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The Map3k12 (Dlk)/JNK3 signaling pathway is required for pancreatic beta-cell proliferation during postnatal development

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Abstract

Unveiling the key pathways underlying postnatal beta-cell proliferation can be instrumental to decipher the mechanisms of beta-cell mass plasticity to increased physiological demand of insulin during weight gain and pregnancy. Using transcriptome and global Serine Threonine Kinase activity (STK) analyses of islets from newborn (10 days old) and adult rats, we found that highly proliferative neonatal rat islet cells display a substantially elevated activity of the mitogen activated protein 3 kinase 12, also called dual leucine zipper-bearing kinase (Dlk). As a key upstream component of the c-Jun amino terminal kinase (Jnk) pathway, Dlk overexpression was associated with increased Jnk3 activity and was mainly localized in the beta-cell cytoplasm. We provide the evidence that Dlk associates with and activates Jnk3, and that this cascade stimulates the expression of *Ccnd1* and *Ccnd2*, two essential cyclins controlling postnatal beta-cell replication. Silencing of *Dlk* or of *Jnk3* in neonatal islet cells dramatically hampered primary beta-cell replication and the expression of the two cyclins. Moreover, the expression of *Dlk*, *Jnk3*, *Ccnd1* and *Ccnd2* was induced in high replicative islet beta cells from *ob/ob* mice during weight gain, and from pregnant female rats. In human islets from non-diabetic obese individuals, DLK expression was also cytoplasmic and the rise of the mRNA level was associated with an increase of *JNK3*, *CCND1* and *CCND2* mRNA levels, when compared to islets from lean and obese patients with diabetes. In conclusion, we find that activation of Jnk3 signalling by Dlk could be a key mechanism for adapting islet beta-cell mass during postnatal development and weight gain.

Keywords Beta-cell mass · Mapk · Obesity · Pregnancy · Postnatal development

Abbreviations

Ccnd	Cyclin D	Cdkn	Cyclin-dependent kinase inhibitor
Cdk	Cyclin-dependent kinase	DLK	Dual leucine Zipper Kinase
		JNK	C-Jun amino terminal kinase
		MAP3K12	Mitogen-activated protein kinase 12
		MAPK10	Mitogen-activated protein kinase 10
		STK	Serine threonine kinase

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Introduction

Pancreatic beta-cell mass expansion is fundamental for achieving appropriate glucose control in response to physiological and pathophysiological conditions [1]. In rodent models of obesity and pregnancy, the expansion of the functional beta-cell mass relies mainly on increased beta-cell proliferation [2, 3]. Although proliferation of human islet cells is still debated, there is some evidence for an increase in pancreatic beta-cell replication in obese individuals [4, 5]. During the postnatal development, the elevated replication rate of pancreatic beta cells that precedes the acquisition of glucose competence is a key process permitting the necessary expansion of the beta-cell mass [6]. In rats, beta-cell replication is substantial from birth until the 10th postnatal day [7]. Thereafter, the replication rate decreases overtime and remains more or less constant throughout the adult life [7]. In human, a similar pattern of beta-cell growth associated with postnatal beta-cell mass expansion has been postulated [8, 9]. The greatest beta-cell replication is observed before 2 years of age with a progressive decline continuing until 5 years [8, 9]. Therefore, the postnatal proliferative capacity is critical for achieving an appropriate adult beta-cell mass, which is important for preventing type 2 diabetes development.

Several independent studies have reported that stimulation of cell cycle gene expression cyclin D2 (*Ccnd2*) and D1 (*Ccnd1*) regulating the cyclin dependent kinases 4 and 6 is a key for postnatal beta-cell growth [7, 10]. The upstream regulation of the two cyclins usually rely on serine threonine kinases (STKs) activation, a group of enzymes comprising hundreds of members including mitogen activated protein kinases (MAPKs) [11]. The c-Jun N terminal kinases (Jnks) group of the MAPKs driven by the Map3k12 also called Dual leucine Zipper Kinase (Dlk) regulates cell proliferation in a mechanism evolving the stimulation of *Ccnd1* [12]. In pancreatic beta cells, several STKs have been identified as key players in the regulation of both proliferation and mass in response to physiological and pathophysiological conditions in adults [13]. Some of them are therapeutic targets of the Glucagon-like peptide 1 (GLP-1) receptor agonists, current antidiabetic medicines that potentiate glucose-induced insulin secretion, stimulate beta-cell proliferation and protect beta cell against apoptosis [14]. The identification of STK regulating *Ccnd2* and *Ccnd1* and thereby beta-cell proliferation during postnatal development would be insightful for identifying new targets for improving beta-cell mass in diabetes. In the present study, using transcriptome analysis and whole STK activity measurement, we found that Map3k12 (Dlk)/Mapk10 (Jnk3) signaling is stimulated in neonate islets and is required for postnatal beta-cell mass proliferation

and expansion by inducing *Ccnd2* and *Ccnd1*. In addition, we found that this signaling is likely involved in beta-cell mass adaptation in response to weight gain and pregnancy.

Materials and methods

Animals

Male and pregnant female Sprague–Dawley rats were obtained from Janvier Laboratories (Le Genest-Saint-Isle, France) and housed under a 12 h/12 h light/dark cycle in a climate-controlled and pathogen-free facility. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the local committee and Veterinary Office. At birth, litters were adjusted to 10–12 pups per dam to standardise the mothers' milk availability. Newborn males and females were nursed until they were euthanised at postnatal day (P)10. Control female mice and obese (*ob/ob*) female mice of the C57BL/6J background were obtained from Elevage Janvier (Le Genest-Saint-Isle, France).

Cell culture

Pancreatic islets from adult and newborn rats were isolated by collagenase digestion. In adults, after ligation at the common bile duct, 10 ml of 2 mg/ml of collagenase solution (Roche, Indianapolis, IN, USA) was injected via the ampulla of Vater. In newborn rats, total pancreas was isolated and was incubated in a 5 ml of 1.5 mg/ml of collagenase for 5 min for lysis. Isolated islets from adult and newborn rats were then purified on a Histopaque density gradient. Islets were dissociated by trypsinization in Trypsin–EDTA 0.5% (ThermoFisher) at 37 °C for 8 min. The insulin-secreting cell line (INS-1E) and dispersed rat islet cells were maintained as previously described [7]. Isolated human islets were obtained from the biotherapies for diabetes unit from the “Centre Hospitalier Régional et Universitaire de Lille”. Human pancreases were harvested from adult brain-dead donors in accordance with French regulations and with the local Institutional Ethical Committee from the “Centre Hospitalier Régional et Universitaire de Lille”. Isolation and preparation of islets in Lille were conducted as described previously [15].

Western blotting

Cells were incubated for 30 min on ice in the lysis buffer (20 mM Tris acetate, 0.27 mM Sucrose, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM Sodium Fluoride, 10 mM BetaGlycérophosphate, 1 mM DTT) supplemented

with proteases and phosphatases inhibitors (Roche). For nuclear extracts preparation, the cells were scraped into 1.5 ml of cold phosphate-buffered saline (PBS), pelleted for 10 s and resuspended in 400 μ l cold Buffer A (10 mM HEPES–KOH pH 7.9 at 4 °C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF) by flicking the tube. The cells were allowed to swell on ice for 10 min, and then vortexed for 10 s. Samples were centrifuged for 10 s, and the supernatant fraction discarded. The pellet was resuspended in 20–100 μ l (according to the starting number of cells) of cold Buffer C (20 mM HEPES–KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 min for high-salt extraction. Cellular debris were removed by centrifugation for 2 min at 4 °C and the supernatant (containing nuclear proteins) was stored at –70 °C. For Western blotting experiments, total protein extract was separated on 10% SDS–Polyacrylamide gel and electrically blotted to nitrocellulose membrane. The proteins were detected after an overnight incubation of the membrane at 4 °C with the specific primary antibodies (all the antibodies are listed in the Table S1). Proteins were visualized with IRDye800 or IRDye700 (Rockland Immochemicals) as secondary antibodies. Quantification was performed using the Odyssey Infrared Imaging System (Rockland Immochemicals) or ImageJ software.

Immunofluorescence

10-day-old and adult rats were killed, and their pancreases were harvested, fixed in 4% formaldehyde, and processed for paraffin embedding and preparation of 10- μ m sections. Beta and alpha cells were visualized by insulin staining using guinea pig anti-insulin antibody (1:100, Dako) and mouse anti-glucagon (1:100, Sigma), respectively. Dlk was detected using the rabbit anti-Dlk primary antibody (1:1000; Genetex) in PBST supplemented with 3% BSA at 4 °C overnight. After being rinsed three times for 10 min each in PBST, slides were incubated with secondary antibody Alexa Fluor (1:500 diluted in PBST plus 1% BSA, Thermofisher) at room temperature for 1 h and were then thoroughly rinsed again. The slides were mounted using a mounting kit containing DAPI (Vectashield, Eurobio-Ingen, H-1200). Cells were observed under a fluorescence microscope. For immunofluorescence of insulin, glucagon and Dlk, whole embryos of littermates control C57BL/6N and KO mice (resulting from the LacZ replacement of the coding exons 1, 2, and a part of 3 of the Map3K12 gene in C57BL/6N mouse) were fixed overnight in 4% paraformaldehyde (PFA) at 4 °C and embedded in paraffin as previously described [16]. Fluorescence images were taken under an Olympus BX51 microscope equipped with DP70 digital camera and the DPManager (DPController) software.

Immunoprecipitation (IP)

INS-1E cells were transfected with the plasmid coding for Dlk cDNA (pcDlk) or small interfering RNA directed against Dlk (siDlk) using Lipofectamine 2000 (Invitrogen) as described [17]. 48 h after transfection, cells were washed with cold PBS and were lysed in 20 mM Tris pH 7.5, 150 mM NaCl 0.1% Triton X-100 buffer (plus protease and phosphatase inhibitors) for 30 min at 4 °C. After centrifugation, the amount of proteins was quantified using bicinchoninic acid protein assay (BCA) reagent (Thermo Fisher Scientific). 500 μ g of proteins were then immunoprecipitated with anti-Dlk (1/1000; Genetex) or anti-Jnk3 (1/1000, Cell Signaling) antibodies that have been pre-coupled to protein G Sepharose (GE Healthcare). Proteins were eluted with SDS sample buffer and subjected to SDS/PAGE and subsequent immunoblotting. Approximately, 5% of the protein was run as input, whereas 30% of the pull-down was run in each lane of the Western blots and blotted with anti-Dlk or anti-Jnk3 antibodies.

Apoptosis and proliferation

Dispersed rat islet cells were seeded on poly-L-lysine-coated glass coverslips. After the treatment, the coverslips were washed with PBS and the cells were fixed with methanol. For measuring proliferation with BrdU, the cells were incubated with BrdU (Sigma) 10 μ M for 24 h. After permeabilization in PBS-saponin solution, non-specific binding was blocked in a solution of PBS-saponin containing 1% BSA. The cells were then incubated with primary antibodies for 1 h (Ki67, BrdU, Insulin, Glucagon) followed by exposure to secondary antibodies. Coverslips were mounted using a mounting kit containing DAPI (Vectashield, Eurobio-Ingen, H-1200) and images were analysed with axioscan, an immune slide automated scanner. Apoptosis was measured by TUNEL assays according to manufacturer's protocol (R&D systems).

Insulin secretion

Dissociated rat islet cells were preincubated with KREBS buffer (127 mM NaCl, 4.7 mM KCl, 1 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM HEPES, 5 mM NaHCO₃) containing 2 mM glucose (basal condition) during 30 min at 37 °C. The medium was then replaced by either the same buffer (basal condition) or a KREBS buffer supplemented with 20 mM of glucose (stimulatory condition). After 45 min, the supernatants were collected and cellular insulin contents were recovered in EtOH acid

(75% EtOH, 1.5% HCl). The amount of insulin in the samples was determined by ELISA (Mercodia).

Transfection

INS-1E and dispersed rat islet cells were transfected with small interfering RNA directed against *Dlk* (siDLK) or *JNK3* (siJNK3) using Lipofectamine RNAiMAX (ThermoFisher) as described in [17]. The siRNAs were purchased from Microsynth. The *Map3k12* (*Dlk*) and *MAPK10* (*Jnk3*) mRNA target sequences were 5'-AUA CGA UGU GGU GAA GAU CUC dTdT-3' and 5' AGC GGA UGU ACU AGC UTT-3', respectively.

Quantitative PCR

Total RNA was extracted using Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's protocol. Total RNA was collected from the pools of pancreatic islets of Postnatal day 1 (P1, four pups), P5 (four pups) and P10 (two pups), while in P15, P20, P23, P31 and adult rats, total RNA was extracted from islets of individual animal. The reverse transcription reaction was performed as previously described [18]. Real-time quantitative PCR assays were carried out on the Bio-Rad MyiQ Real-Time PCR Detection System using iQ SyBr Green Supermix (Bio-Rad) as previously described [18]. The primer sequences for rat and mouse used are: *Ccnd1* (sense 5'-AGCAGAAGTGCAGAGAGGAG-3' and antisense 5'-GAGCTTGTTCCACCAGAAGCA-3'); *Rplp0* (sense 5'-ACCTCCTTCTCCAGGCTTT-3' and antisense 5'-CCTCTTTCTTCCAAGCTTT-3'); *Tbp* (sense 5'-CGT GCCAGAAATGCTGAATA-3' and antisense 5'-CTGGTG GGTCAACACAAGG-3'); *Mapk10* (*Jnk3*) (sense 5'-CGT GGACATATGGTCTGTGG-3' and antisense 5'-AGGTGA GGCCTGCATACTTG-3'); *Map3k12* (*Dlk*) (sense 5'-TTA AATCCAGGCAGAGTGG-3' and antisense 5'-GAGGGC ATTCAGTTCCATGT-3'); *Ccnd2* (sense 5'-GACTGCGGA AAAGCTGTGTA-3' and antisense 5'-TGCTCAATGAAG TCGTGAGG-3'). The primer sequences for Human genes are: *MAP3K10* (*JNK3*) (sense: 5'-GTGGCATTAAAGCACC TCCAT-3' and antisense (5'-ATGACCTCAGGGGCTCTG TA-3'); *MAP3K12* (*DLK*) (sense 5'-GTACTCTCCACACCC CAGGA-3' and antisense 5'-GGCTCTCTCCAGCTTCCCT TT-3'); *RPL27* (sense 5'-ATCGCCAAGAGATCAAAG ATAA-3' and antisense 5'-TCTGAAGACATCCTTATT GACG-3'); *CCND1* (sense 5'-TGAAGGAGACCATCCCC TG-3' and antisense 5'-TGTTCAATGAAATCGTGCGG-3'); *CCND2* (sense 5'-TCCAACTCAAAGAGACCAGC-3' and antisense 5'-TTCCACTTCAACTTCCCCAG-3').

PamChip peptide microarrays for kinome analysis

See supplementary methods.

Statistical analyses

The experiments including more than two groups were analyzed by ANOVA or with the non-parametric equivalent Kruskal–Wallis test followed by post-hoc Bonferroni test (Dunnett's test) when experiments included more than two groups.

Results

Map3k12 (*Dlk*) expression is involved in beta-cell proliferation during the postnatal development of newborn rats

We previously identified through transcriptome analysis a 70-fold higher expression of *Map3k12* in pancreatic islets of 10-day-old rats compared to those of adult animals [7]. *Map3k12* encodes the mitogen activated protein 3 kinase 12, a STK also called dual leucine zipper-bearing kinase (*Dlk*). The expression changes of *Map3k12* (*Dlk*) during the postnatal period was further studied by quantitative PCR and Western Blotting experiments. We found a 2–4-fold increase of the *Map3k12* (*Dlk*) expression between p1 and p10 postnatal days, and declines thereafter, suggesting a transient postnatal activation (Fig. 1a, b). *Map3k12* (*Dlk*) has a multitude of roles that rely on the cellular and environmental context, which may even appear paradoxical. In beta cells, *Map3k12* (*Dlk*) plays a dual role according to its subcellular localization [19]. While the presence of *Map3k12* (*Dlk*) in the nucleus is associated with beta-cell death, the cytosolic localization of the kinase drives opposite cell outcome [19]. In addition, plasmid-based overexpression of *Dlk* has been associated with increased apoptosis in immortalized insulin-producing cells including HIT cells [19] and INS-1 cells (Fig. S1a and S1b). Therefore, we investigated the subcellular localization of overexpressed *Map3k12* (*Dlk*) in islets of 10-day-old rats. Immunofluorescence (IF) and Western Blotting experiments showed that *Map3k12* (*Dlk*) overexpression is enriched in the cytosol fraction of isolated islet cells of neonate rats (Fig. 1c–i). The cytosolic localization of the kinase observed in islet cells from adult rats (Fig. S2), suggests that overexpression of *Map3k12* (*Dlk*) is not associated with a potential change in the nuclear–cytoplasmic distribution of the protein during postnatal development. As beta cells of neonate rats are highly replicative and have immature glucose responsiveness [7], we hypothesized that the transient overexpression of *Map3k12* (*Dlk*) plays a role for beta-cell replication.

To assess a possible involvement of the kinase in beta-cell mass expansion, we measured the proliferation rates of dispersed islet cells from 10 day-old rats in which the expression of *Map3k12* (*Dlk*) was reduced using small interfering

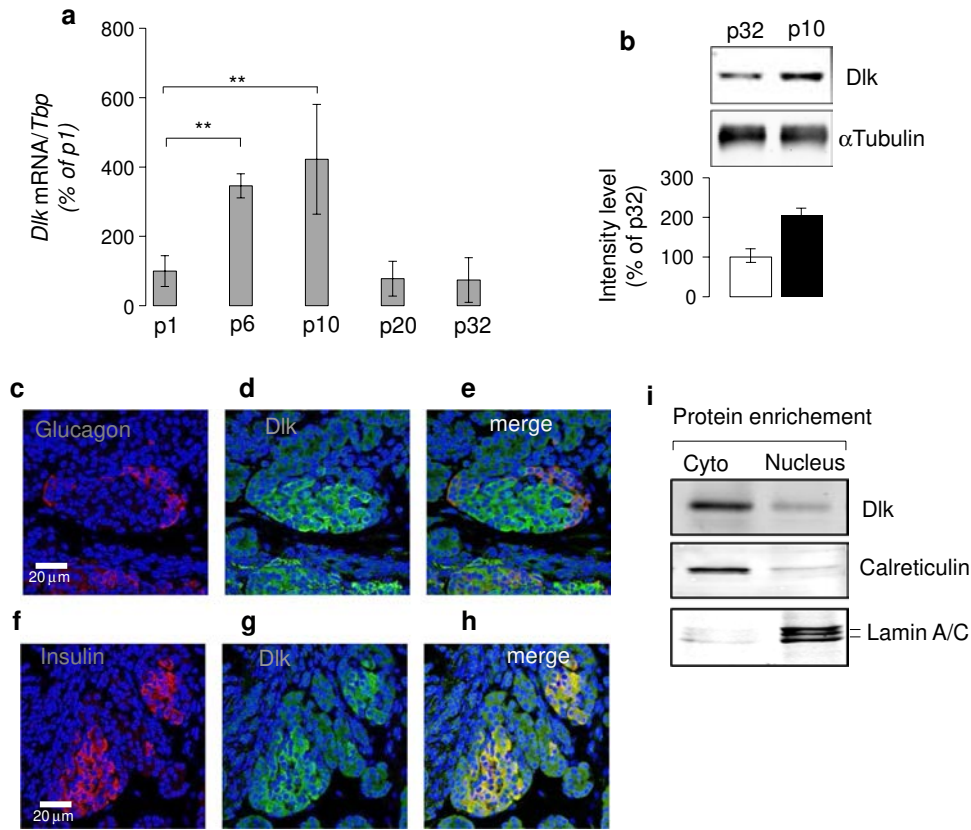


Fig. 1 Postnatal islet induction and beta-cell nuclear localization of Map3k12 (*Dlk*) in neonate rats. **a** The expression of the Map3k12 (*Dlk*) was measured by **a** RT-qPCR and **b** Western blotting in isolated islet of newborn rats between p1, p6, p10 and p20 postnatal days and young adult rats (p32). The mRNA levels of Map3k12 (*Dlk*) were normalized to those of Tata box binding protein (*Tbp*) and were expressed as % changes. The expression of Map3k12 (*Dlk*) at p1 was set to 100%. The data are the mean \pm SEM of 3 independent experiments performed in triplicate (** $p < 0.01$, by ANOVA). **b** Western blotting was performed using total proteins (20 μ g/lane) isolated from islets of 10- and 32-day-old rats using an anti-Dlk (1/1000; Genetex) and anti- α Tubulin (1/5000; Sigma) antibodies. The intensities of the bands were quantified using ImageJ, taking care to avoid saturation and subtracting the background. Values were expressed as the integrals (area \times mean density) of each band (normalized to α Tubulin

band). Values from p32 were set to 100%. **c-h** Representative immunofluorescence images for Map3k12 (*Dlk*), insulin and glucagon in pancreatic islets of 10-day-old rat ($\times 40$ magnification) using anti-Dlk (1/1000; Genetex), anti-insulin (1/100, Dako) and anti-glucagon (1/100, Sigma) antibodies. The scale bars in each picture row correspond to 20 μ m. **c** Map3k12 (*Dlk*) (green; **d, g**), and insulin (red; **f, h**) performed on fixed pancreas of newborn rats of 10 days old. Merged images were achieved to show the potential co-localization of Map3k12 (*Dlk*) with glucagon (**e**) or insulin (**h**). Yellow indicates the co-localization. Blue, DAPI. **i** Detection of Map3k12 (*Dlk*) protein in islets nuclear-enriched fraction of 10-day-old rats. Nuclear and cytosol (Cyto) proteins (20 μ g/lane) were loaded onto 10% SDS-PAGE and then detected by Western blotting using an anti-Dlk (1/1000; Genetex), anti-Lamin a/c (1/1000; Abcam) and anti-Calreticulin (1/1000; Abcam) antibodies

RNAs (Fig. 2a). Map3k12 (*Dlk*) silencing resulted in a marked reduction in beta-cell proliferation, as monitored by ki67 or BrdU and insulin co-staining (Fig. 2b, c and Fig. S3). The loss of beta-cell proliferation by Map3k12 (*Dlk*) silencing was associated with the drop of *Ccnd2* and *Ccnd1* mRNA levels (Fig. 2d). Decreased beta-cell proliferation by siDlk was not associated with a change in beta-cell apoptosis (Figs. 2e, S4) and insulin secretion (Fig. 2f). Moreover, the expression of key beta-cell genes including *Glut2*, *Pdx1*, *MafA*, *Ins2*, *GCK*, *Znt8*, *Kir6.2* and *Nkx6.1* were unaltered by Map3k12 (*Dlk*) silencing (Fig. S5), confirming that the kinase is not involved for specialized beta-cell function. Taken together, our data support the hypothesis that

increased Map3k12 (*Dlk*) expression is required for beta-cell proliferation during postnatal development in a mechanism involving *Ccnd2* and *Ccnd1*.

Map3k12 (*Dlk*) is a strong activator of Mapk signaling including c-Jun amino terminal kinases (Jnk) in different cell types [19, 20], suggesting that Mapk/Jnk activity links Map3k12 (*Dlk*) to beta-cell proliferation in islets of 10-day-old rats. In support of this hypothesis, the measurement of the phosphorylation of hundreds of validated STK substrates by Pamgene technology [18] confirmed the increased activity of the three Mapks/Jnks, including Mapk8 (Jnk1), Mapk9 (Jnk2) and Mapk10 (Jnk3) among the 25 STKs activity that are stimulated in islets of 10-day-old rats when compared

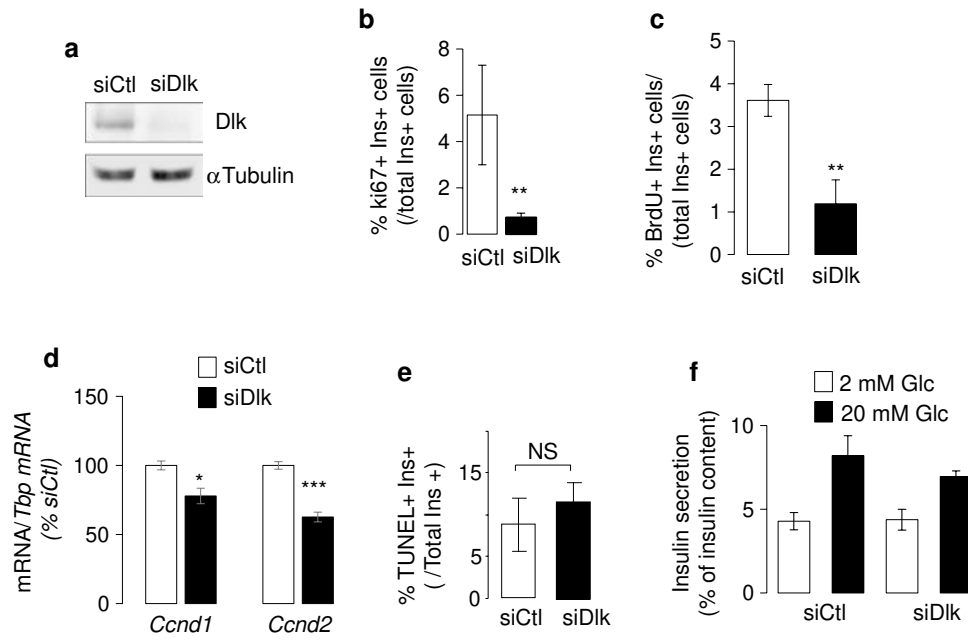


Fig. 2 Effect of Map3k12 (Dlk) silencing in beta-cell proliferation, basal beta-cell death, *Ccnd1* and *Ccnd2* mRNA levels. The expression of the Map3k12 (Dlk) was measured in dispersed islet cells from 10-day-old rats by **a** Western Blotting experiments in dispersed islet cells of newborn rats of 10 days old, which were transfected with control siRNA (siCtl; siRNA directed against GFP) or siRNAs against Map3k12 (Dlk) (siDlk) for 48 h. beta cell proliferation was quantified by determining the percentage of insulin+ (ins+) and **b** Ki67+ cells ($n=4$) or **c** BrdU+ ($n=3$). **d** Effect of siDLK on

the expression of *Ccnd1* and *Ccnd2* in the islet cells of 10-day-old rats. The mRNA levels were normalized to those of *Tbp* and were expressed as % changes. The data are presented as means \pm SEM of 5 independent experiments performed in triplicate as determined by ANOVA, (* $p < 0.05$, *** $p < 0.001$). **e** Beta-cell death was assessed by scoring the percentage of insulin positive cells labelled with TUNEL ($n=3$). **f** insulin secretion after 45 min exposure to 2 or 20 mM glucose ($n=4$). The data are the mean \pm SEM of 3 independent experiments performed in triplicate (** $p < 0.001$, by ANOVA)

to adult rats (Fig. S6). Increased Mapks/Jnk activity was associated with Mapks/Jnks activation as shown by Western blotting using antibodies recognizing the phosphorylated forms of the three Jnk isoforms (Fig. 3a, b). However, in beta cells, Mapk10 (Jnk3) triggers antiapoptotic and trophic effects, whereas Mapk8 (Jnk1) and Mapk9 (Jnk2) are proapoptotic [21]. While they are antagonists, the three Mapks were released from the kinome analysis, because they phosphorylate similar peptide targets. Therefore, we suggested that only Mapk10 (Jnk3) activation couples the effect of Map3k12 (Dlk) to beta-cell proliferation in neonate islets. Several lines of evidences support this hypothesis. First, in agreement with previous data [7], basal beta-cell apoptosis is similar between 10-day-old and adult rats (Fig. S7). Second, Jnk1 and Jnk2 phosphorylation at functional Thr183/ Tyr185 residues was not induced in islet cells from 10-day-old when compared to adult rats (Fig. 3c, d). Therefore, we hypothesized that Map3k12 (Dlk) overexpression directly leads to Mapk10 (Jnk3) activation. In addition, we postulated that the mechanism does not rely on the changes in the expression of Mapk10 (Jnk3) as the Mapk10 (Jnk3) mRNA level was similar in islets of 10-day-old and adult rats (Fig. 3e). To assess the regulation of Mapk10 (Jnk3) by Map3k12 (Dlk), we performed coimmunoprecipitation experiments using

the rat INS-1E as unlimited beta-cell source mimicking beta cells of neonate rats. As previously shown in HIT cells [22], Map3k12 (Dlk) regulates Mapk/Jnk activation in INS-1E cells (Fig. S8). Immunoprecipitation experiments confirmed that Map3k12 (Dlk) associates with Mapk10 (Jnk3) in INS-1E cells (Fig. 3f). Association of Dlk and Jnk3 was further validated by immunoprecipitation of Dlk and Jnk3 in HeLa that overexpressed both proteins (Fig. S9). Moreover, as expected, silencing or overexpression of Dlk modulated the phosphorylation of Mapk10 (Jnk3) in INS-1E cells (Fig. 3g, h). All these results indicate that Map3k12 (Dlk) activates Mapk10 (Jnk3) in beta cells.

Silencing of Mapk10 (Jnk3) in islets of 10-day-old rats reduced beta-cell proliferation as monitored by Ki67 and BrdU (Fig. 4a–c and Fig. S10), suggesting that Mapk10 (Jnk3) activation by Map3k12 (Dlk) accounts for beta-cell proliferation during postnatal development. Moreover, the diminished beta-cell proliferation caused by Mapk10 (Jnk3) silencing was associated with the reduction of *Ccnd2* and *Ccnd1* mRNA (Fig. 4d). These results indicate that the Map3k12 (Dlk)/Mapk10 (Jnk3) signaling are required for beta-cell proliferation by controlling the expression of *Ccnd2* and *Ccnd1*.

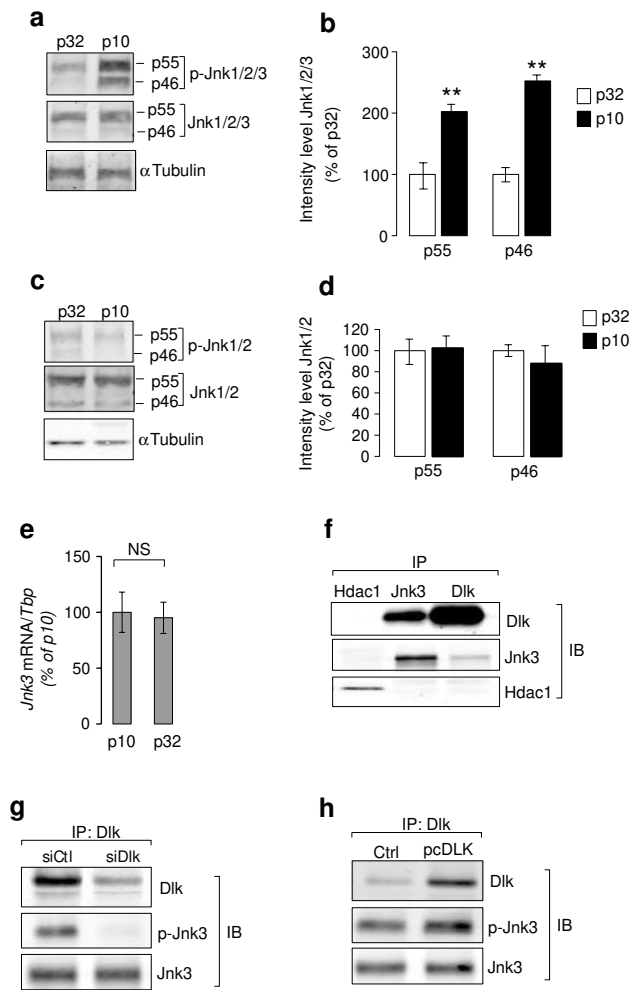


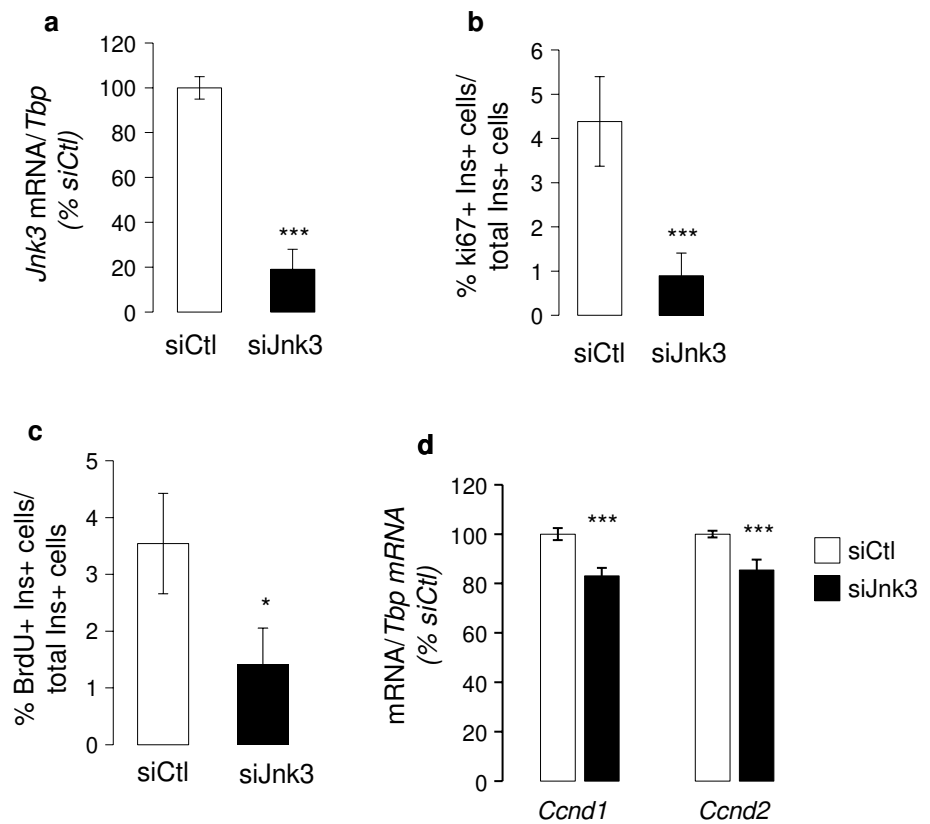
Fig. 3 Mapk10/Jnk3 activation by Map3k12 (Dlk) in neonate islet beta cells. Western Blotting analysis of **a** total and phosphorylated Mapks/Jnks (Jnk1/Jnk2 and Jnk3) and **c** total and phosphorylated Jnk1 and Jnk2 in islets of 10 (p10) and 32 (p32)-day-old rats. Western blotting was achieved loading total proteins from islets of neonate (p10) and adult rats (p32) using either anti-total Jnk1/Jnk2 and p-Jnk1/Jnk2 (phospho Thr183/Tyr185; 1/1000; ThermoFisher), or anti-total Jnk1/Jnk2/Jnk3 and p-Jnk1/Jnk2/Jnk3 (phospho Thr183+Thr183+Thr221, Abcam, dilution 1:1000), or anti- α Tubulin (1/5000; Sigma). **b-d** The intensities of bands were measured using ImageJ, taking care to avoid saturation and after subtracting the background. Values are expressed as the integrals (area \times mean density) of each band corresponding to p-Jnk1/2/3 or p-Jnk1/2 (p55 and p46), normalized to total Jnk1/2/3 or Jnk1/2. Values from p32 were set to 100% (** $p < 0.01$, by ANOVA). **d** The expression of *Mapk10/Jnk3* was measured by RT-qPCR in the islets of rats of p10 and p32. The results were normalized against *Tbp* and the expression levels from p10 were set to 100%. Data are the mean \pm SEM of 3 independent experiments performed in triplicate (NS, $p > 0.05$). **e** Co-immunoprecipitation of Map3k12 (Dlk) and Mapk10 (Jnk3) in INS-1E cells. Dlk and Jnk3 proteins are pulled down by the anti-Dlk (1/1000; Eurogentec) or anti-Jnk3 antibodies (1/1000, Cell Signaling) but not with control anti-Hdac1 antibody (1/1000; Santa Cruz). Phosphorylation level of Jnk3 (Thr183/Tyr185 residue) after Jnk3 immunoprecipitation in response to Map3k12 (Dlk) silencing (siDlk) or **g** Map3k12 (pcDLK) overexpression in INS-1E cells. Immunoblotting were achieved using either anti-Dlk (1/1000; Eurogentec), or anti-Jnk3 (1/1000, Cell Signaling) or anti phospho-Jnk3 (Thr183/Tyr185, 1/1000, Cell Signaling). The figures show the result of a representative experiment out of three

Map3k12 expression is increased in islet cells in response to weight gain and pregnancy conditions

Induction of Map3k12 (Dlk) in highly proliferative beta cells suggests that Map3k12 (Dlk) signaling contributes to beta-cell mass expansion during body growth and development. To verify this hypothesis, we analysed embryos of Dlk-Knockout (KO), in which the Mapk (Jnk) activity including Mapk10 (Jnk3) is totally impaired [16]. Although the homozygous mutant animals (KO) survived during embryonic stages without showing any gross tissue abnormality, they died perinatally, and no KO mutant survived until weaning [16]. Dlk gene disruption results in abnormalities in brain development that are characterized by major defects in axon growth and radial migration of neocortical pyramidal neurons [16]. Besides brain development abnormalities, we observed that beta-cell mass in the KO embryos was reduced (Fig. S11). To further gain insights into the contribution of Map3k12 (Dlk) in the beta-cell expansion, we next measured its expression in insulin-secreting cells from rodent models of pregnancy and obesity. We first measured the expression of Map3k12 (*Dlk*), *Ccnd1* and *Ccnd2* in islet cells from pregnant rats at 14 days of gestation that display increased beta-cell replication [23]. In pregnant rats, it was previously shown that the islet beta-cell replication increases after 10 days of gestation (G10), peaks at 14 days (G14), and decreases thereafter until delivery [3, 24]. We found that the expression of Map3k12 (*Dlk*), *Mapk10* (*Jnk3*), *Ccnd1* and *Ccnd2* raises compared to age-matched non-pregnant female rats starting from G10 and reaches its maximum at G14 (Fig. 5a). As expected from beta-cell replication studies [3, 24], the expression of the three mRNA level decreased after G14 (Fig. 5a).

In obesity rodent models, such as *ob/ob*, the beta-cell proliferation largely contributes to the expansion of the beta-cell mass and rises in response to insulin resistance [2, 25]. In *ob/ob* mice, islet beta-cell growth is prominent in 5–8-week-old animals [26]. We found that the expression of Map3k12 (*Dlk*) is increased in 5- and 8-week-old *ob/ob* mice compared to controls mice (Fig. 5b). The subcellular localization of Map3k12 (Dlk) in islets of 8-week-old *ob/ob* and age-matched control mice were similar, supporting a role for Map3k12 (Dlk) in the beta-cell proliferation during weight gain (Fig. S12). Moreover, induction of Map3k12 (*Dlk*) in 5- and 8-week-old *ob/ob* mice was associated with the rise of *MAPK10* (*Jnk3*), *Ccnd1* and *Ccnd2* (Fig. 5b, c). Beta-cell replication in *ob/ob* mice was associated with induction of genes involved in the adaptive unfolded protein response (UPR) [27–29]. Quantitative PCR analysis revealed that the expression of UPR genes including *Atf4*, *Atf6*, *Dnacj3* (p58), *Edem1* and *Ero1* was unchanged in islet cells from 10-day-old rats in which the expression of Map3K12 (*Dlk*)

Fig. 4 Effect of Mapk10 (Jnk3) silencing in beta-cell proliferation and *Ccnd1* and *Ccnd2* mRNA levels **a** Newborn rat islet cells were transfected with control siRNA (siCtl; siRNA directed against GFP) or siRNAs against Jnk3 for 48 h and *Jnk3* mRNA levels were measured by RT-qPCR. beta cell proliferation was quantified by determining the percentage of insulin+ (ins+) and **b** Ki67+ cells ($n=3$) and **c** BrdU. **d** Effect of siJnk3 on the expression of indicated genes in the islet cells of 10-day-old rats. Data are the means \pm SEM of 3 independent experiments performed in triplicate ($*p < 0.05$, $***p < 0.001$; ANOVA)



was silenced (Fig. S13). This result suggests that Map3K12 (*Dlk*) overexpression is not involved in the activation of adaptive UPR during weight gain.

We next analyzed human islet cells from non-diabetic donors with severe obesity (Table S2) and found also higher *MAP3K12* (*DLK*), *MAPK10* (*JNK3*), *CCND1* and *CCND2* mRNA levels, when compared to normal-weight non-diabetic individuals (Fig. 6). Immunofluorescence pictures that were obtained from pancreatic sections of obese individuals without diabetes and patients with diabetes showed no apparent difference in the DLK localization (Fig. S14). This observation supports that induction of *MAP3K12* (*DLK*) mainly account for the role of kinase and its pathway for the control of human beta-cell mass and thereby, glucose homeostasis during severe weight gain.

Discussion

Postnatal pancreatic development is characterized by an increase in beta-cell mass, enabling adequate insulin supply for early life development. In rodents and possibly in humans, the rise in beta-cell mass before weaning results from an elevated proliferation rate [7, 8]. It has been previously shown that the robust proliferative capacity of beta cells is maximal within the first 10 postnatal days [7]. Here

we found that beta-cell Map3k12 (*Dlk*) mRNA levels raise after birth, reach a peak at the 10th postnatal day, and then decline at the 20th postnatal day. Several evidences have led us to postulate that the stimulation of Map3k12 (*Dlk*) mRNA levels accounts for the beta-cell proliferation in neonate rats. Map3k12 (*Dlk*) has multiple and paradoxical roles upon cell types and environmental context. A trophic role for Map3k12 (*Dlk*) has been previously postulated to induce hepatocytes proliferation upon partial hepatectomy [12, 30]. In addition, Map3k12 (*Dlk*) triggers fibroblast cell proliferation by regulating the expression of cell cycle regulatory proteins including *Ccnd1* [12]. In beta cells, Map3k12 (*Dlk*) may trigger opposite effect according to its subcellular localization [19]. In contrary to the cytosolic presence of Map3k12 (*Dlk*), the nuclear expression of the kinase is pro-apoptotic [19]. Herein, we show that Map3k12 (*Dlk*) is mainly localized in the cytosol of neonate and adult rat islet cells. This observation suggests that the subcellular localization of the kinase is not modified during the postnatal development and that its overexpression is required for beta-cell proliferation to regulate the level of the key cyclins involved in postnatal beta-cell proliferation. Several lines of evidence support this hypothesis. Silencing of Map3k12 (*Dlk*) in isolated islets from newborn rats (10 days old postnatal) dramatically reduced beta-cell proliferation in a mechanism

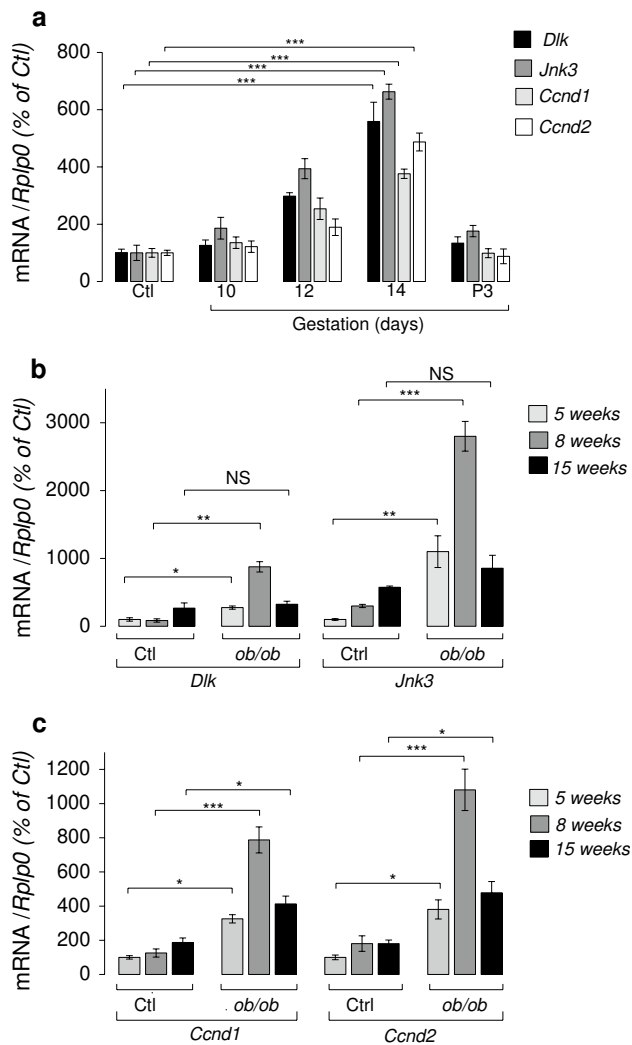


Fig. 5 Measurement of islets Map3k12 (*Dlk*), *Ccnd1* and *Ccnd2* expression changes in mice model of pregnancy and obesity. **a** The expression of Map3k12 (*Dlk*), *Mapk10* (*Jnk3*), *Ccnd1* and *Ccnd2*, was measured by RT-qPCR in the islets of female rats at indicated gestational days 10 (G10), 12 (G12), 14 (G14), and 3 days postpartum (P3). The results are expressed as % of the level in (Ctl). **b** Measurement of Map3k12 (*Dlk*) and *Mapk10* (*Jnk3*) and **c** *Ccnd1* and *Ccnd2* in islets isolated from *ob/ob* mice at the indicated ages and of age-matched controls. The data represent the mean \pm SEM of 4 animals per group. (Significantly different non-pregnant rats *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ by Dunnett's post-hoc test). NS Not significant

involving the reduction of *Ccnd1* and *Ccnd2*. Importantly, the reduction of Map3k12 (*Dlk*) level in isolated islets of 10-day-old rats did not change basal beta-cell apoptosis. This indicates that the observed reduction in proliferation is not the consequence of increased beta-cell death, which is in line with previous results showing that basal apoptosis of newborn and adult rat islet cells is similar [7]. In addition, Map3k12 (*Dlk*) silencing did not enhance glucose-induced insulin secretion, nor the expression of

key genes of beta-cell identity, pointing to the peculiar role of *Dlk* in the control of beta-cell replication during postnatal development.

We also provide some clues supporting a role for Mapk10 (*Jnk3*) in the mechanism whereby Map3k12 mediates the trophic effects on newborn beta cells (Fig. 7). First, Map3k12 (*Dlk*) has been primarily identified as an upstream STK component of the signalling cascade leading to the activation Mapk/Jnk pathway including Mapk10 (*Jnk3*) [21]. In line with this, using a whole STKs activity assessment in islets of neonate rats, we showed that the rise of Map3k12 (*Dlk*) is associated with increased activity of several Mapk, including Mapk10 (*Jnk3*) and the two pro-apoptotic Mapk8 (*Jnk1*) and Mapk9 (*Jnk2*) isoforms. In beta cells, it has been postulated that in physiological conditions, Mapk10 (*Jnk3*) activity, may inhibit the pro-apoptotic activities of Mapk8 (*Jnk1*) and Mapk9 (*Jnk2*) [21]. Our data showing that neither apoptosis nor *Jnk1/Jnk2* activation is modified between neonate and adult islet cells support this hypothesis. In addition, we found that *Mapk10* (*Jnk3*) silencing leads to decreased beta-cell proliferation. Taken together, our observations support the hypothesis that the increased Mapk10 (*Jnk3*) activity results from the rise in Map3k12 (*Dlk*) expression. This provides the first proof of concept supporting a physiological role for Mapk10 (*Jnk3*) in the control of beta-cell proliferation.

Beta-cell proliferation also contributes to the adaptation of the functional beta-cell mass under conditions characterized by a diminished insulin sensitivity such as obesity and pregnancy. In this respect, we postulated that Map3k12 (*Dlk*) signaling may play a pivotal role also in the mechanisms triggered to compensate for insulin resistance in adults. First, we found that the beta-cell number is reduced in Map3k12 KO mice. Second, increased Map3k12 (*Dlk*) mRNA was accompanied by elevated expressions of *Mapk10* (*Jnk3*), *Ccnd1* and *Ccnd2* in rodent models of obesity and pregnancy displaying an elevated beta-cell proliferation rate. As the stoichiometry of the MAPKs components is important to activate the signalling cascade [31], it is possible that induction of *Mapk10* (*Jnk3*) in compensatory islets to weight gain is the consequence of the elevated expression of Map3k12 (*Dlk*). In human, enhanced obesity-driven beta-cell proliferation was monitored in non-diabetic, but not in diabetic donors [5]. This result led to the hypothesis that the lack of proliferation may be causally linked to the reduced beta-cell mass and to diabetes pathogenesis. Our study showing that the *MAP3K12* (*DLK*), *MAPK10* (*JNK3*), *CCND1* and *CCND2* mRNA levels are upregulated in the islets from non-diabetic obese individuals compared to lean or diabetic obese donors, is consistent with *MAP3K12* (*DLK*) signaling being one of the mechanisms regulating human beta-cell proliferation in severe weight gain conditions.

Fig. 6 *MAP3K12 (DLK)* in isolated human islet cells from lean and obese donors. The mRNA levels of *MAP3K12 (DLK)*, *MAPK10 (JNK3)*, *CCND1* and *CCND2* were measured in isolated human islets from lean donors ($N=7$), obese individuals without diabetes donors ($N=7$) and obese patients with diabetes ($N=5$). The data were normalized against the *RPL27* and were expressed as the fold changes over the lean controls. Data are the mean of \pm SEM (significantly different from lean individuals without diabetes *** $p < 0.001$; ** $p < 0.01$; by Dunnett's post-hoc test). *NS* Not significant

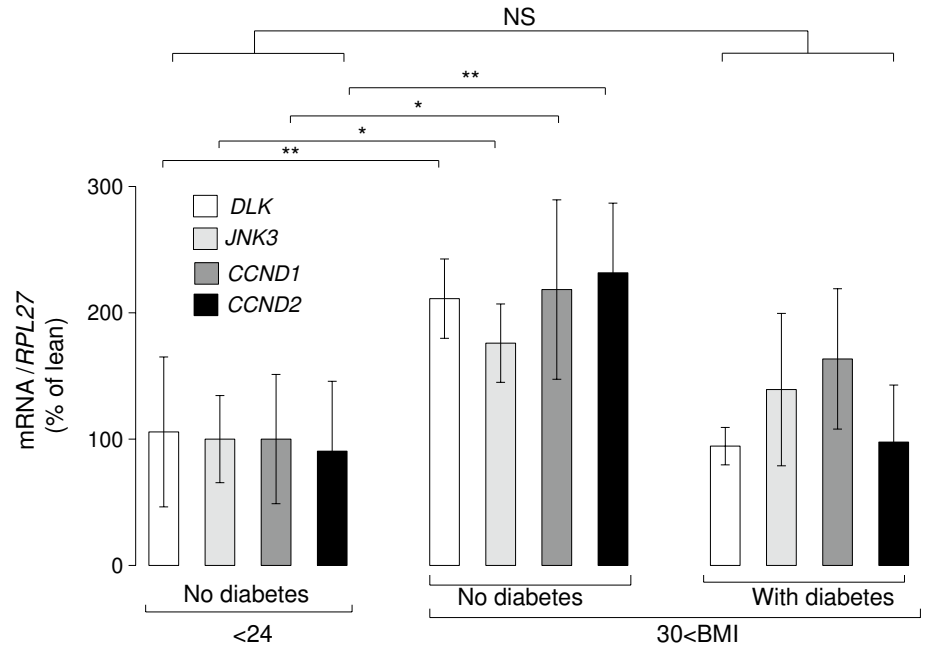
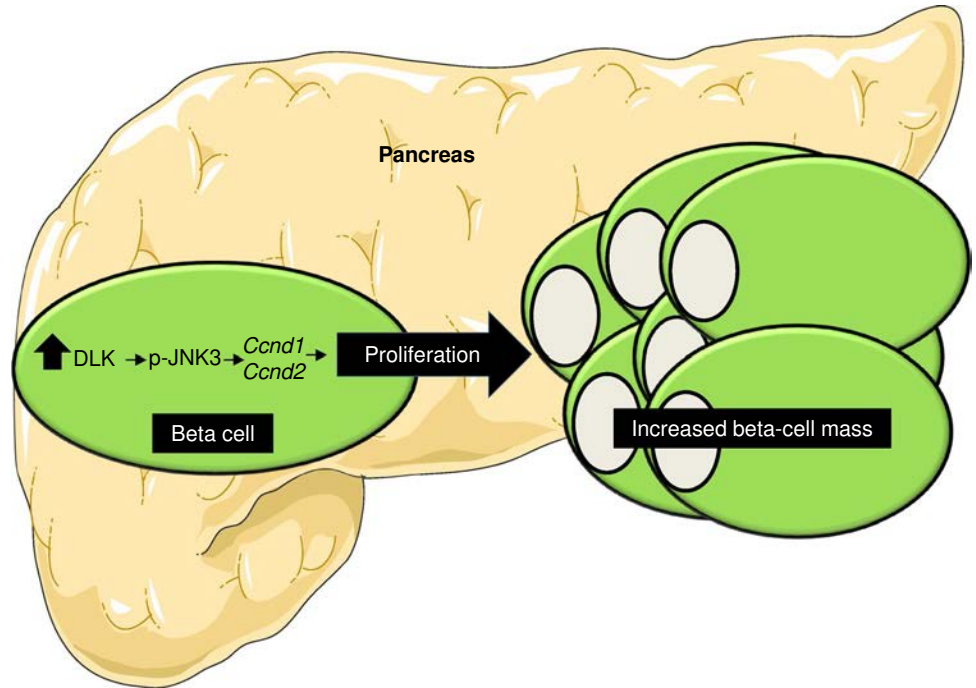


Fig. 7 Schematic representation of beta cell mass regulation by Map3k12 (Dlk)



In conclusion, our study demonstrates that the signaling pathway activated by Map3k12 (Dlk) is a key driver of beta-cell mass expansion during critical life times where the insulin demand is increased such as postnatal development before weaning, pregnancy and obesity in both rodents and human. Abnormalities in this signalling may lead to inadequate functional beta-cell mass and eventually to diabetes development.

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Author contributions MT, JKC, FP, RR, AB, PF and AA contributed to the study design and interpretation of the data. MT, SD, AB, PF and AA wrote and edited the manuscript. MT, VPL, VP, CSP, HE, RB, VG,

CJ, JB, NB, GP performed the experiments and analysed the data. All authors read and approved the submission of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no duality of interest associated with the manuscript.

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