European Review for Medical and Pharmacological Sciences 2020; 24: 10028-10035

The phosphorylation of hCDC14A modulated by ZIPK regulates autophagy of murine pancreatic islet β-TC3 cells upon glucose stimulation

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Abstract. – OBJECTIVE: Depletion of islet β cells plays a crucial role in the onset of diabetes mellitus. Cell autophagy, as a self-healing process, contributes to maintaining metabolic homeostasis and can protect islet β cells from apoptosis upon starvation or high glucose stress. However, the underlying regulatory network of the autophagic process in islet β cells has not been fully explored.

MATERIALS AND METHODS: Murine β-TC3 cells treated with different concentrations of glucose, and wild-type or the Ser484 mutant human cell division cycle gene 14A (hCDC14A) was transfected. Cell viability, proliferation and autophagy as well as islet secretion were studied. The mTOR and AMPK signaling pathways were investigated by western blots. Zipper-interacting protein kinase was studied using mass spectrometry and immunoprecipitation.

RESULTS: Overexpression of wild-type hCDC14A, but not the Ser484 mutant hCDC14A, promoted cell viability, proliferation and autophagy accompanied by enhanced islet secretion and reduced cell apoptosis via mTOR pathway inhibition as well AMPK pathway activation in β-TC3 cells and vice versa. Furthermore, Zipper-interacting protein kinase (ZIPK), also known as DAPK3, was found to interact with hCDC14A primarily for Ser484 phosphorylation, and ZIPK knockdown could affect the phosphorylation of hCDC14A and weaken cell death or cell cycle modulation.

CONCLUSIONS: Taken together, our results may provide new insight into the role of hCDC14A in the autophagy of islet β cells and suggest the potential therapeutic value of hCDC14A phosphorylation in the prevention and treatment of diabetes.

Key Words:
hCDC14A, Phosphorylation, Islet β cells, Autophagy, ZIPK, Diabetes.

Introduction

Diabetes mellitus (DM) is a common chronic disease worldwide and a severe public health issue, with 285 million patients reported in 2010 and a prediction of 439 million by 2030 according to the International Diabetes Federation[1]. Type 2 DM (T2DM) is caused by peripheral tolerance to insulin and an inadequate secretory response by the pancreatic β cell population. Several pathophysiological conditions are believed to contribute to β cell dysfunction, including glucotoxicity and lipotoxicity. The persistent stimulation of β cells by glucose and free fatty acids eventually results in the depletion of insulin stores, progressive hyperglycemia and β cell mass deterioration[2]. With regard to T2DM, environmental abnormalities outside the pancreatic islets have been primarily studied to assess the pathogenesis of islet β cell failure in many recent studies. However, the molecular alterations in T2DM islet cells that may be responsible for disease development have not been fully illustrated[3].

Autophagy is a highly regulated catabolic pathway responsible for the degradation and recycling of cellular components upon various cellular stresses[4]. The principal role of autophagy is to reallocate nutrients from unnecessary processes to more pivotal processes required for survival[5]. Autophagy is an evolutionarily conserved process by which autophagosomes composed of double membrane vesicles engulf cellular proteins and organelles for delivery to the lysosome for degradation[6]. ATG proteins (ULK1, BECN1, ATG3/5/7/12, and LC3, etc.) are widely

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acknowledged to contribute to autophagy through several signaling pathways, including the PI3K/Akt/mTOR[7], AMPK[8], and p53 pathways[9]. Current studies have revealed that islet β cells with deficient autophagy show reduced cell growth and insulin secretion and display a phenotype of oncotic mitochondria, rough endoplasmic reticulum and Golgi bodies[10]. In contrast, the utilization of rapamycin in rats with T2DM can prevent β cells from undergoing apoptosis and alleviate the symptoms of T2DM via autophagic activation[11,12]. Therefore, as self-repairing machinery is involved in the process of cell proliferation and regeneration, autophagy is important for β cell survival in severe conditions. However, the role and regulatory targets of autophagy in islet β cells have not been fully elucidated.

Our previous study reported that human cell division cycle gene 14A (hCDC14A) played an important role in βcell function and cell cycle regulation[13]. In this study, we focused on the connection between hCDC14A and autophagy and further investigated the post-translational regulatory mechanism of hCDC14A in murine β-TC3 cells under the stress of different glucose cultures.

Materials and Methods

Cell Culture

Mouse β-TC3 cells (CHI Scientific, Boston, MA, USA) were grown in DMEM (HyClone, Logan, UT, USA) with high, normal or low concentrations of glucose and 10% FBS (HyClone, Logan, UT, USA). Cells were grown on plastic at the liquid interface at 37°C in 5% CO_2 saturated humid air. Wild-type hCDC14A (F: 5'-ATGGCAGCGGAGTCAGGG-3', R: 5'-TCAGAAGGCTTCCTTGG-3') was cloned and inserted into the pEGFP-C2 vector (ClonTech Laboratories, Mountain View, CA USA), to prepare the Ser484 and Ser583 mutant hCDC14A using mutagenic PCR (48°C annealing temperature for 30 s and 68°C extension temperature for 5 min). Wild-type Zipper-interacting protein kinase (ZIPK) was cloned and inserted into MSCV2.2 (Cat. No. 80139, Addgene, Watertown, MA, USA). hCDC14A (F: 5'-GAAAGAAGAUAGUGCACUACA-3', R: 5'-UAGUGCACUAUCUUCUUUCUU-3') and ZIPK (F: 5'-CACGAUAGGUGCUCUCCUACG-3', R: 5'-UAGGAGAGCACCUAUCGUGGG-3') siRNAs were purchased from Sangon Biotech (Shanghai, China). The nucleotides were transfected into cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. A final concentration of 2.5 μM rapamycin (Sigma-Aldrich, St. Louis, MO, USA) or 1×phosphorylation inhibitor cocktail (Roche, Basel, Switzerland) was used in this study. Cells were harvested after 48 h of transfection or treatment for the next experiments.

Cell Counting Kit-8 (CCK-8) Assay

β-TC3 cells after 48 h of transfection were subcultured into 96-well plates at 80% density, followed by the addition of 10 μl of CCK-8 solution (Solarbio, Beijing, China) to incubate for an additional 1 h. Absorption values of 450 nm were examined using a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) to construct the regression equation and calculate the IC_{50}. The IC_{50} values were evaluated for cell proliferation.

Immunoblotting Assay

For this assay, 10^7 β-TC3 cells were added to 100 μl of RIPA buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitor cocktail (Solarbio, Beijing, China), and the protein concentration was quantified using BCA methods. Aliquots of proteins (40 μg) were added into the lanes of 10% Sodium dodecyl sulfate (SDS) polyacrylamide gel, and the proteins were separated through electrophoresis and transferred onto nitrocellulose membranes. Subsequently, the membranes were blocked with 5% nonfat dry milk in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) and 0.05% Tween-20 for 1 h at room temperature (RT). The blocked membranes were incubated with Coomassie blue stain (Thermo Fisher Scientific, Waltham, MA USA) for 2 h and washed with water for 24 h at RT. The bands of interest were selected for further mass spectrum assays (APTbiotech, Shanghai, China). For Western blot assays, the membranes were then incubated with primary antibodies against hCDC14A (1:2000, C2238, EMD Millipore, Billerica, MA, USA), Beclin-1 (1:2000, Novus, Centennial, CO, USA), LC3II (1:2000, CST, Beverly, MA, USA), protein kinase AMP-activated catalytic subunit alpha 1 (AMPKα) (1:2000, #5832, CST), p-AMPKα (1:1000, #2535, CST), mechanistic target of rapamycin kinase (mTOR) (1:2000, #2972, CST), p-mTOR (1:1000, #5536, CST), ZIPK (1:1000, sc-514223, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Green fluorescent protein (GFP)

(1:2000, ab290, Abcam, Cambridge, MA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, Beyotime Biotechnology, Shanghai, China) overnight at 4°C, followed by incubation with the appropriate secondary antibodies (horseradish peroxidase-conjugated rabbit anti-mouse diluted with 1: 10000 and donkey anti-rabbit diluted with 1: 5000, Beyotime) for 30 min at RT. The expression was determined by the enhanced chemiluminescence method using the Amersham Imager 600 system (GE Healthcare Life Sciences, Pittsburg, PA, USA), whereas the density of the immunoblots was measured with Quantity One 4.62 software (Bio-Rad Laboratories, Hercules, CA, USA).

Flow Cytometric Assay

Cell apoptosis was assessed using an Annexin V FITC apoptosis detection kit (Dojindo Molecular Technologies, Kumamoto, Tokyo, Japan). Cells were washed with PBS twice and suspended to a density of 10^6 cells by 1× binding buffer, and AV-FITC and PI were added, followed by incubation in the dark at RT for 15 min. FITC and PE channels were used to detect the signals by Beckman FC500 with 10,000 events acquired.

For the cell cycle assay, the cells were fixed with 75% cold ethanol at 4°C overnight, washed and resuspended in PBS, and incubated with RNase A at 37°C for 1 h. Then, the cells were screened by 400 mesh gauze and incubated with PI in the dark at 4°C for 30 min. PE channels were used to detect the signals by Beckman FC500 with 10,000 events acquired. Flow cytometric data were analyzed using FlowJo 10.5 (BD Bioscience, Franklin Lakes, NJ, USA).

Enzyme-Linked Immunosorbent (ELISA) Assay

Briefly, the culture supernatant was harvested and centrifuged at 1000×g for 15 min. Insulin concentrations were determined with a commercially available kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The color change was measured by a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 450 nm.

Immunofluorescence Staining Assay

β-TC3 cells were fixed within a 4% solution of paraformaldehyde, washed with PBS, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and blocked with 0.5% horse serum in PBS. Immunostaining of samples was performed using the appropriate antibodies overnight at 4°C. After 4 washes with PBS (5 min per wash), the appropriate secondary antibodies (1:20000; Jackson ImmunoResearch, West Grove, PA, USA) were incubated for 2 h at RT, followed by further incubation with DAPI for 15 min and 4 washes with PBS. After the samples were dried in air, slices were dropped with coated mounting medium and coated with cover glasses. The positive staining was statistically analyzed using Image J (National Institutes of Health, Bethesda, MD, USA).

Immunoprecipitation Assay

β-TC3 cells were fixed within a 4% solution of paraformaldehyde and quenched by 0.125 M glycine, 1 ml IP lysis buffer (50 mM NaCl, 50 mM Tris, [pH 7.4], 1% NP-40) with protease inhibitor was added, and the mixture was transferred to a 1.5 ml tube after thawing. The cell lysates were mixed with 1 μg GFP or ZIPK antibodies and 40 μl of a suspension of protein A beads (Thermo Fisher Scientific, Waltham, MA, USA) for rotating overnight at 4°C. The immunoprecipitants were washed three times with washing buffer containing 250 mM NaCl and denatured with 1% SDS at 100°C for 10 min. Eluate from the IP pellets was then Western blotted with the appropriate antibodies.

Statistical Analysis

The data are presented as the mean ± SEM. All statistical analyses were performed using SPSS 20.0 statistics software (IBM, Armonk, NY, USA). Group comparisons were analyzed with one-way ANOVA. A p-value less than 0.05 was considered a significant difference.

Results

Autophagy of β-TC3 Cells Impacted by hCDC14A Under Low/High Glucose Stimulation

Initially, we investigated the physiological reactions of pancreatic β cells treated with hCDC14A upon glucose stimulation. β-TC3 cells were cultured in medium with low (1 g/L), normal (3 g/L) or high (4.5 g/L) glucose (LG, HG) and further transfected with hCDC14A overexpression (OE) or knockdown (KD) constructs. The cell proliferation was elevated in β-TC3 cells with HG but reduced

in LG compared to that of the untreated cells. Moreover, the growth rate of β-TC3 cells could be stimulated by hCDC14A OE but repressed by hCDC14A KD (Figure 1A). In contrast, cell apoptosis was compromised in the β-TC3 cells with HG but enhanced in LG compared to that of the untreated cells. Likewise, hCDC14A OE was beneficial for cell survival, while hCDC14A KD caused β-TC3 cell death (Figure 1B). Furthermore, we observed G2/M phase arrest following LG treatment and G1/G0 phase arrest following HG treatment compared to the control. Similarly, hCDC14A OE resulted in G1/G0 phase arrest, while hCDC14A KD led to G2/M phase arrest (Figure 1C, **Supplementary Figure 1**). We found that hCDC14A overexpression could induce the response of β-TC3 cells to insulin secretion, while hCDC14A silencing diminished the production and secretion of insulin by β-TC3 cells (Figure 1D). Finally, HG but not LG stimulation could upregulate the protein levels of Beclin-1 and LC3II, and the phosphorylation of AMPK was increased while the phosphorylation of mTOR was decreased upon HG stress, which suggested that HG could induce the autophagic activation of β-TC3 cells. Interestingly, the expression of hCDC14A *per se* was substantially elevated upon HG but not LG stress, and hCDC14A OE intensified while hCDC14A KD inhibited the effect of autophagy (Figure 1E). Taken together, the data above showed that hCDC14A could strengthen the viability and function of β-TC3 cells upon HG stress.

The Effect of Ser484 Phosphorylation of hCDC14A on Autophagic Activation

Our previous study[13] revealed that the phosphorylation of hCDC14A played an important role in target gene regulation. Therefore, the two known phosphorylated sites, Ser484 and Ser583[14], were mutated in the exogenous hCDC14A vectors (**Supplementary Figure 2**). Compared to the wild-type (WT) and Ser583 mutant (MT) hCDC14A, Ser484 MT hCDC14A failed to affect the AMPK or mTOR signaling pathways and autophagic initiation of β-TC3 cells (Figure 2A). Furthermore, an immunofluorescence assay

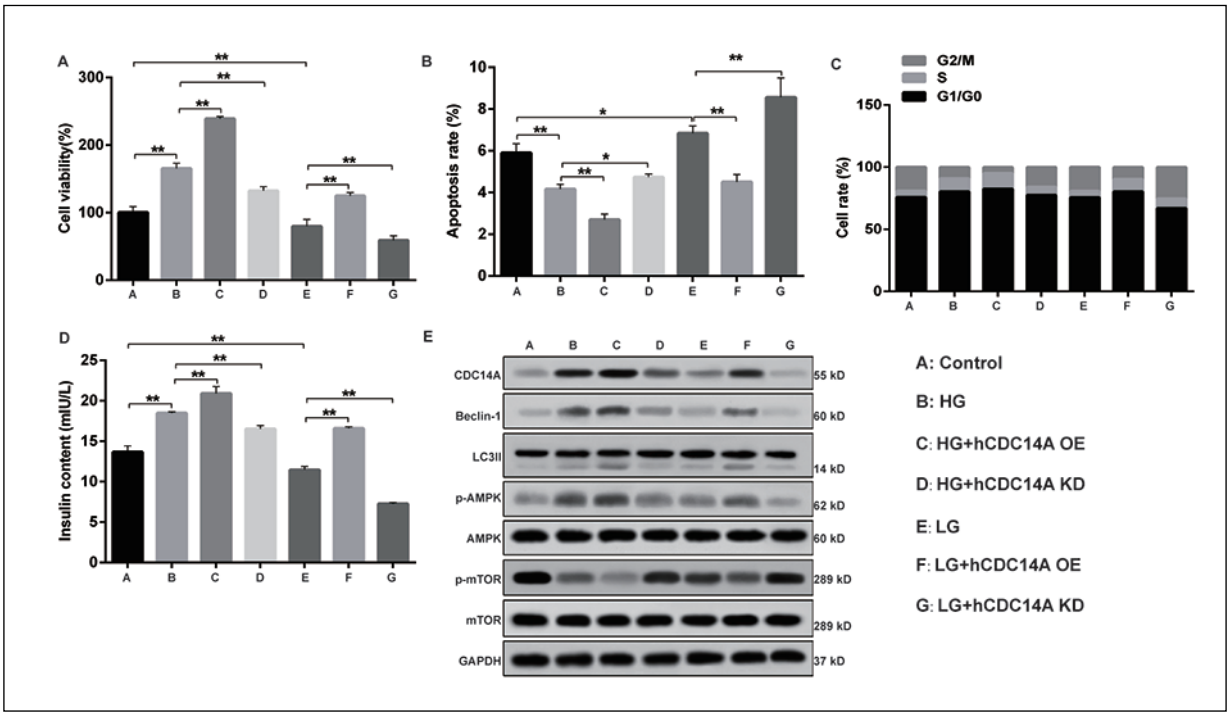

Figure 1. Autophagic activity regulated by hCDC14A under low/high glucose stress in **β-TC3 cells.** The cell proliferation (**A**), apoptosis (**B**), cell cycle (**C**) and insulin secretion (**D**), as well as the protein levels of the autophagy-associated biomarkers Beclin-1 and LC3II and the (phosphorylated) AMPK and mTOR, (**E**) of β-TC3 cells that underwent low- and high-glucose stress and hCDC14A overexpression or knockdown. The labels for groups A to G are listed. HG: high glucose; LG: low glucose; OE: overexpression; KD: knockdown. Each experiment was repeated three times. Data are presented as the mean ± SEM of triplicate individual experiments. "*" and "**" represent *p*-values less than 0.05 and 0.01 by one-way ANOVA.

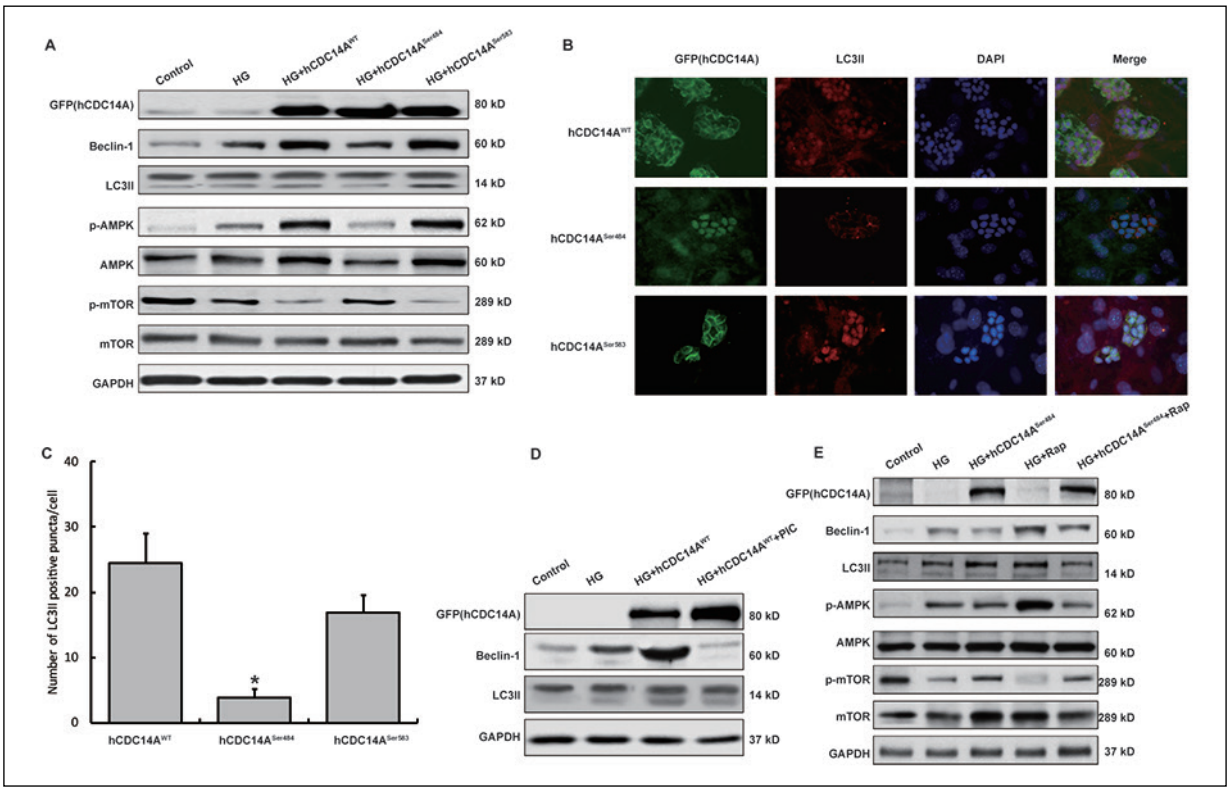

Figure 2. Contribution of Ser484 of hCDC14A to autophagy in **β-TC3 cells** under high glucose stress. The protein levels of the autophagy-associated biomarkers Beclin-1 and LC3II and (phosphorylated) AMPK and mTOR (**A**), the location of hCDC14A and LC3II (**B**) and the statistical analysis of LC3II-positive puncta (**C**) of β-TC3 cells that underwent high-glucose stress and overexpression of the wild-type and the Ser484 and Ser583 mutant hCDC14A. The levels of autophagy-associated biomarkers in β-TC3 cells treated with a phosphorylation inhibitor cocktail (**D**) or rapamycin (**E**). PIC: phosphorylation inhibitor cocktail; Rap: rapamycin. Each experiment was repeated three times. The images of immunofluorescence are shown at 200× magnification. Data are presented as the mean ± SEM of triplicate individual experiments. "*" represents a *p*-value less than 0.05 by one-way ANOVA.

showed that hCDC14A with the Ser484 MT was strongly distributed in the nucleus along with diminished LC3II puncta compared to the WT and Ser583 MT hCDC14A in β-TC3 cells (Figure 2B, C), which implied that autophagic activation depended on the Ser484 phosphorylation of hCDC14A. To validate this hypothesis, we further treated β-TC3 cells with rapamycin or phosphatase inhibitor cocktail, and autophagic activity was investigated. We observed that WT hCDC14A inhibited by phosphatase inhibitors failed to upregulate Beclin-1 and LC3II (Figure 2D). Certainly, the phosphorylation of AMPK and mTOR was suppressed by phosphatase inhibitors (data not shown), which could not be attributed to hCDC14A. Ser484 MT hCDC14A could compromise rapamycin-mediated autophagy (Figure 2E). Thus, Ser484 phosphorylation of hCDC14A played a crucial role in autophagic activation in β-TC3 cells.

The Role of ZIPK in Ser484 Phosphorylation of hCDC14A Induced by HG

Finally, for determination of the key kinases contributing to hCDC14A phosphorylation, β-TC3 cells transfected with WT or Ser484 MT hCDC14A were pulled down by GFP for the mass spectrum assay, and we found that ZIPK was a potential target protein interacting with hCDC14A (Figure 3A, B). In contrast, ZIPK IP studies in β-TC3 cells were conducted and showed that ZIPK failed to bind with Ser484 MT hCDC14A (Figure 3C), which indicated that ZIPK might contribute to Ser484 phosphorylation of hCDC14A. As expected, ZIPK KD with WT hCDC14A neither promoted autophagy (Figure 3D) nor modulated the cell cycle arrest (Figure 3E, **Supplementary Figure 3**) of β-TC3 cells with HG compared to WT hCDC14A. Additionally, ZIPK OE with Ser484 MT hCDC14A

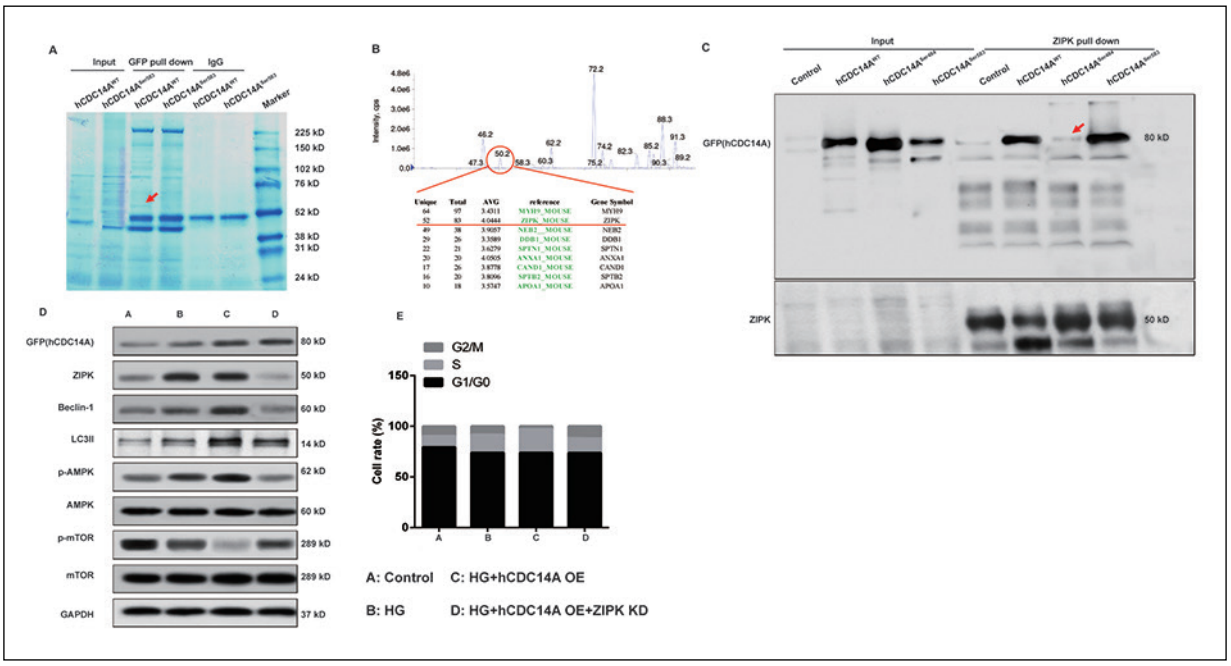

Figure 3. The interaction of ZIPK with hCDC14A for Ser484 phosphorylation. Blue staining of GFP pulldown in β-TC3 cells transfected with the wild-type and the Ser484 and Ser583 mutant hCDC14A fused with GFP (**A**). The red arrow represents the band of interest for the mass spectrum. The peptide signals by mass spectrometry are shown (**B**). ZIPK is one candidate protein within the peak at 50.2 kD. Reverse IP showing ZIPK pulldown in β-TC3 cells transfected with wild-type, Ser484 mutant and Ser583 mutant hCDC14A fused with GFP (**C**). The red arrow represents the compromised interaction between ZIPK and the Ser484 mutant hCDC14A. The levels of autophagy-associated biomarkers (**D**) and the cell cycle (**E**) in β-TC3 cells treated with hCDC14A overexpression and ZIPK knockdown. The labels of groups A to D are listed. HG: high glucose; OE: overexpression; KD: knockdown. Each experiment was repeated three times. Data are presented as the mean ± SEM of triplicate individual experiments.

did influence β-TC3 cells slightly compared to ZIPK KD with WT hCDC14A, which might be attributed to normal functioning by endogenous ZIPK and hCDC14A in β-TC3 cells (data not shown). Due to lack of a commercial phosphorylated antibody of hCDC14A, the data above indirectly indicated that ZIPK impacted autophagy by regulating hCDC14A phosphorylation at the Ser484 site.

Collectively, we determined that hCDC14A could induce autophagic activation in islet β cells under glucose stress through hCDC14A Ser484 phosphorylation regulated by ZIPK.

Discussion

Islet β cells are a particular type of cell for insulin production and secretion. The endoplasmic reticulum (ER) of β cells has a high capacity for protein synthesis and folding processes for secretory proteins, such as insulin, cell surface receptors and integral membrane proteins. However, the sensitive folding environment in the ER can be disturbed by pathological insults, such as viral infections, environmental toxins, inflammatory cytokines and mutant protein expression, and is highly prone to misfolded protein accumulation when biosynthetic needs are chronically increased due to insulin production in response to food uptake. Indeed, baseline ER stress levels are higher in β cells than in other types of cells[15,16]. Due to these observations, ER stress signaling has increasingly become a focus in diabetes research.

Previous studies[17,18] have shown the direct connections between ER stress and autophagy in yeast and reveal that autophagy plays a pro-survival role in mouse embryo fibroblasts after ER stress induction. The observations of the protection of β cell cultures from HG or after palmitate-induced cell death by autophagy suggest implications for β cell homeostasis and pathogenesis of diabetes. Interestingly, islet β cells are particu-

larly susceptible to ER stress under both HG and LG stimulation. HG induces increased insulin synthesis, which will lead to an increased amount of unfolded protein and activation of phosphorylated mitogen-activated protein kinase 1 (ERK), while LG leads to decreased synthesis of insulin through eukaryotic translation initiation factor 2 subunit alpha (eIF2α) phosphorylation[19,20], which is consistent with our data (Figure 1). Nevertheless, the effects on cell growth, apoptosis, insulin secretion and autophagy induced by HG induction of β cells are stronger than those of starvation stress, which implies that short-term starvation of β cells results in less cell death.

hCDC14A was originally shown to dephosphorylate the substrates of cyclin-dependent kinases, including tumor protein (p53), (proteasome 26S subunit, non-ATPase 9/cyclin dependent kinase inhibitor 1B) p27/KIP1 and phosphatase and tensin homolog (PTEN), and govern the cell cycle transition[21]. Our previous study[13] revealed that hCDC14A *per se* can also be phosphorylated at Ser351 and Ser363 by polo-like kinase 1 and promote cell proliferation and insulin secretion in β cells. However, this protective role of hCDC14A in β cells has never been connected with autophagy. In addition, the regulatory mechanism underlying the given phosphorylation sites of Ser484 and Ser583 in hCDC14A has not been reported. With the same model of murine β-TC3 cells in this study, we observed an enhanced effect of hCDC14A on autophagy upon HG and LG induction (Figure 1), which suggests that hCDC14A is beneficial for the survival of β cells *via* autophagic activation. Moreover, we determined that the Ser484 of hCDC14A contributes to the enhancement of autophagy (Figure 2), which suggests that Ser484 phosphorylation is essential for hCDC14A protein structure alteration and functional activation. Unfortunately, the crystal structure of hCDC14A has not been deciphered. In addition, rapamycin-induced autophagy was partially compromised by the Ser484 MT hCDC14A (Figure 2E), indicating that hCDC14A exerts a synergistic but not dependent effect on the signaling pathways of autophagy induced by rapamycin.

Finally, ZIPK mediates Ser484 phosphorylation of hCDC14A (Figure 3). We previously reported[22] that ZIPK could interact with hCDC14A by yeast two-hybrid assay but did not know the reason for this interaction. Interestingly, we also found that ZIPK, as a potential therapeutic target for the treatment of diabetic vascular complications, plays a protective role in HG-treated aortic smooth muscle cells[23]. Herein, we linked these data and finally confirmed this hypothesis using a mass spectrum assay (Figure 3B) and reversed IP (Figure 3C). Unexpectedly, ZIPK can induce cell apoptosis and autophagic death *via* caspase-dependent and caspase-independent pathways[24,25] and acts as a molecular switch bridging apoptotic and autophagic cell death pathways. In our results, the autophagy and cell cycle modulation suppressed by ZIPK KD was consistent. However, it is difficult to assess the apoptotic status of β-TC3 cells simultaneously treated with ZIPK and hCDC14A. The repressed cell apoptosis induced by hCDC14A and the enhancement of apoptosis by ZIPK are contradictory to the effect of Ser484 phosphorylation of hCDC14A regulated by ZIPK. We speculate that hCDC14A OE is likely to counteract the effect of cell proliferation and apoptosis caused by ZIPK, which needs to be further investigated.

Conclusions

Overall, this study reveals the role of hCDC14A in rat β-TC3 cells exposed to glucose stimulation, and further indicates a novel mechanism underlying hCDC14A regulated by ZIIPK. We conclude that hCDC14A contributes to autophagic activation upon high glucose stress in pancreatic islet β cells *via* Ser484 phosphorylation by ZIPK. Our results provide insights into the underlying mechanism of β cells against diabetic stress and provide a potential therapeutic target for diabetes treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

HH performed all the experiments and analyzed the data. JY, DS, LW and FH helped with the experimental performance. XH, HG, XX, SZ and PZ helped with the experimental design and data analysis. JC designed the whole project and drafted and revised the manuscript. This project is supported by the Natural Science Foundation for Young Scientists of China (Grant No. 81700705), the Natural Science Foundation for Zhejiang Province of China (Grant No. LY17H070001) and the Natural Science Foundation for Zhejiang Province of China (Grant No. LSY19H160001).

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