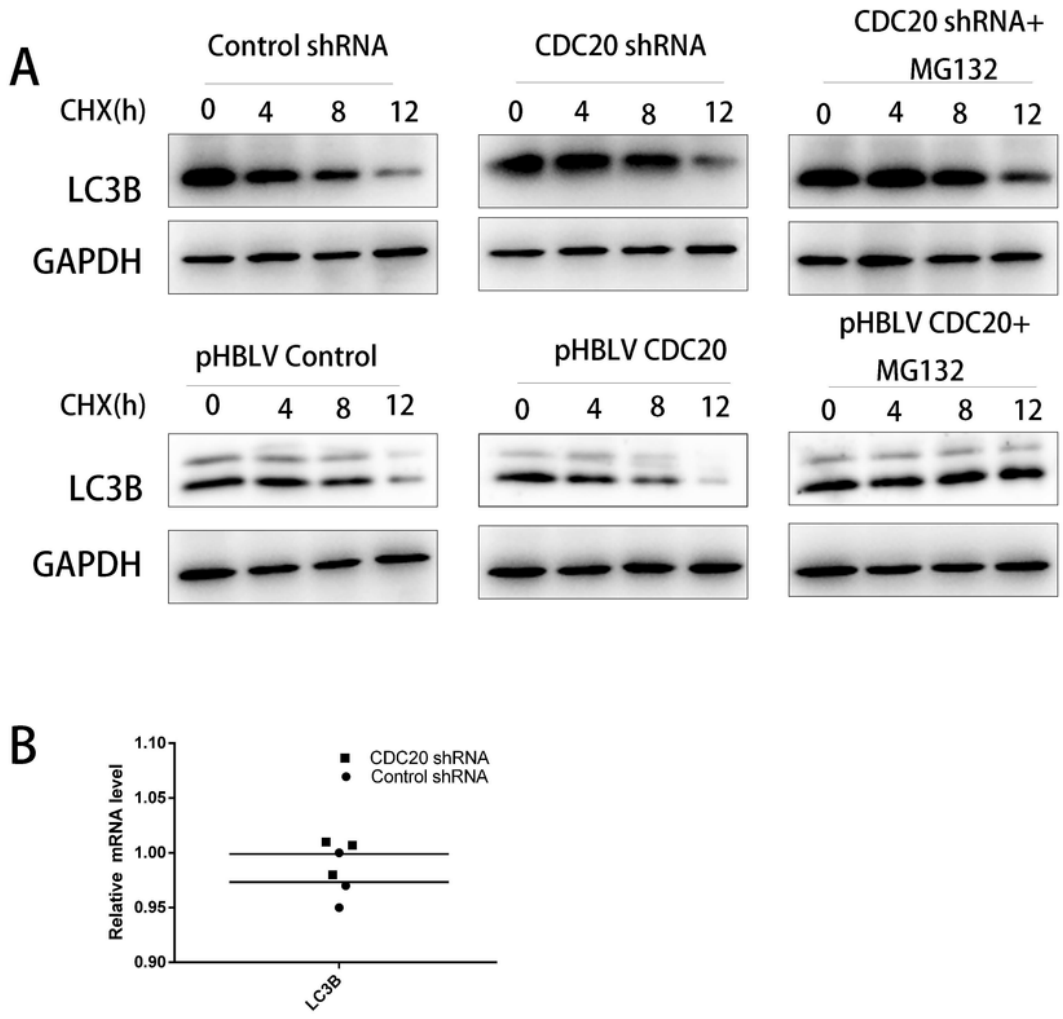

Figure 5

Cdc20 affects autophagy through degradation of LC3B a. THP-1 cells were infected with Control shRNA and Cdc20 ShRNA.Kasumi cells were infected with pHBLV Control or pHBLV Cdc20, then treated with cycloheximide (CHX, 10 μM) and/or MG132 (10 μM) and harvested at then indicated time

points.Representative immunoblotting analyses of LC3B protein levels for each group.b. LC3B, P62, LC3A mRNA level was assessed by quantitative real-time PCR.

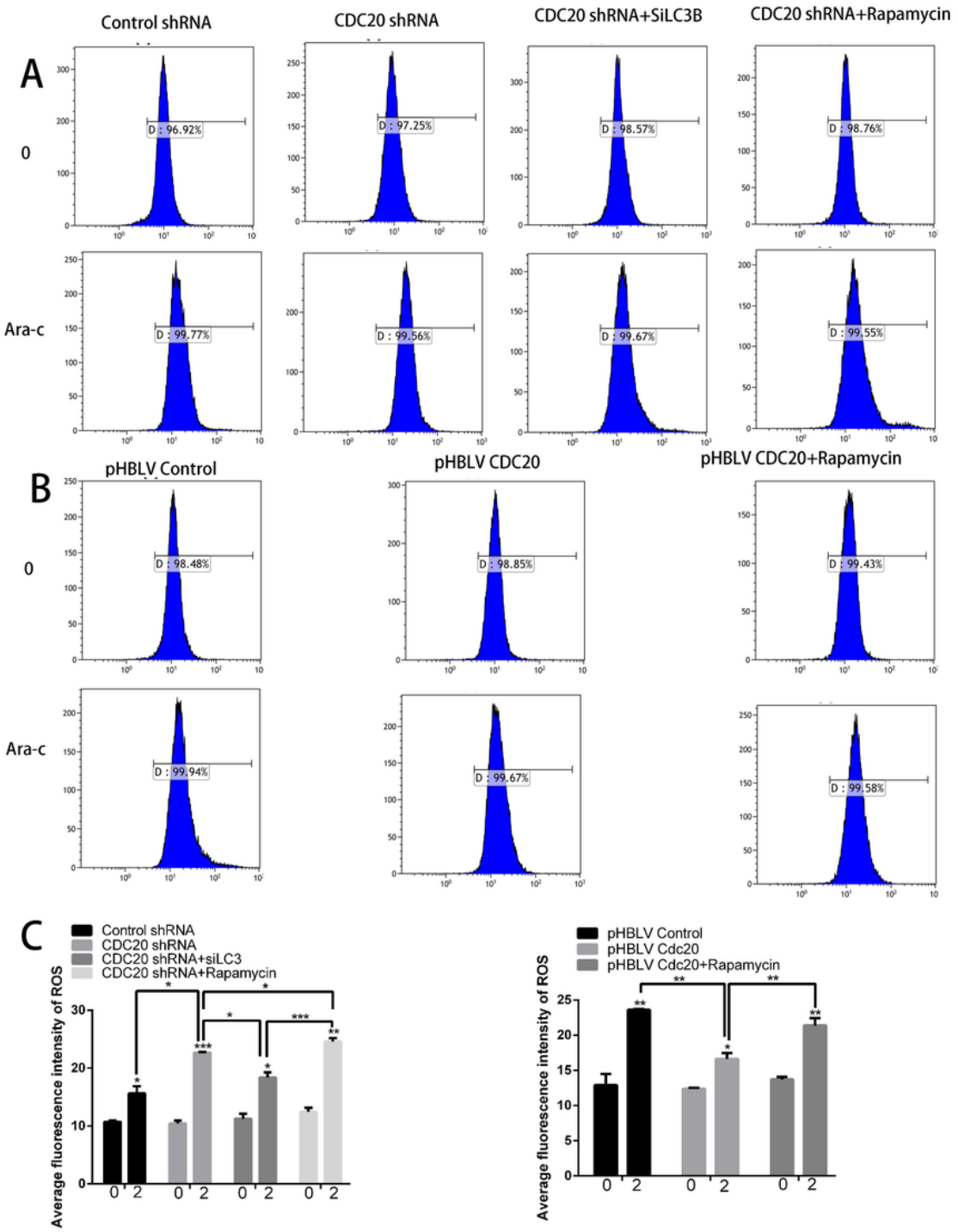

Figure 6

After exposure to Ara-c(2μmol/L) for 24 h, the intracellular ROS levels of Cdc20 shRNA transfected and Cdc20-overexpressing cells were measured by flow cytometry.a Cdc20 shRNA transfected THP-1 cell.b. Cdc20-overexpressing kasumi cell.cThe results of ROS fluorescence intensity of Cdc20 shRNA

transfected cells and Cdc20-overexpressing cells from three independent experiments. Data were presented as mean ± SD. *P<0.05,**P<0.01 and ***P<0.001.

Figure 7

Structure of Human Cdc20