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Mint3 is dispensable for pancreatic and kidney functions in mice

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ABSTRACT

Munc-18 interacting protein 3 (Mint3) is an activator of hypoxia-inducible factor-1 in cancer cells, macrophages, and cancer-associated fibroblasts under pathological conditions. However, exactly which cells highly express Mint3 in vivo and whether Mint3 depletion affects their physiological functions remain unclear. Here, we surveyed mouse tissues for specific expression of Mint3 by comparing Mint3 expression in wild-type and Mint3-knockout mice. Interestingly, immunohistochemical analyses revealed that Mint3 was highly expressed in islet cells of the pancreas, distal tubular epithelia of the kidney, choroid plexus ependymal cells of the cerebrum, medullary cells of the adrenal gland, and epithelial cells of the seminal gland. We also studied whether Mint3 depletion affects the physiological functions of the islets and kidneys. Mint3-knockout mice did not show any abnormalities in glucose-tolerance and urine-biochemical tests, indicating that Mint3 depletion was compensated for in these organs. Thus, loss of Mint3 might be compensated in the islets and kidneys under physiological conditions in mice.

1. Introduction

Vesicle transport is essential for secretion of peptide hormones and precisely regulated by many adapter proteins. Munc-18 interacting protein (Mint3; also known as amyloid beta precursor protein binding family A member 3, or APBA3) is a member of the Mint-family proteins and was originally identified as an amyloid-beta precursor protein (APP)-binding proteins [1,2]. Mint3 has the unique N-terminal without apparent domain structure and the C-terminal region with one phosphotyrosine-binding domain and two PDZ domains conserved among other Mint family proteins, Mint1 and Mint2 [1,2]. Mint3 localizes in the cytosol and binds to the cytoplasmic domain of membrane proteins at the Golgi apparatus and on secretory vesicles via its C-terminus [3–9]. Among the Mint-family proteins, Mint1 and Mint2 are predominantly expressed in the central nervous system and bind to Munc-18 (an essential component of the synaptic vesicle-fusion protein complex) via a Munc-18 interaction domain at the N-terminus of Mint1 and Mint2 [1,10,11]. Mint1-and Mint2-deficient mice showed defects in neurotransmission, and combined depletion of Mint1 and Mint2 caused decreased survival of mice [12–14]. In contrast, Mint3 does not have a Munc-18-interacting domain at the N-terminus [1,15], and Mint3 knockout (KO) mice showed no apparent defects in the central nervous system [12,16]. Thus, the molecular function and pathophysiological roles of Mint3 was unclear.

Previously, we have revealed that Mint3 activates the hypoxia-responsive transcription factor, hypoxia inducible factor-1 (HIF-1), even during normoxia in cancer cells, activated macrophages, and cancer-associated fibroblasts [7,17–19]. The unique N-terminal region of Mint3 which lacks Munc-18-interacting domain binds to a HIF-1 negative regulator, FIH-1, and thereby activates HIF-1. Mint3 depletion attenuates tumor growth, metastasis, septic shock, and influenza-related pneumonia [16,20–25], suggesting that Mint3 might be a good molecular target for cancer and inflammatory diseases. However, although Mint3 expression is detected in various tissues at the mRNA level [2,15], exactly which cells in tissues express Mint3 and how Mint3 functions in these cells under physiological conditions remain

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unclear.

In this study, we explored Mint3 expression in adult mouse tissues by immunohistochemistry using wild-type and Mint3-KO mice. We also examined whether Mint3 depletion affects the physiological functions of cells that normally express high levels of Mint3.

2. Materials and methods

2.1. Animals

Animals were maintained under specific pathogen-free conditions, and experiments were performed according to the institutional animal ethical and safety guidelines for gene manipulation experiments (The Institute of Medical Science, University of Tokyo; permit number: PA13-115). Mint3-KO mice [16] (Riken CDB Accession number: CDB0589K) were backcrossed into a C57BL/6 J (CLEA Japan, Tokyo, Japan) background for at least 12 generations. Male and female mice were analyzed at 8–12 weeks of age.

2.2. Histological analysis

Mice were anesthetized with butorphanol (Meiji Seika, Tokyo, Japan), medetomidine (Fujita Pharmaceutical Company, Tokyo, Japan), and midazolam (Sandoz, Tokyo, Japan). After anesthetization, mice were exsanguinated and perfused with 2% paraformaldehyde in 0.1 M phosphate buffer (pH7.4) through the heart. Tissues (brain, heart, lung, liver, pancreas, stomach, intestines, spleen, kidney, adrenal gland, muscle, testis, epididymis, seminal gland, ovary, uterus, vagina, and mesenteric lymph nodes) were resected, immersed in the same fixative overnight, and routinely embedded in paraffin wax. The paraffin-embedded specimens were cut into 4-μm thick slices and mounted on glass slides. The sliced sections were routinely deparaffinized with xylene and rehydrated with a graded series of ethanol, distilled water, and phosphate-buffered saline (PBS) with a pH of 7.4. Some sections were stained with hematoxylin and eosin (H&E) to observe the tissue morphology, and other sections were used for immunostaining. For immunostaining of Mint3, Lin7a, and aquaporin 1, sections were completely dipped in 10 mM sodium citrate buffer (pH 6.0) and microwaved for 5 min, followed by cooling at room temperature (RT) for 30 min for antigen retrieval. Sections were incubated with 0.3% hydrogen peroxide in PBS for 30 min. They were then treated with 5% normal goat serum in PBS for 1 h and immunostained with a primary antibody overnight at 4 °C, followed by a secondary antibody (Dako EnVision + Dual Link System-HRP; Agilent, California, USA) at RT for 1 h. Subsequently, the sections were visualized using diaminobenzidine (DAB; Thermo Fisher Scientific, Inc., USA) and finally observed under a light microscope. Detailed information for the antibodies used in this study is provided in Table 1.

2.3. Immunoblotting

Tissue were lysed in RIPA buffer (Sigma–Aldrich, St. Louis, MO, USA) by sonication and centrifuged at 20,000 *g* for 30 min at 4 °C. The supernatants were collected and total protein content measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Lysates were subjected to immunoblotting as previously described [26], using anti-Mint3 mouse antibody (#611380; BD Biosciences, San Jose, CA, USA), anti-Mint1 mouse antibody (SC-137022; Santa Cruz Biotechnology, Dallas, TX, USA), anti-Mint2 mouse antibody (SC-377060; Santa Cruz Biotechnology), and *anti*-β-actin mouse antibody (#0111–24554; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Table 1
List of antibodies used in this study.

antigen (host)	manufacturer	catalogue number	antigen retrieval	dilution
Mint3 (mouse)	BD Biosciences	611380	citrate buffer	1:50
Insulin (rabbit)	Proteintech	15848-1-AP	none	1:1000
Glucagon (rabbit)	Proteintech	15954-1-AP	none	1:1000
Lin7a (rabbit)	Gene Tex	GTX117114	citrate buffer	1:100
Aquaporin 1 (rabbit)	Merck Millipore	AB2219	citrate buffer	1:100
FIH-1 (rabbit)	Novus Biologicals	NB100-428	none	1:100

2.4. Glucose-tolerance and urine-biochemical tests

The amount of water that mice drank each day was calculated by weighing the bottle of drinking water every 24 h. For glucose-tolerance tests, mice were starved for 16 h. Then, glucose (1 g/kg body weight) in saline was intraperitoneally injected into the mice, and blood-glucose levels from tail vein blood were measured using the Precision Xceed instrument (Abbott Japan, Matsudo, Japan) at 0, 15, 30, 60, and 120 min after glucose injection. For the urine-biochemical tests, mouse urine samples were analyzed by Oriental Yeast Co., Ltd. (Tokyo, Japan) using a pyrogallol red method for total protein levels; an immunoturbidimetry for albumin levels; a urease–glutamate dehydrogenase method for urea nitrogen levels; an enzymatic method for creatinine, uric acid, calcium, inorganic phosphorus, and magnesium levels; an ion-selective electrode method for sodium, potassium, and chloride levels; and a hexokinase–glucose-6-phosphate dehydrogenase method for glucose levels. Urine-biochemical testing was performed using a Hitachi 7170 Chemistry Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.5. Statistical analyses

Statistical analyses were performed using GraphPad Prism software 7 (GraphPad Software, Inc., La Jolla, CA, USA). The Mann–Whitney *U* test was used for statistical evaluations. Data are represented as the mean ± standard deviation of the mean (S.D.); *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Specific expression of Mint3 in mouse tissues

Firstly, Mint3 expression was examined in various mouse tissues by immunostaining using wild-type (WT) and Mint3 KO mice. Specific staining of Mint3 was observed in the pancreas, kidneys, adrenal glands, brain, and seminal glands (Fig. 1). In WT pancreas tissue, Mint3 was immunostained in many endocrine cells of the islets (black arrows in Fig. 1a) whereas this staining was abolished in Mint3-KO pancreas tissue (black arrows in Fig. 1b). In the cortex of the kidney, some convoluted tubules and Bowman's capsules in WT mice were immunostained with the Mint3 antibody (black and white arrows in Fig. 1c). In Mint3-KO mice, staining deposits were also observed in Bowman's capsules (white arrows in Fig. 1d), indicating that non-specific staining occurred. In the adrenal glands of WT mice, the zona fasciculata of the cortex (white arrows in Fig. 1e) and the medulla cells (black arrow in Fig. 1e) were stained with the Mint3 antibody. However, in Mint3 KO mice, the zona fasciculate was also positively stained (white arrows in Fig. 1f) and staining of the medulla disappeared (black arrow in Fig. 1f), indicating that only medullary cells specifically expressed Mint3 in the adrenal gland. Ependymal cells of the choroid plexus (black arrows in Fig. 1g) and epithelial cells of the seminal gland (white arrows in Fig. 1h) were immunostained in WT mice, but not in Mint3-KO mice (black and white arrows in the insets of Fig. 1g and h). We could not detect specific Mint3 staining in other types of tissues, such as heart, lung, liver, stomach, intestine, spleen, muscle, testis, epididymis, ovary, uterus, vagina, and mesenteric lymph nodes (data not shown).

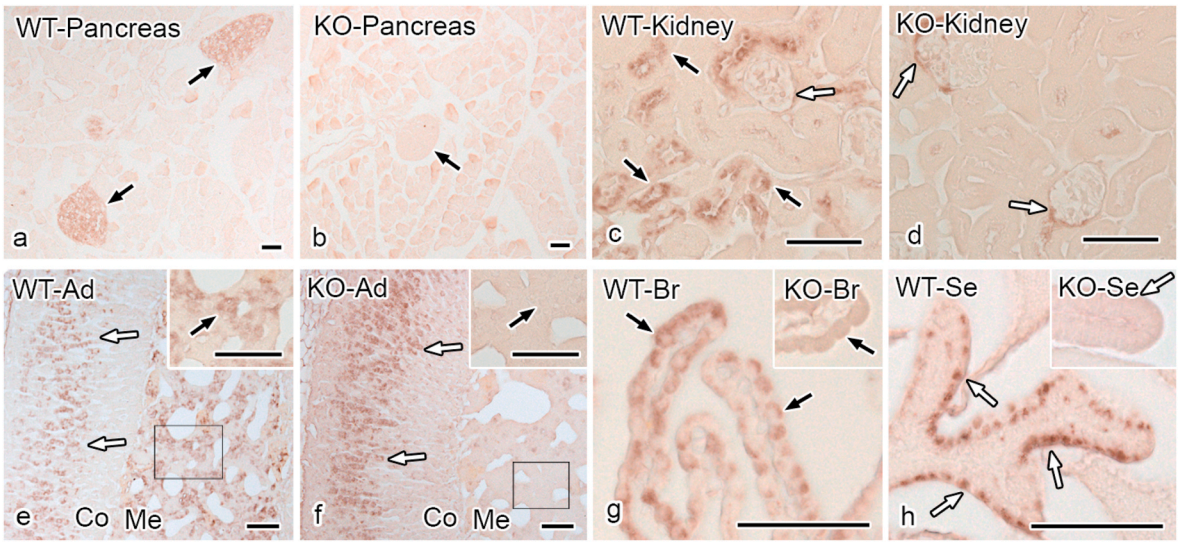

Fig. 1. Specific expression of Mint3 in mouse tissues. (**a, b**) Pancreatic expression of Mint3. **a:** Mint3 expression in the pancreas of a WT mouse. **b**: Mint3 expression in a Mint3-KO mouse. Black arrows: pancreatic islets. (**c, d**) Mint3 expression in the kidney of a WT mouse (**c**) or a Mint-3-KO mouse (**d**). Black arrows: specific Mint3 localization in convoluted tubules. White arrows: non-specific Mint3 detection in Bowman's capsules. (**e, f**) Mint3 expression in the adrenal glands (Ad) of a WT mouse (**e**) or a Mint3-KO mouse (**f**). Co: cortex. Me: medulla. Inset of (**e**): higher-magnification view of the boxed area. Inset of (**f**): higher-magnification view of the boxed area. White arrows: non-specific Mint3 localization in the zona fasciculate of the cortex. Black arrowheads: specific Mint3 localization in the medulla. (**g**) Choroid plexus of a ventricle in the brain (Br) of WT and Mint3-KO mice. Inset of (**g**): same magnification view of Mint3-KO mouse tissues. Black arrows: ependymal cells. (**h**) Seminal gland (Se). Inset of (**h**): same magnification view of Mint3-KO mouse tissues. White arrows: epithelial cells. Scale bars = 50 μm.

3.2. *Mint3 expression in pancreatic islets*

Among Mint3-expressing tissues detected by immunostaining, we focused on the pancreas and kidney for further characterization of Mint3-expressing cells. Firstly, Mint3 expression in the pancreas was confirmed by immunoblotting (Fig. 2a). Mint3 is expressed in various tissues, while Mint1 and Mint2 are predominantly expressed in the central nervous system [1,10,11]. Thus, we examined whether the loss of Mint3 affected the expression of Mint1 and Mint2 in the pancreas. Neither Mint1 nor Mint2 was detected in the pancreas of WT and Mint3-KO mice as revealed in the immunoblotting analysis (Fig. 2b). Next, to demonstrate which pancreatic islet cells in WT mice express Mint3, H&E staining and immunohistochemical staining of Mint3, glucagon, and insulin were examined in serial sections of paraffin specimens. In H&E-stained pancreas tissues, islets were normally observed with surrounding exocrine cells (Fig. 2c). The cytoplasm of the endocrine cells in the islets was immunopositive with the Mint3 antibody (black arrows in Fig. 2d), whereas the exocrine cells were immunonegative (white arrows in Fig. 2d). The α cells (producing glucagon) were localized to the edges of the islets (black arrowheads in Fig. 2e), and the β cells (producing insulin) were localized to other areas of the islets (white arrowheads in Fig. 2f). These results suggested that Mint3 was primarily expressed in the β cells of the islets. Mint3 binds to and suppresses FIH-1 [7,17–19]. Although Mint3 was primarily expressed in the islets, FIH-1 was expressed in both the islets and the exocrine cells in the pancreas (Fig. 2g and h). Thus, Mint3 and FIH-1 were co-expressed at least in the islets of the pancreas.

3.3. *Mint3 expression in the kidney*

Next, we confirmed Mint3 expression in the kidney (Fig. 3a), and loss of Mint3 did not affect the expression of Mint1 and Mint2 in the kidney (Fig. 3b). Next, we examined which types of tubules in mouse kidney express Mint3. H&E staining and immunostaining with markers for proximal tubules (aquaporin 1, AQP1) or distal convoluted tubules (Lin7a) [30,31] was performed in serial paraffin sections of WT mouse kidneys. In the kidney cortex, proximal convoluted tubules (arrowheads in Fig. 3c) and distal convoluted tubules (arrows in Fig. 3c) were morphologically distinguished by H&E staining (Fig. 3c). In those serial sections, Mint3 was present in the cytoplasm of distal, but not proximal convoluted tubules (arrows in Fig. 3d). These Mint3-immunopositive tubules were immunostained with an *anti*-Lin7a antibody (arrowheads in Fig. 3e), whereas apical area of the Mint3-immunonegative tubules were immunostained with an *anti*-AQP1 antibody (arrowheads in Fig. 3f). These data indicated that Mint3 was expressed in the epithelial cells of the distal convoluted tubules of the kidney cortex. Although Mint3 was expressed primarily in the distal convoluted tubules, FIH-1 was expressed in both distal and proximal convoluted tubules (Fig. 3g and h). Thus, Mint3 and FIH-1 were co-expressed in the distal convoluted tubules of the kidney.

3.4. *Mint3 depletion did not affect glucose tolerance*

Subsequently, we examined whether Mint3 depletion affects the physiological functions of Mint3-expressing cells in the pancreas and kidney. Insulin-producing β cells in the islets play a critical role in glucose metabolism. Dysfunction of β cells causes diabetes with polyposia and hyperglycemia [27]. Thus, we examined whether Mint3 depletion affects glucose metabolism. Mint3-KO mice showed similar water drinking compared to WT mice (Fig. 4a). Glucose-tolerance testing showed no significant differences in fasting blood-glucose levels and blood-glucose levels after glucose intake between WT and Mint3-KO mice (Fig. 4b). These data indicated that Mint3 deletion does not affect glucose metabolism in mice.

3.5. *Mint3 depletion did not affect the urine composition*

In the kidney, distal tubular epithelia are involved in the resorption of electrolytes and water from the primary urine [28]. Thus, we finally addressed whether Mint3 depletion affects the urine composition. Biochemical analyses of urine from WT and Mint3-KO mice showed that Mint3 depletion did not significantly affect the concentrations of total protein, albumin, urea nitrogen, creatinine, uric acid, sodium, potassium, calcium, inorganic phosphorus, magnesium, and glucose in the urine (Table 2). Taken together, these data indicate that Mint3 depletion did not affect renal functions in mice.

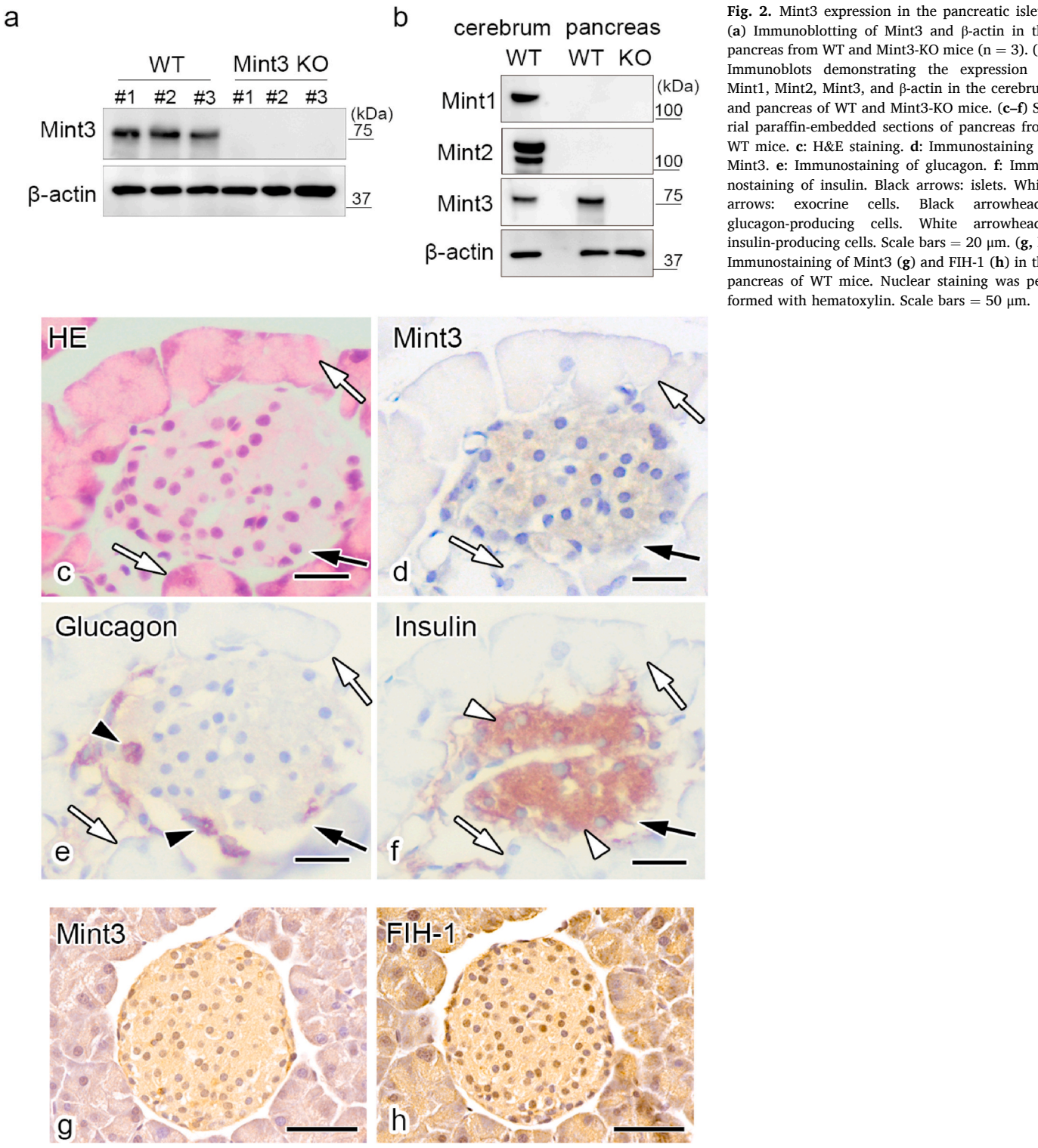

Fig. 2. Mint3 expression in the pancreatic islets. (**a**) Immunoblotting of Mint3 and β-actin in the pancreas from WT and Mint3-KO mice (n = 3). (**b**) Immunoblots demonstrating the expression of Mint1, Mint2, Mint3, and β-actin in the cerebrum and pancreas of WT and Mint3-KO mice. (**c–f**) Serial paraffin-embedded sections of pancreas from WT mice. **c**: H&E staining. **d**: Immunostaining of Mint3. **e**: Immunostaining of glucagon. **f**: Immunostaining of insulin. Black arrows: islets. White arrows: exocrine cells. Black arrowheads: glucagon-producing cells. White arrowheads: insulin-producing cells. Scale bars = 20 μm. (**g, h**) Immunostaining of Mint3 (**g**) and FIH-1 (**h**) in the pancreas of WT mice. Nuclear staining was performed with hematoxylin. Scale bars = 50 μm.

4. Discussion

The findings of this study revealed that Mint3, which promotes cancer and inflammatory diseases [16,20–25], was highly expressed in some secretory and fluid-regulating cells under physiological conditions in adult mice. In cells, Mint3 localizes to the Golgi apparatus and secretory vesicles by binding to the cytoplasmic region of membrane proteins such as APP and furin via its phosphotyrosine-binding and PDZ domains, implying its relevance to vesicle trafficking [3–9]. Among the Mint-family proteins, Mint1 and Mint2 are predominantly expressed in the central nervous system [1,10,11], and Mint1-and Mint2-deficient mice showed defects in neurotransmission [12–14]. In this study, specific Mint3 expression was also confirmed in ependymal cells of the choroid plexus that produces cerebral fluid (Fig. 1g). As previously reported [12,16], Mint3-KO mice did not show hydrocephalia or any apparent abnormalities in the central nervous system, indicating that Mint3 depletion might not cause adverse effects in the central nervous system. Interestingly, Mint3 was highly expressed in the choroid plexus

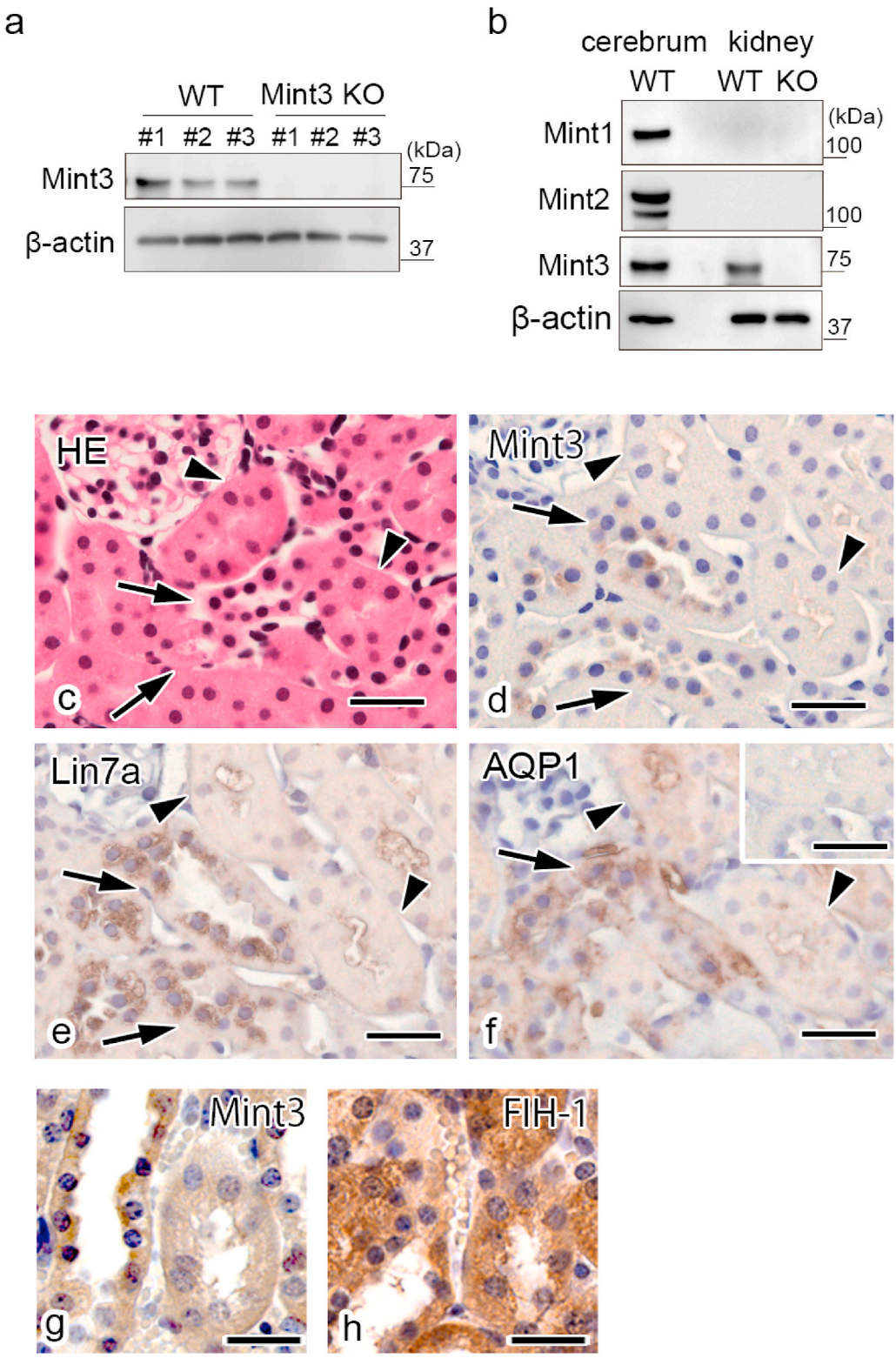

Fig. 3. Mint3 expression in the kidney. (**a**) Immunoblotting of Mint3 and β-actin in the kidney from WT and Mint3-KO mice (n = 3). (**b**) Immunoblots demonstrating the expression of Mint1, Mint2, Mint3, and β-actin in the cerebrum and the kidney of WT and Mint3-KO mice. (**c–f**) Serial paraffin sections of the renal cortex from WT mice. **c**: H&E staining. **d**: Immunostaining of Mint3. **e**: Immunostaining of Lin7a. **f**: Immunostaining of AQP1. (**d–f**) Nuclear staining with hematoxylin. Insets: negative control without a primary antibody. Arrowheads: proximal convoluted tubules. Arrows: distal convoluted tubules. Scale bars = 20 μm. (**g, h**) Immunostaining of Mint3 (**g**) and FIH-1 (**h**) in the kidney cortex of WT mice. Nuclear staining was performed with hematoxylin. Scale bars = 15 μm.

and distal renal tubules, which have similar structures and contribute to the chemical stability of the fluid. However, Mint3-KO mice showed no abnormality in the urine-biochemical tests (Table 2). Thus, the fluid regulation by Mint3 might be dispensable at least in adult mice.

Islet and adrenomedullary cells secrete peptide hormones via secretory vesicles. Because Mint3 was highly expressed in these cells, we hypothesized that Mint3 depletion might affect hormone secretion in these cells. However, Mint3-KO mice did not show any abnormalities in terms of fasting blood-glucose levels and glucose levels after glucose injection, compared with WT mice (Fig. 4), indicating that Mint3 depletion is dispensable for hormone secretion. Although a previous report demonstrated that Mint1 is expressed in rat islets [29], we could not detect Mint1 expression in the mouse pancreas by immunoblotting. In addition, Mint2 was not detected by immunoblotting in the pancreas and kidney of WT and Mint3-KO mice. These results demonstrate that the expression of Mint1 and Mint2 was low, at least in the kidney and

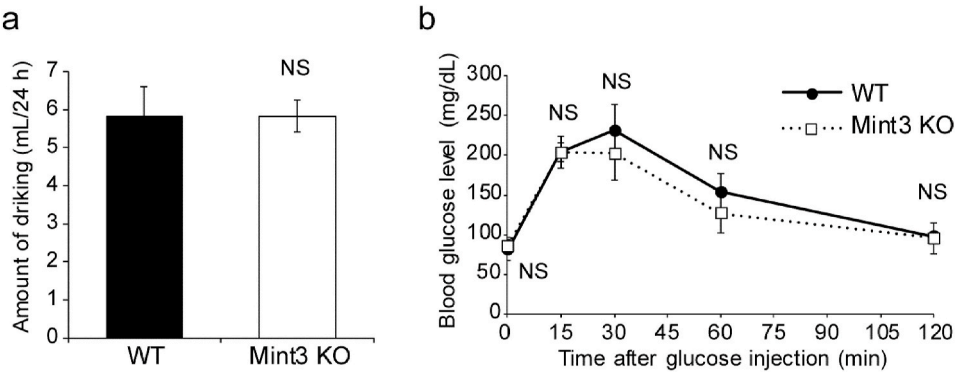

Fig. 4. Glucose tolerance in WT and Mint3-KO mice. (**a**) Water consumption in WT and Mint3-KO mice over the course of 24 h. (**b**) Blood-glucose levels of WT and Mint3-KO mice after glucose injection. n = 6. The data shown indicate the means ± S.D. NS, not significant.

Table 2
Biochemical analyses of urine samples from WT and Mint3 KO mice.

	WT	Mint3 KO	P value
TP (mg/dL)	475.1 ± 69.9	294.9 ± 296.8	0.1508
ALB (μg/mL)	4.1 ± 0.9	5.12 ± 1.3	0.2492
UN (mg/dL)	3736.6 ± 288.8	3348.6 ± 579.6	0.3095
CRE (mg/dL)	37.52 ± 3.6	38.978 ± 4.6	0.6905
UA (mg/dL)	3.7 ± 0.9	4.4 ± 0.8	0.3457
Na (mEq/L)	138.8 ± 18.0	104 ± 34.2	0.1376
K (mEq/L)	188 ± 22.4	167.7 ± 83.2	0.8413
Cl (mEq/L)	147 ± 29.9	127.2 ± 76.9	0.5476
Ca (mg/dL)	1.82 ± 0.2	2.78 ± 1.1	0.1376
IP (mg/dL)	184.4 ± 44.4	248.2 ± 36.8	0.056
Mg (mg/dL)	83.8 ± 12.5	96.6 ± 3.9	0.115
GLU (mg/dL)	25 ± 4.4	33.2 ± 9.3	0.1425

n = 5. Data indicates mean ± S.D. TP, total protein. ALB, albumin. UN, urea nitrogen. CRE, creatinine. UA, uric acid. Na, sodium. K, potassium. Ca, calcium. IP, inorganic phosphorus. Mg, magnesium. GLU, glucose.

pancreas of the mice. Mint3 might play a different role than that of Mint1 and Mint2, and another molecule (s) might compensate for the loss of Mint3 in these tissues. Although the expression of Mint3 was limited to certain cell types, FIH-1 was expressed in most cell types in the pancreas and kidney. Thus, Mint3 might control FIH-1 activity in limited cells of the pancreas and kidney. Epithelial cells of the seminal glands, where Mint3 was also expressed (Fig. 1h), secreted proteins, fructose, mucin, and other nutrients that support sperm fertility. Male Mint3-KO mice did not show apparent defects in fertility [12,16], indicating that Mint3 depletion might not affect male fertility.

Previous studies have demonstrated that the expression of Mint3 in macrophages promotes inflammatory responses during endotoxin shock, influenza, pneumonia, and vesicular stomatitis virus-mediated pneumonia models [16,25,30]. However, macrophages were Mint3-immunonegative in normal tissues examined in this study, implying that Mint3 expression might be low in macrophages under physiological conditions. In human specimens, cancer cells and cancer-associated fibroblasts in cancer tissues were Mint3-immunopositive, while most epithelial cells and fibroblasts in normal tissues were Mint3-immunonegative, as demonstrated by immunohistochemical analysis [17,31]. Hence, the expression of Mint3 might be induced in several cell types under pathological conditions.

In conclusion, the data generated in this study revealed the specific expression of Mint3 in mouse tissues and that Mint3 depletion did not affect the functions of pancreatic islets and the kidney in cells where Mint3 was highly expressed. Loss of Mint3 might be compensated at least in renal and pancreatic islet functions under the physiological conditions and this property might be useful to avoid severe adverse effects when Mint3 is targeted in cancer and inflammatory diseases.

CRediT authorship contribution statement

Yoohwa Chung: Investigation. **Yurika Saitoh:** Methodology, Investigation, Writing - original draft. **Tetsuro Hayashi:** Methodology, Investigation. **Yuya Fukui:** Investigation. **Nobuo Terada:** Methodology, Formal analysis. **Motoharu Seiki:** Supervision. **Yoshinori Murakami:** Supervision. **Takeharu Sakamoto:** Conceptualization, Supervision, Writing - original draft, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

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