

MTORC1 inhibition drives crinophagic degradation of glucagon



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ABSTRACT

Objective: Crinophagy is a secretory granule-specific autophagic process that regulates hormone content and secretion in endocrine cells. However, despite being one of the earliest described autophagic processes, its mechanism of action and regulation in mammalian cells remains unclear.

Methods and results: Here, we examined mammalian crinophagy and its modulation that regulate hormone secretion in a glucagon-producing mouse pancreatic α -cell line, alpha TC1 clone 9 (α TC9), and *in vivo*. Western blot, electron microscopy, and immunofluorescence analyses were performed to study crinophagy and glucagon secretion in α TC9 cells and C57BL/6 mice, in response to the mammalian target of rapamycin complex 1 (MTORC1) inhibitor rapamycin. Amino acid depletion and pharmacological inhibition of MTORC1 increased the shuttling of glucagon-containing secretory granules into lysosomes for crinophagic degradation to reduce glucagon secretion through a macroautophagy-independent mechanism. Furthermore, MTORC1 inhibition reduced both intracellular and secreted glucagon in rapamycin-treated mice, in response to hypoglycaemia.

Conclusion: In summary, we have identified a novel crinophagic mechanism of intracellular glucagon turnover in pancreatic α -cells regulated by MTORC1 signalling.

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Keywords Autophagy; Crinophagy; Diabetes; Glucagon; Lysosomes; MTORC1; Rapamycin

1. INTRODUCTION

Autophagy is a degradative process that recycles intracellular contents such as proteins, lipids, and damaged organelles within lysosomes to maintain cellular and systemic homeostasis [1]. Depending on the cargo and mode of delivery of the intracellular components to the lysosomes, there are four main forms of autophagic processes in mammals: macroautophagy, microautophagy, chaperone-mediated autophagy, and crinophagy [1]. Of all these types, mammalian crinophagy is the least understood. Discovered in the 1960s [2] and named by Christian de Duve, crinophagy involves the direct fusion of secretory granules with late endosomes/lysosomes, resulting in the rapid digestion of contents and redeployment of their products [3]. Crinophagy occurs in all cells that secrete peptide hormones and enzymes such as exocrine, endocrine, and neuroendocrine cells [4]. Although some components of macroautophagy have been implicated in secretory vesicle degradation, their involvement in crinophagy remains controversial [3,5–7]. While the physiological role of crinophagy may be to limit excessive hormonal secretion or to provide amino acids under starvation [8], its over- or under-activation could underlie the abnormal endocrine and metabolic defects observed in humans [9–12]. Therefore, a better mechanistic understanding of mammalian crinophagy and revealing the possible

pharmacologic targets of this cellular process may hold promise for future crinophagy-directed therapies.

Glucagon is a glycoprotein hormone that is normally secreted by the pancreatic α -cells in response to hypoglycemia. However, its dysregulation plays a significant role in the pathogenesis of both type I and type II diabetes mellitus (DM) [13], as excessive glucagon secretion can occur in these conditions. This glucagon dysregulation contributes to hyperglycaemia through glucagon's glycogenolytic and gluconeogenic effects on the liver [13]. The importance of impaired glucagon secretion in DM has led to intensive research aimed at preventing hyperglucagonemia and blocking its hyperglycaemic action [14]; either by inhibiting glucagon secretion from the pancreatic α -cells or inhibiting glucagon effects by blocking glucagon receptor activation. Although the latter approach has shown promising results [15], the former approach has been hampered by a lack of understanding of intracellular glucagon turnover.

In the present study, we examined the mechanism and regulation of glucagon turnover by a novel form of crinophagy, in a mouse pancreatic α -cell line, alpha TC1 clone 9 (α TC9), and *in vivo*. We also demonstrated a central role for the mammalian target of rapamycin complex 1 (MTORC1) in mediating glucagon crinophagy and its pharmacological inhibition as a novel strategy to regulate glucagon release.

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This study, thus, serves as proof-of-concept for further research directed towards crinophagy modulation in endocrine and metabolic disorders.

2. MATERIAL AND METHODS

2.1. Cell culture and chemicals

Alpha TC1 clone 9 (α TC9) is a pancreatic α -cell line cloned from the alpha TC1 cell line which was derived from an adenoma created in transgenic mice expressing the SV40 large T antigen oncogene, under the control of the rat preproglucagon promoter [16]. The cells were grown in regular media containing DMEM and 1 g/L glucose, supplemented with 10% FBS, 50 U/mL penicillin, and 50 U/mL streptomycin [16,17]. For nutrient starvation experiments, the regular media was replaced with Hanks' balanced salt solution (HBSS). For siRNA transfection, cells were transfected using RNAiMAX (Thermo Fisher Scientific, 13,778,150) with *Atg5* (Thermo Fisher Scientific, s62452), *beclin* (Thermo Fisher Scientific, s80166), siRNA's (10 nM) for 48 or 72 h followed by treatment with rapamycin. HBSS (Cat#H6648), rapamycin (Cat#R0395), TAT-beclin (Cat# T1331), trehalose (Cat#T9531), torin (Cat#475991), and PP242 (Cat#P0037) were purchased from SIGMA-ALDRICH.

2.2. Transmission electron microscopy (TEM)

Cells were seeded onto four-chambered coverglass (Lab-Tek Chambered Coverglass System; Nalgene-Nunc, Rochester, NY) at a density of 2×10^4 cells/mL (14,000 cells/well). Cells were fixed in 2.5% glutaraldehyde, osmified using 1% osmium tetroxide, and underwent dehydration in varying concentrations of ethanol before embedding in Araldite. Ultrastructural images of cells in ultrathin sections, mounted on copper grids, were acquired using the JEM-1010 transmission electron microscope (JEOL, Japan).

2.3. In vivo experiments

Mice were housed and maintained at a 12-h light/dark cycle at the vivarium of SGPGIMS, Lucknow, in accordance with the institutional animal ethics guidelines. Rapamycin was dissolved in 100% ethanol and stored at -20°C . The stock solution was further diluted in an aqueous solution of 5.2% Tween 80 and 5.2% PEG 400, with a final concentration of 2% ethanol [18]. Six to eight weeks old male C57BL/6 wild-type mice were injected with rapamycin (2 mg/kg) every day for 9 days. Vehicle control mice received an equivalent amount of 5.2% Tween 80 and 5.2% PEG 400, with a final concentration of 2% ethanol. Insulin tolerance test (ITT) was performed on day 8 between injections. Sacrifice was performed on day 9, after the last injection for fed and fasted serum glucagon measurement and tissue collection for immunoblotting and confocal microscopy. Tat-beclin 1 peptide (20 mg/kg) or vehicle control was administered intraperitoneally daily for 7 days, in 6 to 8-week-old C57BL/6 mice, and the pancreas was collected 6-h after the last injection.

2.4. Confocal microscopy

Immunofluorescence experiments were performed in chambered slides and paraffin-embedded sections of the mouse pancreas. In brief, formalin-fixed cells were permeabilized with 0.1% Triton X-100 (SIGMA-ALDRICH, X100) in PBS, for 5–10 min, and blocked with 3% BSA-PBS for 30 min, at room temperature. Cells were incubated with the primary antibody (1:200 in 3% BSA-PBS) overnight at 4°C , followed by fluorochrome-labeled secondary antibodies (Molecular probes), and cell imaging was performed using an LSM710 Carl Zeiss (Carl Zeiss Microscopy GmbH, Germany) confocal microscope. The primary antibodies used were anti-LC3B (Cell Signaling Technology,

#2775), anti-glucagon (Sigma–Aldrich, #G2654), anti-SQSTM1/p62 (Cell Signaling Technology, #7695), anti-GABARAP (Cell Signaling Technology, #13733), and anti-LAMP1 (DSHB, 1D4B). Colocalization studies were performed using ImageJ software with the JACoP plugin. At least 5–10 different fields per section from 5 different animals in each group were analyzed for calculating Pearson's coefficient.

2.5. RNA isolation and qRT-PCR

Total RNA was isolated and qRT-PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, 204,141), according to the manufacturer's instructions. KiCqStart® SYBR® Green Primers were purchased from Sigma–Aldrich, USA. Mouse glucagon primer sequence was as follows:

Forward: 5'-AGGCGAGACTTCCCAGAAGA-3';

Reverse: 5'-AGTGACTGGCAGAGATGTT-3'.

2.6. Glucagon measurement

Glucagon secretion in the α TC9 cell-conditioned media was assayed using Glucagon EIA kit (Cat# RAB0202) from SIGMA-ALDRICH, USA.

2.7. Western blotting

Cells or tissue samples were lysed using CellLytic™ M Cell Lysis Reagent (Sigma, C2978) and immunoblotting was performed as per manufacturer's guidelines (Bio-Rad Laboratories, USA) using ECL Select developing reagent (GE Healthcare, RPN2235). Image acquisition was carried out using Chemi Doc (Bio-Rad Chemi Doc™ MP System, 1,708,280). Densitometry analysis was performed using ImageJ software (NIH, Bethesda, MD, USA). Antibodies used were anti-beclin (Cell Signaling Technology, #3495), anti-ATG5 (Cell Signaling Technology, #12994), anti-phospho MTOR (Cell Signaling Technology, #5536), anti-MTOR (Cell Signaling Technology, #2983), anti-phospho P70S6K (Cell Signaling Technology, #9204), anti-P70S6K (Cell Signaling Technology, #2708), anti-glucagon (ABCAM, #ab92517), and ACTB (Cell Signaling, #4970). Secondary antibodies were also from Cell Signaling Technology, USA.

2.8. Lysosomal fraction purification

Pure lysosomal fractions from tissue and cell homogenates were prepared using LYSIS01 - Lysosome Isolation Kit from Sigma–Aldrich, USA. Fraction purity was ascertained using appropriate marker proteins.

2.9. Statistics

Results are expressed as mean \pm SD. The statistical significance of differences was assessed by unpaired Student's t-test or one-way ANOVA with post-hoc Dunnett's multiple comparisons test used for comparisons among 3 or more groups. $P < 0.05$ is considered as statistically significant.

3. RESULTS

3.1. Amino acid depletion induces crinophagy and MTORC1 inhibition in α -cells

Amino acid depletion has been shown to increase crinophagy in pancreatic β -cells [5]. To determine whether a similar process occurs in pancreatic α -cells, we examined glucagon secretion and autophagy under starvation conditions, by growing α TC9 cells in HBSS, which contains D-glucose and essential inorganic ions but lacks amino acids and serum [19]. We observed a time-dependent decrease in glucagon secretion in α TC9 cells grown in HBSS (Figure 1A). We also found a time-dependent decrease in the levels of intracellular glucagon protein

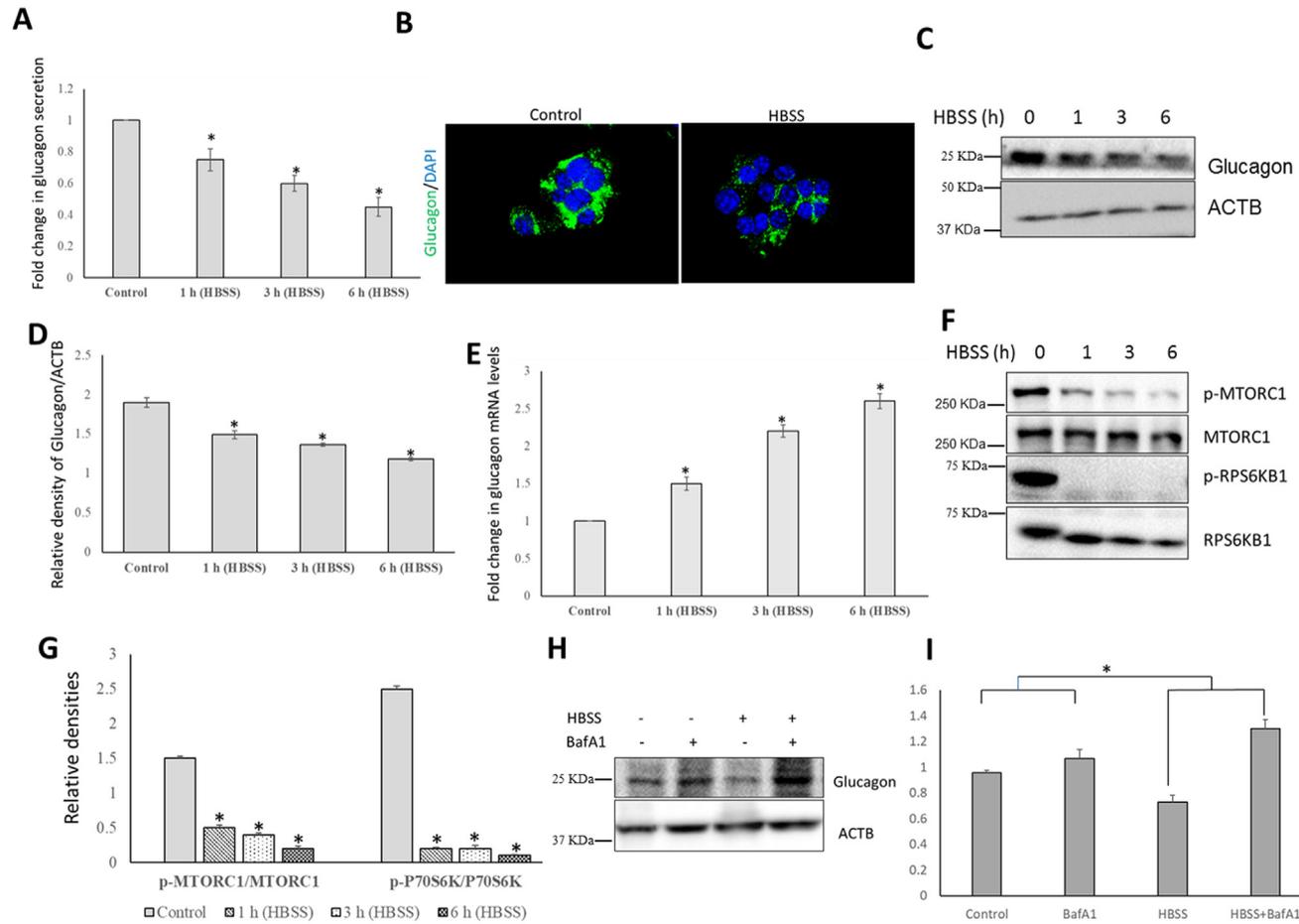


Figure 1: Amino acid withdrawal induces glucagon turnover in lysosomes. (A) Histogram showing fold change in glucagon secretion in the culture media of α TC9 cells cultured in HBSS for different periods. Values are means \pm SD (n = 5, *p < 0.05). Control cells were cultured in regular media. (B) Representative immunofluorescence image showing intracellular glucagon staining in α TC9 cells cultured in HBSS for 6 h. (C, D) Representative immunoblot and densitometric analysis of intracellular glucagon protein levels in α TC9 cells cultured in HBSS for a different time period. Values are means \pm SD (n = 5, *p < 0.05). (E) qRT-PCR based gene expression analysis of glucagon mRNA from α TC9 cells cultured in HBSS for a different time period. Values are means \pm SD (n = 5, *p < 0.05). Of note, immunoblotting with commercial anti-glucagon antibodies identified a ~25 kDa immunoreactive band which represents a proglucagon species [33] and has been referred to as glucagon in this manuscript, in accordance with the antibody datasheet provided by ABCAM. (F, G) Representative immunoblot and densitometric analysis of MTORC1 signaling in α TC9 cells cultured in HBSS for a different period. Values are means \pm SD (n = 5, *p < 0.05). (H, I) Representative immunoblot and densitometric analysis of glucagon levels in α TC9 cells cultured in HBSS (3 h) with or without lysosomal inhibitor BafA1 (50 nM/3 h) showing crinophagic flux. Values are means \pm SD (n = 5, *p < 0.05 shows a significant increase in glucagon content in HBSS + BAF A1 vs. HBSS alone as compared to BafA1 vs. Control alone).

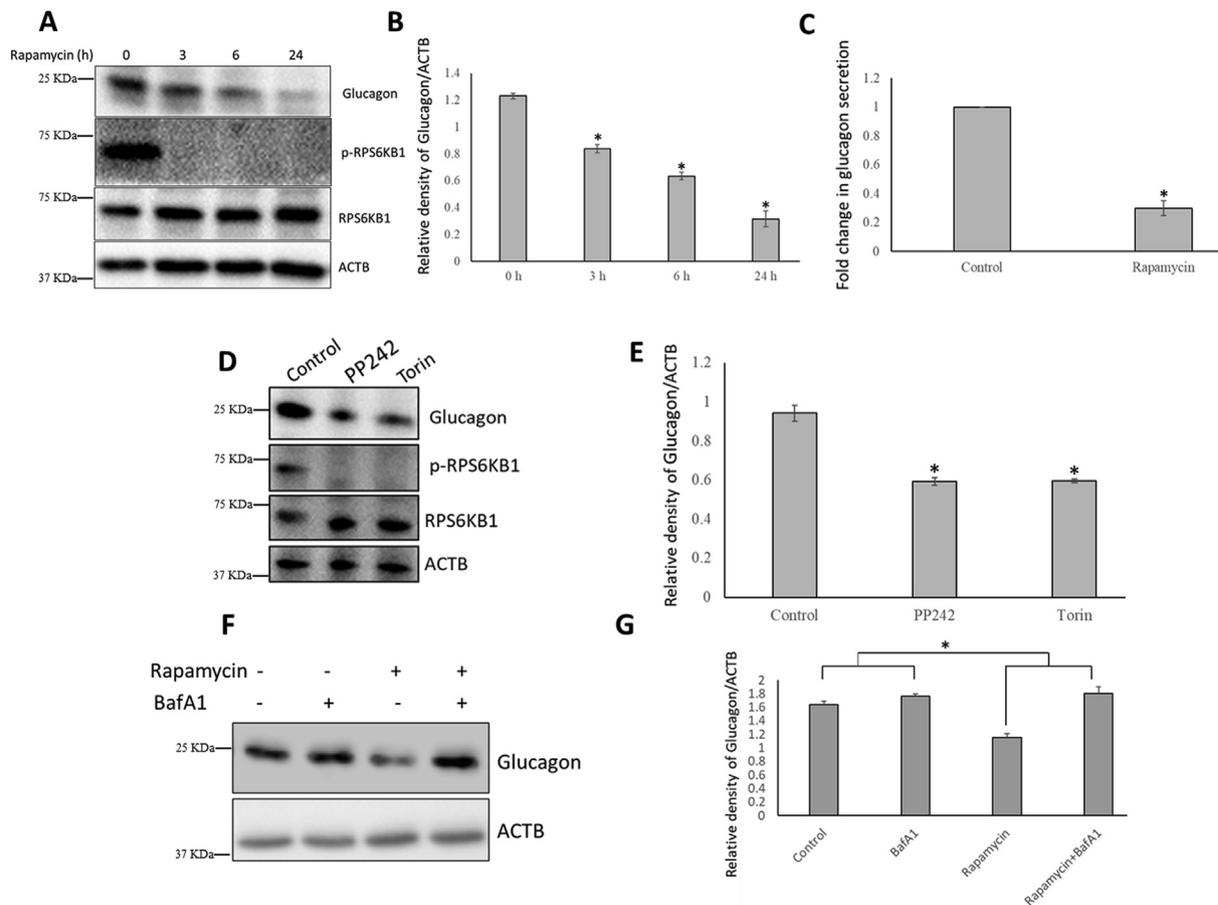


Figure 2: Pharmacological inhibition of MTORC1 induces lysosomal degradation of glucagon. (A, B) Representative immunoblot and densitometric analysis of glucagon levels and MTORC1 signaling in α TC9 cells treated with 300 nM rapamycin for indicated durations. Values are means \pm SD (n = 5, *p < 0.05). (C) Histogram showing fold change in glucagon secretion in the culture media of α TC9 cells treated with 300 nM rapamycin for 24 h. Values are means \pm SD (n = 5, *p < 0.05). (D, E) Representative immunoblot and densitometric analysis of glucagon levels and MTORC1 signaling in α TC9 cells treated with PP242 (500 nM), or Torin1 (500 nM) for 24 h. Values are means \pm SD (n = 5, *p < 0.05). (F, G) Representative immunoblot and densitometric analysis of glucagon levels in α TC9 cells cultured with rapamycin (300 nM/24 h) with or without lysosomal inhibitor BafA1 (10 nM/24 h) showing crinophagic flux. Values are means \pm SD (n = 5, *p < 0.05 shows a significant increase in glucagon content in Rapamycin + BAF1A vs. Rapamycin alone as compared to BafA1 vs. Control alone).

in α -cells grown in HBSS by western blotting and immunostaining (Figure 1B–D). This reduction in glucagon secretion and storage was not caused by a decrease in glucagon gene transcription and/or mRNA stability as its mRNA levels were increased in α TC9 cells cultured in HBSS (Figure 1E), and suggested that post-translational degradation could reduce glucagon secretion. HBSS, which lacks amino acids, is known to inhibit MTORC1 that, in turn, can induce autophagy [19]. Thus, we examined the effect of HBSS on the phosphorylation of MTORC1 and its substrate RPS6KB1/P70S6K1, and observed that their phosphorylation was decreased in α TC9 cells grown in HBSS (Figure 1F, G). To determine whether the HBSS-mediated decrease in glucagon protein was caused by lysosomal degradation rather than decreased glucagon protein translation, we treated both control and HBSS-treated cells with the lysosomal inhibitor, bafilomycin A1 (BafA1). Our results showed that the restoration of intracellular glucagon level by BafA1 was more pronounced in HBSS-treated cells than in control cells (Figure 1H,I) implicating a direct involvement of lysosomes in glucagon turnover, in response to MTORC1 inhibition. Although autophagy genes are required for the capture of insulin-containing secretory granules into autophagosomes [20], their role in pancreatic α -cell crinophagy has not been investigated. Accordingly,

we observed decreased lysosomal localization of MTORC1 upon HBSS treatment (Suppl Figure 1A,B) together with decreased intracellular SQSTM1 levels, suggesting there could be increased macroautophagy induction (Suppl Figure 1A). Surprisingly, the genetic silencing of macroautophagy genes, *Atg5* and *beclin1*, did not affect either basal or HBSS-induced glucagon degradation in α TC9 cells, suggesting that macroautophagy was not involved in glucagon degradation under these conditions (Suppl Figure 1C,D). Taken together, these findings suggested that amino acid starvation inhibited MTORC1 and degraded glucagon through another lysosome-dependent degradation mechanism, i.e., crinophagy that was independent of macroautophagy.

3.2. Pharmacological inhibition of MTORC1 is sufficient to trigger glucagon crinophagy

We next examined whether pharmacological inhibition of MTORC1 under normal growth conditions could induce glucagon crinophagy in α TC9 cells. Interestingly, the MTORC1 inhibitor, rapamycin reduced the intracellular glucagon levels in a time-dependent manner (Figure 2A,B). Concomitantly, glucagon secretion in the culture media, during both basal and K^+ induced stimulatory conditions, was reduced by rapamycin treatment (Figure 2C and Suppl Figure 2). Similar results

were observed using two other MTORC1 inhibitors, PP242 and torin1 (Figure 2D,E), suggesting that MTORC1-specific signalling was directly involved in glucagon turnover. Additionally, BafA1 completely abolished rapamycin-induced glucagon turnover to demonstrate the involvement of lysosomes in MTORC1-mediated glucagon crinophagy (Figure 2F,G).

Evidence for crinophagy-mediated glucagon turnover was further strengthened by colocalization studies. In immunofluorescence studies, rapamycin treatment significantly increased the colocalization of glucagon and LAMP1, indicating that the former resided within the lysosomal compartments of α TC9 cells (Figure 3 A and B). TEM provided further evidence of increased localization of glucagon secretory granules within the lysosomal compartments following rapamycin treatment (Suppl Figure 3A,B). In contrast, most of the glucagon secretory vesicles/granules in control cells were in close proximity to the cell plasma membrane (Suppl Figure 3A,B).

3.3. Glucagon turnover by rapamycin-induced crinophagy does not involve macroautophagy

As rapamycin is a known macroautophagy inducer, we further examined whether some of rapamycin's effects on glucagon degradation involved macroautophagy. Accordingly, we co-treated α TC9 cells with different macroautophagy inducers in addition to rapamycin. Unlike rapamycin, macroautophagy inducers such as trehalose [21]

and Tat-beclin 1 peptide [22] did not reduce intracellular glucagon content despite increasing the levels of the autophagosomal marker, LC3B-II (Figure 4A,B). Additionally, siRNA-mediated knockdown of key macroautophagy proteins, *Atg5* and *beclin1* did not affect rapamycin-induced glucagon turnover (Figure 4C,D) despite their ability to inhibit macroautophagy as indicated by p62/SQSTM1 levels. Furthermore, we did not observe the colocalization of glucagon with LC3B by immunostaining, suggesting that the crinophagy of glucagon did not employ autophagosomes typically involved in macroautophagy (Figure 4E,F). These data indicate that macroautophagy was not involved in glucagon degradation in pancreatic α -cells. Instead, MTORC1 signalling regulated glucagon turnover through crinophagy that was distinct from macroautophagy. Furthermore, the co-inductions of crinophagy and macroautophagy by MTORC1 inhibition most likely served distinct functions in pancreatic α -cells.

3.4. Rapamycin treatment *in vivo* inhibits glucagon secretory responses to hypoglycaemia and increases lysosomal localization of glucagon

To validate the observed *in vitro* effects of MTORC1 inhibition on glucagon secretion *in vivo*, we evaluated the effect of pharmacologic inhibition of MTORC1 on glucagon secretion by intraperitoneal administration of rapamycin in mice. Using insulin-induced hypoglycaemia to physiologically stimulate glucagon release from

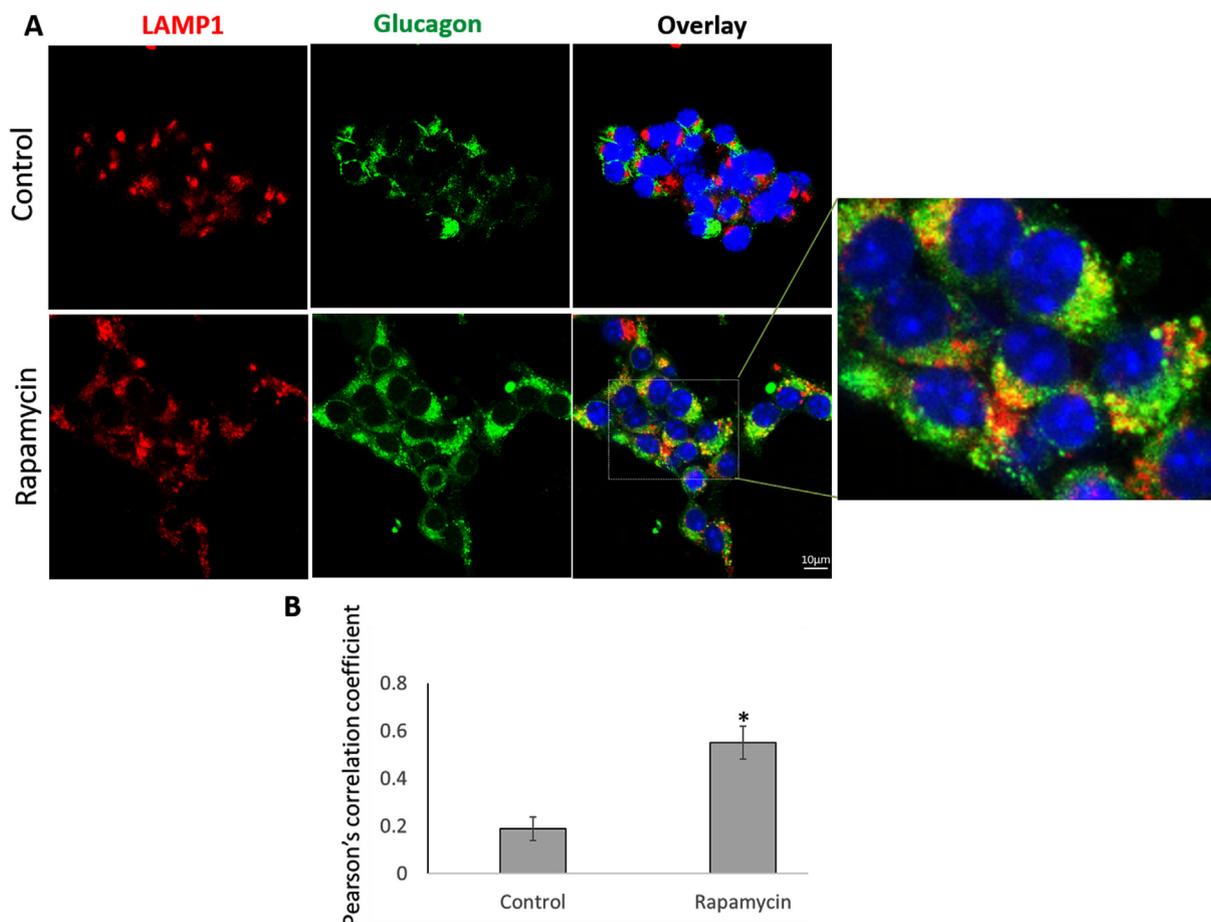


Figure 3: Rapamycin-induced MTORC1 inhibition leads to glucagon crinophagy in α TC9 cells. (A, B). Immunofluorescence imaging and Pearson's correlation coefficient values of colocalized glucagon (green) and lysosomes (red) in control and rapamycin (300 nM/3 h) treated α TC9 cells. Cell nuclei are stained with DAPI (blue). Values are means \pm SD (n = 5; *p < 0.05).

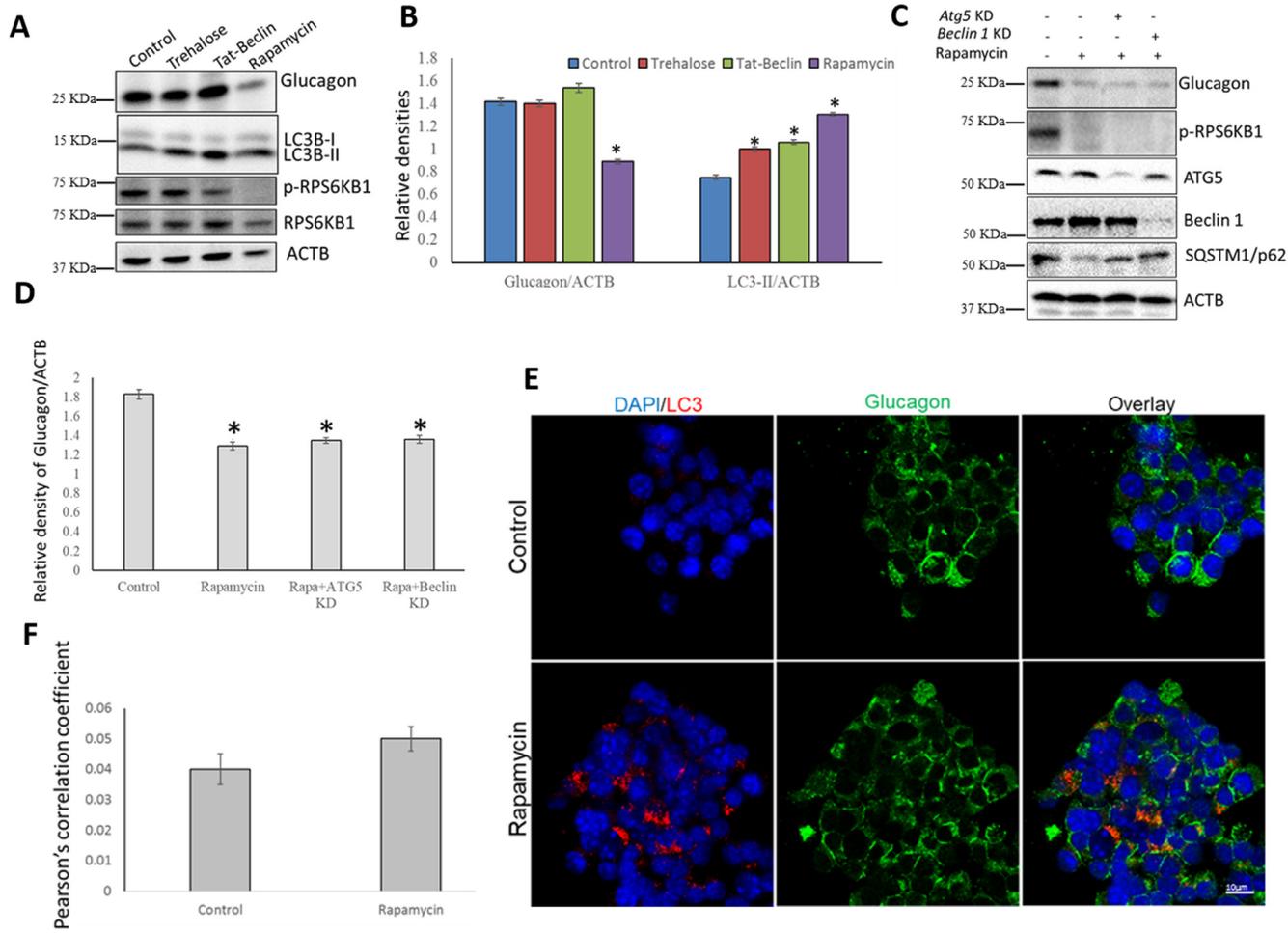


Figure 4: MTORC1 inhibition promotes glucagon crinophagy independent of macroautophagy. (A, B) Representative immunoblot and densitometric analysis of glucagon levels in α TC9 cells treated with rapamycin (300 nM), trehalose (100 mM) and Tat-beclin peptide (25 μ M) for 24 h. Values are means \pm SD ($n = 5$, $*p < 0.05$). (C, D) Representative immunoblot and densitometric analysis of glucagon levels in α TC9 cells treated with rapamycin (300 nM/24 h) with or without genetic silencing of macro-autophagy genes *ATG5* or *Beclin*. Values are means \pm SD ($n = 5$, $*p < 0.05$ vs. control). (E, F) Immunofluorescence imaging and Pearson's correlation coefficients of control and rapamycin (300 nM/3 h) treated α TC9 cells stained with glucagon and autophagosome marker LC3B antibody.

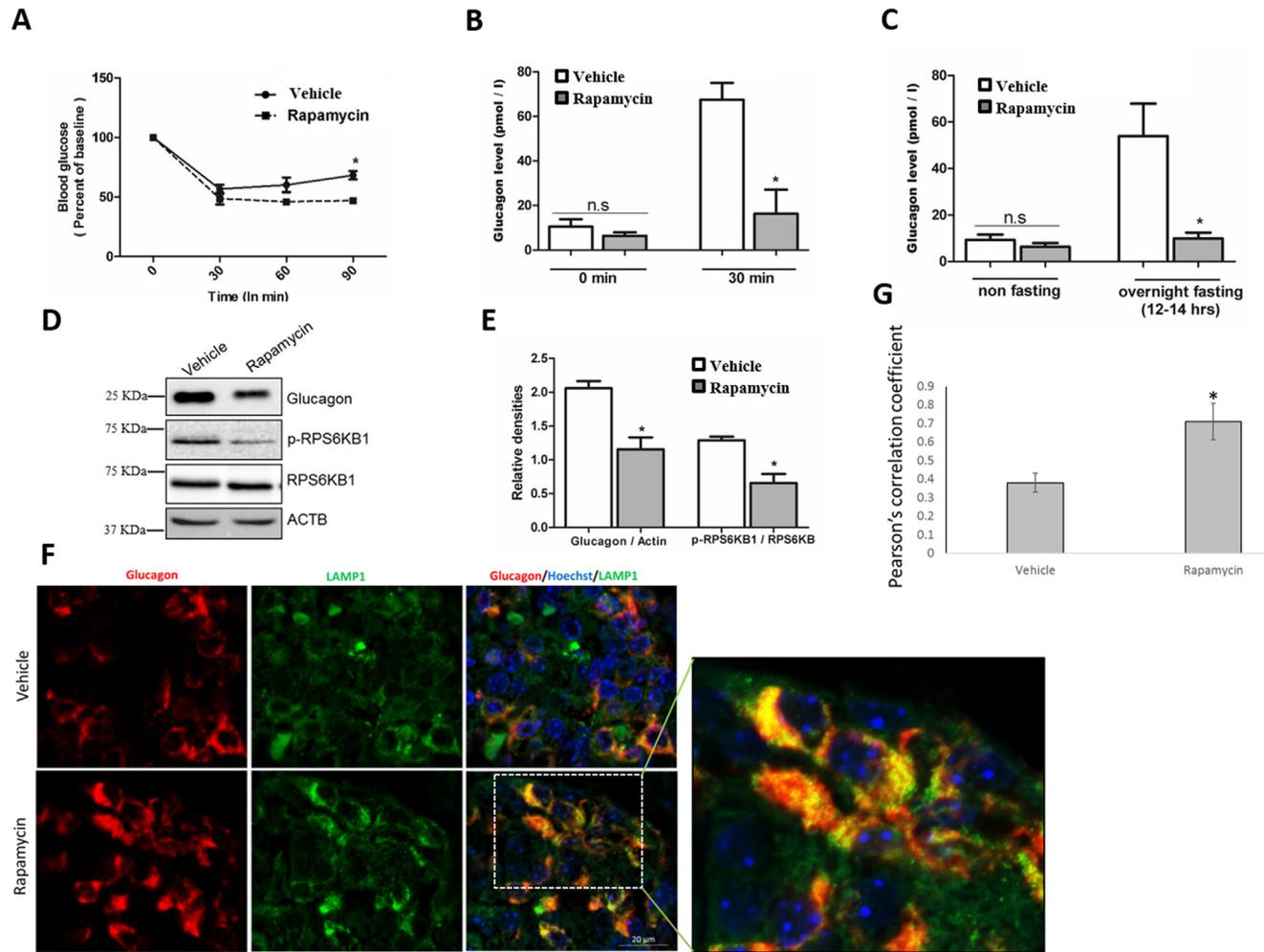


Figure 5: Rapamycin induces crinophagy and regulates glucagon secretion *in vivo*. (A) Rapamycin (2 mg/kg) or vehicle control was administered intraperitoneally every alternate day for 9 days (*i.e.*, on days 1, 3, 5, 7, and 9 for a total of 5 injections) in 6- to 8-week-old C57BL/6 mouse before tissue collection. For ITT, glucose levels were measured after intraperitoneal injection of insulin (1 U/kg) mice on day 8 (*i.e.*, one day before the last injection) (n = 5). Data are shown as means ± SEM. *p ≤ 0.05. (B) Glucagon levels were measured at 0 and 30 min after insulin injection in the same group of mice at day 8 (n = 5). Data are shown as means ± SEM. *p ≤ 0.05. (C) Fed and fasted glucagon levels in 6–8 weeks old C57BL/6 mice that were preinjected with vehicle or rapamycin every alternate day for 9 days (n = 5). Data are shown as means ± SEM. *p ≤ 0.05. (D, E) Representative immunoblot in islet tissue lysates from mice injected with vehicle or rapamycin every alternate day for 9 days showing the levels of intracellular glucagon and RPS6KB1 (n = 5). Data are shown as means ± SD, *p ≤ 0.05. (F, G) Immunofluorescence imaging and Pearson's correlation coefficient values of colocalized glucagon (red) and lysosomes (green, LAMP1) in islet sections from mice injected with vehicle or rapamycin every alternate day for 9 days. Cell nuclei are stained with DAPI (blue). Values are means ± SD (n = 5, *p < 0.05).

pancreatic α -cells, we observed that rapamycin-treated mice displayed lower serum glucose than control mice, 90 min after insulin injection, suggesting that rapamycin decreased the counter-regulatory response to hypoglycaemia (Figure 5A). Furthermore, serum glucagon levels in response to hypoglycaemia were significantly compromised by rapamycin treatment (Figure 5B). Additionally, fasting-induced glucagon secretion was also blunted in rapamycin-treated mice (Figure 5C). Also, the intracellular glucagon content in the pancreas was significantly reduced after rapamycin treatment (Figure 5D,E) and correlated with MTORC1 inhibition measured by decreased RPS6KB1 phosphorylation ratio (Figure 5D,E). We also noted that glucagon colocalized more with lysosomal marker LAMP-1 in islet sections of rapamycin-treated mice compared to control mice (Figure 5F,G). Furthermore, the immunoblots showed significantly more glucagon enriched in the lysosomal fraction of rapamycin-treated mice pancreas (Suppl Figure 4). Although macroautophagy flux increased on rapamycin treatment (Suppl Figure 5), there was no evidence of increased colocalization of glucagon with the macroautophagy marker, LC3B, in the islets of the mice treated with rapamycin vs. vehicle (Suppl Figure 6A,B). Additionally, there was no effect of a macroautophagy inducer, Tat-beclin 1 peptide [22], in altering the intracellular glucagon levels (Suppl Figure 6C,D) suggesting that the macroautophagy machinery most likely were not involved in the delivery of glucagon secretory granules to lysosomes *in vivo*. Taken together, these findings showed that MTORC1 inhibition decreased islet intracellular glucagon content and secretion by crinophagy both *in vitro* and *in vivo*.

4. DISCUSSION

Peptide hormone production and secretion are regulated at several key steps such as gene transcription, post-translational maturation, packaging into secretory granules, and secretion by exocytosis. However, during conditions of limited demand for hormone secretion and/or starvation, the excess hormones stored within secretory granules can be redirected towards the endosomal-lysosomal compartments for degradation [8]. This cellular process of secretory granule degradation by lysosomes has been termed as “crinophagy” [23]. Although this process in mammals was first discovered in the 1960s [2], there has been little progress in our understanding of the mechanism(s) underlying crinophagy because of the lack of good model systems to study and pharmacologically manipulate crinophagy. Studies performed in pancreatic β -cells have shown that the degradation route for insulin-containing secretory granules in β -cells may involve multiple autophagic routes — including direct fusion of secretory granules with lysosomes (crinophagy) and engulfment of secretory granules by autophagosomes (macroautophagy) or directly by lysosomes (microautophagy) [4,24]. Furthermore, a type of crinophagic activity was observed in pancreatic β -cells, termed as “starvation-induced nascent granule degradation” (SINGD) which regulated insulin release by inhibiting macroautophagy in DM [5,10]. The activation of crinophagy likely protects the hormone-producing cells by degrading and recycling excess secretory material under stress or nutrient deprivation [8,25]. However, over activation of crinophagy can be detrimental owing to its inhibitory actions on cell survival and hormone secretory pathways [8,25]. Therefore, determining the cellular processes involved in crinophagy in individual endocrine organs is important for understanding hormone secretion under different nutritional conditions and dysfunctional responses that occur in endocrine/metabolic disorders.

There has been increased interest in the role of glucagon in diabetes and obesity as it is a major contributor toward hepatic glucose output and its oversecretion leads to hyperglycaemia in humans [14]. In this study, we examined the mechanism of crinophagy in a pancreatic α -cell line that produces glucagon. We observed that similar to insulin, intracellular glucagon was degraded in the lysosomal compartments of α -cells during amino acid starvation. MTORC1, which is a central regulator of macroautophagy [26], also regulated crinophagy in pancreatic α -cells both *in vitro* and *in vivo*. However, we found that crinophagy was distinct from macroautophagy, as it did not require several key autophagy components such as *Atg5* and *beclin1*. Furthermore, MTORC1-independent inducers of macroautophagy such as trehalose and Tat-beclin1 peptide did not decrease intracellular glucagon content.

In response to nutrient deprivation, there is increased delivery of newly synthesized insulin granules to lysosomes by crinophagy concomitant with lysosomal MTORC1 recruitment and activation to suppress macroautophagy [5]. Conceptually, the early switch from macroautophagy to crinophagy is important to reduce insulin secretion in response to fasting, and to degrade insulin granules that provide nutrients for cell survival. As the α -cells secrete glucagon in response to fasting, it might be expected that α -cells would preserve their granules and make nutrients for survival through macroautophagy. However, our results suggest that amino acid starvation led to intracellular glucagon degradation rather than increased secretion. A possible reason for this occurrence could be the particular starvation conditions of amino acid deprivation used in our *in vitro* study. During physiological fasting, there presumably is an initial decrease in serum glucose level rather than amino acids, which triggers glucagon release from α -cells rather than its degradation. Furthermore, an increase in glucose vs. amino acids is also known to have a different effect on glucagon secretion wherein glucose suppresses but amino acids increase glucagon secretion [27]. Therefore, amino acid starvation conditions used in this study resulted in decreased glucagon secretion. Similarly, short-term fasting and insulin-induced hypoglycaemic conditions result in glucagon release, as these starvation conditions do not decrease the intracellular amino acids' levels within α -cells, thereby indicating different regulation of glucagon turnover and secretion in response to different nutrient starvation.

Macroautophagy was previously reported to be decreased in β -cells during amino acid starvation [5]. Interestingly, we showed that macroautophagy was increased in the α -cells under these conditions. Additionally, we observed reduced lysosomal localization of MTORC1 and decreased MTORC1 activity after amino acid deprivation in α -cells whereas MTORC1 was activated in β -cells under the same conditions [5]. However, it is significant that despite these differences, amino acid starvation led to increased crinophagy of glucagon granules in α -cells and insulin granules in β -cells [5]. Presently, it is unclear why these two types of islet cells behave differently during amino acid starvation with respect to macroautophagy; however, it is noteworthy that increased macroautophagy in α -cells did not contribute to the increased degradation of glucagon. A limitation of the present study is the lack of complete macroautophagy gene knockout models. Therefore, we cannot fully rule out the involvement of residual amounts of macroautophagy genes such as *Atg5* and *beclin1* in glucagon degradation; given some minor leakiness of siRNA-based knockdown approach. Nevertheless, considering the corroborative results obtained by glucagon-LC3B colocalization, macroautophagy inducer experiments both *in vitro* and *in vivo*, and the relative effects of autophagy

gene knockdown on SQSTM1 levels vs. glucagon levels, it seems unlikely that macroautophagy plays a significant role in glucagon degradation through lysosomes.

Macroautophagy has been implicated in mediating insulin secretion from β -cells [5]; however, further studies will be required to determine whether it is similar for glucagon secretion. Notably, hypoglycaemia triggers glucagon secretion by a reduction in the cytoplasmic ATP/ADP ratio, leading to diminished K_{ATP} channel activity, increased P/Q type Ca^{++} channels opening, and Ca^{2+} dependent glucagon secretory granules exocytosis [28]. Therefore, it is possible that hypoglycaemia-induced ATP decline could trigger an AMPK driven macroautophagy to enable glucagon release. Concerning amino acid starvation, however, MTORC1-driven macroautophagy in α -cells most likely serves other pro-survival functions and not glucagon turnover and secretion.

The highly conserved small GTPase, RAB7, and SNARE protein, SNAP29, have been observed to play important roles in insulin degradation by crinophagy [6,29]. Thus, it is possible that these or other specific proteins present in the secretory granules may be critical to determine the fate of glucagon release vs. lysosomal shuttling. In this regard, a recent study identified stathmin-2, which is present in the membrane of glucagon-containing secretory granules, as a critical protein that diverted glucagon towards endosomal–lysosomal pathways for degradation [30]. Further studies linking MTORC1 inhibition to the regulation of secretory granule sorting through RAB7 and SNARE family proteins and their potential interaction with lysosomes need to be investigated in the regulation of glucagon crinophagy.

MTORC1 was recently shown to control nutrient-dependent regulation of glucagon secretion and to maintain α -cell mass in mice [18]. Taken together, these results may offer an explanation for the clinical observation of persistent hypoglycemia in patients after islet transplantation when they were administered rapamycin for chronic immunosuppression. This treatment would inhibit MTORC1 activity in α -cells [31,32] and thus induce crinophagy-mediated glucagon degradation to reduce glucagon secretion. Our results also raise the possibility that MTORC1 inhibitors with more specificity for α -cells could be employed to decrease hyperglucagonemia in type I diabetes and some type II diabetes patients. In summary, we have identified a novel mechanism that controls glucagon degradation and secretion through MTORC1-regulated crinophagy. These findings raise the exciting possibility that MTORC1 inhibition in α -cells may be a new therapeutic approach to counteract dysregulated glucagon secretion in diabetes.

AUTHOR CONTRIBUTIONS

R.A.S. conceived the experiments, researched the data, revised, and wrote the manuscript. R.A.S., B.H.B. and P.M.Y. researched the data, revised, and approved the manuscript. R.A.S, S. R., S.X. A.T, S.R., and W.Y. performed the experiments.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2021.101286>.

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