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## Bone morphogenetic protein 4 inhibits insulin secretion from rodent beta cells through regulation of calbindin1 expression and reduced voltage-dependent calcium currents

Christensen, Gitte L.; Jacobsen, Maria L. B.; Wendt, Anna; Mollet, Ines; Friberg, Josefine; Frederiksen, Klaus S.; Meyer, Michael; Bruun, Christine; Eliasson, Lena; Billestrup, Nils

*Published in:*  
Diabetologia

*DOI:*  
[10.1007/s00125-015-3568-x](https://doi.org/10.1007/s00125-015-3568-x)

2015

[Link to publication](#)

### *Citation for published version (APA):*

Christensen, G. L., Jacobsen, M. L. B., Wendt, A., Mollet, I., Friberg, J., Frederiksen, K. S., Meyer, M., Bruun, C., Eliasson, L., & Billestrup, N. (2015). Bone morphogenetic protein 4 inhibits insulin secretion from rodent beta cells through regulation of calbindin1 expression and reduced voltage-dependent calcium currents. *Diabetologia*, 58(6), 1282-1290. <https://doi.org/10.1007/s00125-015-3568-x>

*Total number of authors:*  
10

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**Bone Morphogenetic Protein 4 inhibits insulin secretion from rodent beta cells through regulation of Calbindin1 expression and reduced voltage dependent calcium currents.**

*Authors: Gitte L. Christensen<sup>1</sup>, Maria L. B. Jacobsen<sup>1</sup>, Anna Wendt<sup>2</sup>, Ines G. Møller<sup>2</sup>,  
Josefine Friberg<sup>1</sup>, Klaus S. Frederiksen<sup>3</sup>, Michael Meyer<sup>4</sup>, Christine Bruun<sup>5</sup>,  
Lena Eliasson<sup>2§</sup> and Nils Billestrup<sup>1§</sup>*

(1) Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark, (2) Lund University Diabetes Center, Lund University, Malmö, Sweden, (3) Biopharmaceuticals Research Unit, Novo Nordisk A/S, Måløv, Denmark, (4) Department of Cellular Physiology, Ludwig-Maximilians-Universität, Munich, (5) Department of Incretin and Islet Biology, Novo Nordisk A/S, Måløv, Denmark.

<sup>§</sup> Corresponding authors: Lena Eliasson, Lund University Diabetes Center, Lund University, Malmö, Sweden, [Lena.Eliasson@med.lu.se](mailto:Lena.Eliasson@med.lu.se), +46-40391153 and Nils Billestrup, Department of Biomedical Sciences, University of Copenhagen, Nørre Alle 20, DK-2100 Copenhagen, [Billestrup@sund.ku.dk](mailto:Billestrup@sund.ku.dk), phone: +45-29607952.

Running title: Mechanisms in BMP4 inhibition of insulin secretion

Keywords: Diabetes, beta cells , insulin secretion, exocytosis, BMP4, Calbindin1

Word count: Abstract: 237 Main text: 3999 Figures: 4 Tables: 1

Online only supplementary information: 2 Tables, 2 Figures

Abbreviations:

BMP: Bone Morphogenetic protein

CDF: Chip definition file

ECM: Extracellular matrix

FDR: False discovery rate

GSIS: Glucose stimulated insulin secretion

HBSS: Hanks' balanced salt solution

Id1: Inhibitor of differentiation 1

Ins1: Insulin 1

KO: Knock out

KRHB: Krebs Ringer Hepes Buffer

NCS: Newborn calf serum

TGF- $\beta$ : Transforming Growth Factor- $\beta$

VDCC: Voltage dependent calcium channel

WT: Wild type

## **Abstract**

**Aims/hypothesis:** Type 2 diabetes is characterized by progressive loss of pancreatic beta cell mass and function. Therefore, it is of therapeutic interest to identify factors with potential to improve beta cell proliferation and insulin secretion. Bone morphogenetic protein 4 (BMP4) expression is increased in diabetic animals and BMP4 reduce glucose-stimulated insulin secretion (GSIS). Here we investigate the molecular mechanism behind this inhibition.

**Methods:** The BMP4 mediated inhibition of GSIS was investigated in detail using single cell electrophysiological measurements and live calcium imaging. BMP4 mediated gene expression changes were investigated with microarray profiling, q-pcr and western blotting.

**Results:** Prolonged exposure to BMP4 reduced GSIS from rodent pancreatic islets. This inhibition was associated with decreased exocytosis due to a reduced  $\text{Ca}^{2+}$ -current through voltage-dependent  $\text{Ca}^{2+}$ -channels. To identify proteins involved in the observed inhibition of GSIS we investigated the global gene expression changes induced by BMP4 in neonatal rat pancreatic islets. The expression of the  $\text{Ca}^{2+}$ -binding protein Calbindin1 was induced significantly by BMP4. Overexpression of Calbindin1 in primary islet cells reduced GSIS and the effect of BMP4 on GSIS was lost in islets from Calbindin1 knock out mice.

**Conclusions/interpretation:** We find BMP4 treatment to markedly inhibit GSIS from rodent pancreatic islets in a Calbindin1-dependent manner. Calbindin1 is suggested to mediate the effect of BMP4 by buffering  $\text{Ca}^{2+}$  and decreasing  $\text{Ca}^{2+}$ -channel activity resulting in diminished insulin exocytosis. BMP4 and Calbindin1 are both potential pharmacological targets for the treatment of beta cell dysfunction.

In response to insulin resistance, the pancreatic beta cells will initially compensate by increasing mass and function to maintain normal blood glucose levels [1]. In individuals who progress to develop type 2 diabetes the beta cells eventually fail to adapt and progressive loss of functional beta cells starts [1]. Although current type 2 diabetes drugs targeting insulin resistance or insulin secretion are initially efficient, beta cell mass and function tends to decline over time [2, 3]. Unidentified factors may limit the ability of the beta cells to adapt to insulin resistance. In our search for factors, with inhibitory effects on beta cells, we have recently described the inhibition of beta cell proliferation and insulin secretion by bone morphogenetic protein 4 (BMP4) [4].

BMPs belong to the Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) protein family, known to play central roles in pancreas and islet development [5-10]. While the role of BMPs in the developing pancreas and beta cell has gained much attention [8, 11, 12], less is known about their function in the postnatal pancreas. Increasing evidence indicates BMP2 and 4 to be inflammatory markers in various tissues under diabetic conditions [13-15]. We have recently shown that BMP2 and 4 are expressed in pancreatic islets, and are upregulated during diabetes progression in islets from *db/db* mice and by proinflammatory cytokines *in vitro* [4]. Although culture of pancreatic islets in the presence BMP2 and 4 negatively affects beta cell function, it appears that the effect of BMPs *in vivo* is more complex. Beta cell specific deletion of the BMP receptor 1A (BMPR1A) results in impaired glucose-induced insulin secretion (GSIS), whereas transgene BMP4 expression increase GSIS [16]. In contrast, deficiency of ID1, a central BMP regulated transcription factor, resulted in enhanced insulin secretion and protect from diet-induced glucose intolerance, suggesting that BMPs and ID1 normally exert inhibitory effects on adult beta cell function [17]. BMPs are released from and affect several metabolic relevant tissues, including fat, liver and kidney adding to the complexity of the role of BMPs in metabolism [14, 18, 19]. To characterize the direct effects of BMPs on beta cells we have recently reported BMP4

mediated inhibition of beta cell proliferation and repression of GSIS from mouse, rat and human islets [4]. Here, we further characterize the effects of BMP4 on insulin secretion by single cell electrophysiological measurements and evaluation of gene regulation to identify the molecular mechanism behind the observed inhibition of insulin secretion.

## **Research Design and Methods**

### *Rat islet isolation and culture*

Neonatal rat islets of Langerhans were isolated from 4-day-old Wistar rat pups (Taconic, Lille Skensved, Denmark) as previously described [20]. The isolated islets were pre-cultured for 7-10 days in RPMI 1640 with ultraglutamine (Lonza, Vallensbaek, Denmark) supplemented with 10% newborn calf serum (NCS) (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies, Taastrup, Denmark), in 5% CO<sub>2</sub> at 37°C. For experimental setups, rat islets cultured as intact and free-floating in medium supplemented with 2% human serum (Lonza (BioWhittaker) or as islets dispersed into single cells by 0.2% trypsin (Gibco), 10 mmol/L EDTA (Gibco) in HBSS. Dispersed islets were cultured on coverslips coated with bovine corneal extracellular matrix (ECM), (Biological Industries, Kibbutz Beit, Haemek, Israel) in the medium described above containing 2% human serum.

### *Mouse islet isolation and culture*

Pancreatic islets from 10-12 weeks old female NMRI mice were isolated using collagenase digestions as previously described [21]. The islets were handpicked in albumin-coated Petri dishes (1 mg albumin/ml HBSS) and cultured for 1 day in medium I (RPMI 1640 with 10 mmol/L glucose and 10% fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin) and further 3 days in medium II (RPMI 1640 with 10 mM glucose and 2% fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin) in the

absence or presence of BMP4 (50 ng/ml). After culture, mouse islets were fixated for transmission electron microscopy (TEM) or dispersed into single cells, using  $\text{Ca}^{2+}$ -free buffer, for electrophysiological experiments.

Pancreatic islets from 12-19 week old 129SV/C57/6cr1 WT and Calbindin1 KO mice [22] were isolated by bileduct perfusion of the pancreas with liberase (Roche, Hvidovre, Denmark). Digestion was stopped by addition of HBSS buffer containing  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  (Gibco) and 3 g/L BSA and 0.5 g/L D-glucose. Islets were filtered through a 400  $\mu\text{m}$  pore size mesh, followed by 100  $\mu\text{m}$  and 70  $\mu\text{m}$  mesh strainers (BD Falcon, Albertslund, Denmark). Retained islets were handpicked under a dissection microscope. Islets were cultured for 1 day in medium I. The following day islets were transferred to medium II, in which they were kept throughout stimulation. Female mice were used for electrophysiological experiments and male mice for insulin secretion assays.

#### *Microarray analysis*

800 intact, free-floating rat islets were cultured in 5.5 cm Sterilin dishes for 5-10 days and exposed to BMP4 for 96 hrs. Total RNA was extracted using TRIzol (Gibco). One microgram of total RNA was labelled by One-Cycle Target labeling kit (Affymetrix, Santa Clara, Ca, USA) following the instructions of the manufacturer. Hybridization cocktails were hybridised to Rat Genome 230 2.0 GeneChip® arrays (Affymetrix) at 45°C for 17 hours (60 RPM) in a Hybridization Oven 640 (Affymetrix). GeneChips® were washed and stained in a GeneChip® fluidics station 450 using the fluidics protocol “EukGE-WS2v5\_450” (Affymetrix). Chips were scanned in a GeneChip® scanner 3000 (Affymetrix). Microarray data were normalized and gene expression measures derived using the RMA algorithm and the Bioconductor package “Affy” (<http://www.bioconductor.org>). Custom CDF (chip definition file) from brainarray.mbni.med.umich.edu was used. Qlucore Omics Explorer 3.0 (Qlucore AB, Sweden) was used for the statistical analysis of the normalized data. For

comparing BMP4 to vehicle treatment, the microarray data were variance filtered ( $\sigma/\sigma(\max)>0.1$ ), and the two groups compared (t-test, FDR=5% (Benjamini Hochberg correction for multiple testing)).

#### *Analysis of Calbindin1 and Insulin1 mRNA expression by real-time qPCR*

800 intact neonatal rat islets cultured for five days were exposed to 50 ng/ml BMP4 for the indicated time periods and total RNA extracted using TRIzol (LifeTechnologies). cDNA synthesis was performed by TaqMan Reverse Transcription Reagents (Applied Biosystems, CA, USA). TaqMan Gene Expression probes against rat *Calbindin1* (Rn00583140\_m1), *Insulin 1* (Rn02121433\_g1) and *Ppia* (Rn\_00690933\_m1) were from Applied Biosystems. The samples were run on ABI PRISM 7900 HT Taqman (Applied Biosystems). Each sample was run in duplicates or triplicates and expression was normalized to the internal control, *Ppia*.

#### *Analysis of Calbindin1 protein expression by western blotting*

1000 intact neonatal rat islets were cultured for 5-10 days prior to exposure to 50 ng/ml BMP4 for 24, 48, 72 or 96 hrs. SDS-PAGE and western blotting was performed as described previously [23]. Primary antibodies were rabbit anti-Calbindin1 (Cell Signaling Technology, AH Diagnostics, Aarhus, DK) and mouse-anti- $\beta$ -actin ( Abcam, Cambridge, UK). Chemiluminiscense was detected by Lumi-GLO (Cell Signaling Technology) and visualized by use of Las 3000 (Fuji Film). Densitometric scanings were performed in Image J (Freeware, NIH, Bethesda, Maryland, USA).

#### *Glucose stimulated insulin secretion*

7-10 days after isolation, forty neonatal rat islets were transferred to medium containing 2% human serum, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin and stimulated with



50 ng/ml BMP4 for 0-96 hours. Mouse islets were stimulated the day after isolation. For each condition 20 islets were transferred to Krebs Ringer Hepes buffer (KRHB) (115 mmol/L NaCl, 4.7 mmol/L KCl, 2.6 mmol/L CaCl<sub>2</sub>, 1.2 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol/L MgSO<sub>4</sub>, 10 mmol/L HEPES, 0.2% BSA, 2 mmol/L glutamine, 5 mmol/L NaHCO<sub>3</sub>, 1% P/S, pH 7.4), Containing 2 mmol/L glucose and incubated for 90 minutes prior to the GSIS experiment. Islets were sequentially exposed to 2 mmol/L glucose, 20 mmol/L glucose and 20 mmol/L glucose plus 10 μmol/L forskolin (Sigma-Aldrich, Brøndby, Denmark) for 30 minutes and buffer was collected from each condition. Insulin content was determined using an in-house insulin Elisa assay. Results were corrected for DNA content using Quant-IT™ PicoGreen® dsDNA Reagent and Kit (Invitrogen, Life Technologies).

#### *Production of Calbindin1 lenti virus*

Mouse Calbindin1 cDNA in entry vector pENTR(tm) 221 (Invitrogen) was transferred to the pLenti6.2/v5DEST Gateway® Vector (Invitrogen). Lenti virus was produced in HEK293ft cells using the ViraPower™ Lenti viral Expression Systems (Invitrogen, Carlsbad, CA, USA) and lenti virus was harvested by ultra centrifugation. Titters were determined in HT1080 cells (Invitrogen). The virus was used at a MOI 5 for 6 hours.

#### *Insulin release in single cells overexpressing Calbindin1 using lenti virus*

Dispersed rat islet cells were cultured on coverslips coated with bovine corneal extracellular matrix (ECM) in 4 well containers for 4 days prior to transduction with Calbindin1 or GFP lenti virus for 6 hours at a MOI of 5. 96 hours after transduction, GSIS was evaluated as described above.

#### *Electrophysiology*

Capacitance measurements and ion-current measurements were performed on single beta cells in a mixture of dispersed islets cells using the patch-clamp technique as previously described [21]. Beta cells were identified by size and inactivation properties of the voltage dependent  $\text{Na}^+$  channel [24, 25]. Exocytosis was evoked by a train of ten 500-ms depolarization from -70 mV to 0 mV with a frequency of 1Hz and measured as changes in membrane capacitance. The VDCC-currents to voltage relationship was determined by employing a protocol where the membrane was depolarized from -70 mV to between -40 mV to +40 mV for 50 ms.

#### *Live calcium imaging*

Islets were loaded with 4  $\mu\text{M}$  Fura 2-AM (TefLabs) for 40 minutes followed by 30 minutes de-esterification in imaging buffer at pH 7.4 (mM: KCl 3.6,  $\text{MgSO}_4$  0.5,  $\text{CaCl}_2$  2.5, NaCl 140,  $\text{NaHCO}_3$ ,  $\text{NaH}_2\text{PO}_3$  0.5, HEPES 5). Imaging was performed with a Polychrome V monochromator (TILL Photonics, Graefeling, Germany) and a Nikon Eclipse Ti Microscope (Nikon, Tokyo, Japan) with a ER- BOB-100 trigger on an iXON3 camera and iQ2 (Andor Technology, Belfast, UK) software. Recording was performed at one frame per second at 37°C under perfusion 1ml/min. A region was marked around each islet and the light intensity was recorded in that region to get the integrated light intensity per unit area ( $\mu\text{m}^2$ ) at 340nm (exposure 150 ms) and 380 nm (exposure 100 ms). These measured intensities were then used to calculate the ratio of Fura-2 bound (340 nm) and unbound (380 nm) to calcium at one frame per second.

## **Results:**

#### *BMP4 inhibits glucose stimulated insulin secretion*

The effect of BMP4 on GSIS was investigated using neonatal rat islets. Pretreatment with 50 ng/ml BMP4 for 0-4 days resulted in a decrease in GSIS observed after 48 hours (Fig.

1a). Acute stimulation with BMP4 (0h) or pre-stimulation for up to 24 hours had no effect on insulin secretion. In addition doses as low as 2 ng/ml inhibits insulin secretion [4]. BMP4 stimulation had no effect on total islet insulin content (Fig. 1b) or *Ins1* mRNA levels (Fig. 1c). We did not observe any effect on the number, size or insulin granule proximity to the cell membrane using high resolution electron microscopy of adult mouse islets (Supplementary figure 1).

#### *BMP4 inhibits exocytosis and voltage dependent $Ca^{2+}$ -channel current*

The fact that BMP4 inhibits GSIS without affecting total insulin content or total number of insulin granules, indicates that BMP4 may affect the secretory machinery. For further detailed electrophysiological analysis of insulin secretion we used dispersed primary mouse islet cells. We investigated exocytosis measured as an increase in membrane capacitance and found reduced depolarization-evoked increase in membrane capacitance in BMP4 treated primary mouse beta cells (Fig. 2a-e). The most pronounced effect was observed during the latter depolarizations (Depol 2-10; Fig 2d). This was further confirmed by a continued reduced exocytotic response to a second train of depolarizations performed 2 minutes later (Fig. 2e). Exocytosis is highly dependent on  $Ca^{2+}$ -influx through voltage-dependent  $Ca^{2+}$ -channels (VDCC) [26]. We therefore determined the VDCC-influx to voltage relationship (Fig. 2f-g). Beta cells exposed to BMP4 show ~50% reduced  $Ca^{2+}$ -influx at 0 mV. BMP4 increased the  $Ca^{2+}$ -sensitivity, i.e. the increase in membrane capacitance per  $Ca^{2+}$ -charge unit entering the cell, which suggests that the BMP4-dependent decrease in exocytosis is caused by a reduced  $Ca^{2+}$ -current rather than through a direct effect on exocytosis (Figure 2h). Moreover, BMP4 can be suggested to have a direct stimulatory effect on exocytosis that is less pronounced than the effect on the  $Ca^{2+}$ -current. To gain further insight into the mechanism on how BMP4 may cause reduced exocytosis we performed live calcium imaging in primary adult mouse islets. Evaluation of calcium

fluctuations was determined by differences in the Fura-2 340/380 ratio. Representative traces are shown in Figure 2i-j. All traces can be seen in supplementary Figure 1. Temporal fluctuation of intracellular calcium in response to glucose were organized into 2 classes: class 1 islets represent islets with separate 1<sup>st</sup> and 2<sup>nd</sup> phases with a clear 1<sup>st</sup> phase peak followed by a lowering of calcium and a 2<sup>nd</sup> phase with distinct regularly spaced calcium oscillations; class 2 islets present a rise in calcium with no clear first and second phases and no distinct oscillation in 2<sup>nd</sup> phase. In control islets we observed 64% and in BMP4 treated islets 26% to have a distinct 1<sup>st</sup> peak and 2<sup>nd</sup> phase oscillation in presence of high glucose (see Supplementary Table 1). In addition, there was a significant reduction in the first lowering of  $\text{Ca}^{2+}$  (Figure 2j and k) and the 1<sup>st</sup> phase peak amplitude at 16.7 mM glucose (Figure 2j and m) and the response to depolarizing  $\text{K}^+$  (Figure 2j and o). The response time after increasing glucose concentrations was decreased by BMP4 treatment (Figure 2 j and l) whereas the response time to lowering of glucose was increased (Figure 2n). We did not observe significant changes in the amplitude or frequency of calcium oscillations.

#### *BMP4 mediates upregulation of Calbindin1*

Since the effects of BMP4 on GSIS are first observed after 48 hours exposure, we hypothesize that gene regulation is required for this effect. To unravel the mechanism of BMP4 mediated inhibition of GSIS we therefore performed a gene expression array comparing 3 independent set of vehicle and BMP4 (96h) treated neonatal rat islets. With a false discovery rate (FDR) of 5%, we find 102 genes to be regulated by BMP4 (Supplementary Table 2). The genes with more than 2-fold up- or downregulation are shown in Table 1. Seven of the gene regulations have been verified on independent rat islet samples (denoted with a \* in Table 1).

We paid particular attention to genes known to be involved in hormone secretion and  $\text{Ca}^{2+}$ -handling based on the effects of BMP4 on insulin secretion, exocytosis and  $\text{Ca}^{2+}$ -channel

activity. We observed no regulation of the BMP receptors or L-type calcium channel subunits. One of the most regulated genes is Calbindin1, an EF-hand  $\text{Ca}^{2+}$ -binding protein which has previously been shown to regulate  $\text{Ca}^{2+}$ -currents through VDCC and inhibit GSIS in beta cells [27-29]. BMP4 increased the expression of Calbindin1 mRNA 6-fold in neonatal rat islets resulting in a 2.5 fold increase in Calbindin1 protein level after 96 hours (Figure 3a and b) As in primary neonatal rat islets, BMP4 increased the expression of Calbindin1 mRNA in adult mouse islets (Figure 3c).

#### *Overexpression of Calbindin1 impairs GSIS*

To determine the role of Calbindin1 in the BMP4 mediated effect on GSIS we induced overexpression of Calbindin1 in dispersed neonatal rat islet cells. Overexpression of Calbindin1 significantly reduced GSIS (Fig. 4a). Transfection efficiency was more than 80%, resulting in a robust upregulation of Calbindin1 protein (Fig. 4b).

#### *BMP4 inhibition of GSIS and exocytosis is dependent on Calbindin1 upregulation*

We further investigated the effect of BMP4 on GSIS from pancreatic islets isolated from Calbindin1 knock out (KO) mice and wild type (WT) littermates. Like in neonatal rat islets, we observed significant inhibition of GSIS by BMP4 treatment in WT adult mouse islets (Fig. 4c), whereas no inhibition was observed in islets from Calbindin KO mice (Fig. 4c). In accordance with this, we find that BMP4 does not reduce exocytosis in islets from Calbindin1 KO mice, but rather showed a non-significant increase in exocytosis (Fig. 4d-f).

### **Discussion:**

Here we investigate the mechanism behind BMP4 mediated inhibition of GSIS in neonatal rat and adult mouse islets of Langerhans. This effect is not caused by decreased *Insulin1*

mRNA expression or protein content, or by number, size or localization of insulin granules, but rather seems to be due to diminished  $\text{Ca}^{2+}$ -influx through VDCC resulting in decreased exocytosis. Metabolism of glucose increase intracellular ATP levels resulting in closure of ATP dependent  $\text{K}^+$  channels and membrane depolarization. Consequential opening of VDCC trigger  $\text{Ca}^{2+}$ -entry which elicits insulin exocytosis (reviewed in [30, 31]). Hence,  $\text{Ca}^{2+}$ -entry is essential for the amount of insulin released. We observe a BMP4 dependent decrease in VDCC  $\text{Ca}^{2+}$ -current leading to a reduction in depolarisation induced exocytosis and GSIS.  $\text{Ca}^{2+}$ -sensitivity of exocytosis was not decreased, rather increased, indicating that the reduced  $\text{Ca}^{2+}$  current is the main determinant of the reduced exocytosis. The reduced  $\text{Ca}^{2+}$ -influx was also confirmed by lack of response to  $\text{K}^+$  in live  $\text{Ca}^{2+}$ -measurements. Both observations are indicative of reduced depolarization evoked  $\text{Ca}^{2+}$  -influx or increased  $\text{Ca}^{2+}$ -buffering after BMP4 treatment. An increased buffering would agree with the continued and more pronounced reduction in late exocytosis evoked by the latter depolarizations and the second train (Figure 2d-e). Interestingly, our microarray analysis identified the  $\text{Ca}^{2+}$ -binding protein Calbindin1 to be upregulated by BMP4. We re-find this regulation on independent samples and in mouse islets. Although neonatal and adult islets have been suggested to respond different to glucose and other stimuli, BMP4 inhibit GSIS and upregulate Calbindin1 in both model systems. Overexpression of Calbindin1 reduced GSIS. The effect of BMP4 on GSIS was lost in islets from Calbindin1 KO mice (Fig. 4c). The exocytotic response to BMP4 in wild type islets showed the same trend as previously observed (Fig 4d and 2a-e), whereas this regulation was lost in islets from KO mice. There was a trend towards an increased exocytosis in response to BMP4 in the Calbindin1 KO mice, but this was not significant neither was it reflected in the GSIS. The observation that BMP induced inhibition of GSIS is not observed in islets from Calbindin1 KO mice points to the increased Calbindin1 expression being causative to the observed BMP4 mediated decline in GSIS. Indeed, the

BMP4 induced decrease in VDCC-influx is remarkably similar to the effect observed upon overexpression of Calbindin1 in a pancreatic beta cell line (Figure 2b-h and [29]). In addition to a  $\text{Ca}^{2+}$ -scavenging effect, accumulating evidence suggests that Calbindin1 reduce  $\text{Ca}^{2+}$ -currents through an association to L-type VDCCs [29]. A glucose and  $\text{Ca}^{2+}$ -dependent translocation of Calbindin1 to the plasma membrane has previously been suggested to facilitate the interaction with the L-type  $\text{Ca}^{2+}$ -channel [28, 29]. This could explain the lack of effect on the capacitance increase evoked by the first depolarisation (Figure 2c and 4e), as the first influx of  $\text{Ca}^{2+}$  would induce translocation of Calbindin1 to the plasma membrane.

Glucose induced  $\text{Ca}^{2+}$ -oscillations occur in fewer BMP4 treated islets compared to control cells possibly due to reduced  $\text{Ca}^{2+}$ -influx through VDCC. This is further supported by the blunted  $\text{Ca}^{2+}$ -response to high glucose and depolarizing  $\text{K}^+$  after BMP4 treatment (Fig. 3i-o and Supplementary Table 1). The lack of  $\text{K}^+$ -induced  $\text{Ca}^{2+}$ -influx was also observed in a Calbindin1 overexpressing cell line, indicating that this is an effect caused by the increased Calbindin1 expression [27, 29]. Finally, the oscillations persist longer in BMP4 treated islets when glucose is lowered back to 2.8 mM (Figure 2n and Supplementary Table 1), indicating a failure of the beta cells to repolarize to baseline; again suggesting dysfunctional  $\text{Ca}^{2+}$  handling after BMP4 treatment.

Interestingly, Calbindin1 expression is increased in pancreatic islets from diabetic rats and mice [32, 33]. Concomitant upregulation of BMP2 (but not BMP4) and Calbindin1 in islets of Langerhans has been observed in a type 2 diabetic mouse model [33]. BMP2 also upregulate the expression of Calbindin1 and reduce GSIS in rat islets (data not shown). Generally BMP2 and 4 may be considered as inflammatory markers in several metabolic tissues. Under diabetic conditions the expression of BMP2 or 4 have been reported to increase in arteries, kidney, bones and islets [4, 14, 18, 19]. The increased expression is reflected by increased circulating levels of BMP4 in type 2 diabetic patients in one study

[13]. Thus, systemic inhibition of BMP2 and 4 appear as a possible strategy for broad targeting of a mediator of low grade inflammation associated with type 2 diabetes. Interestingly, it was recently reported that systemic administration of the natural BMP inhibitor noggin lowered blood glucose in *db/db* mice [14].

In conclusion, we have provided insight into mechanisms involved in BMP4 mediated inhibition of insulin secretion and gene regulation in islets of Langerhans and identified Calbindin1 as a mediator of BMP4 induced beta cell dysfunction.

### **Acknowledgements:**

We acknowledge the technical assistance from Helle Fjordvang and Lene Grønne Pedersen, University of Copenhagen, Sabine Mach, Ludwig-Maximilians-Universität, Britt-Marie Nilsson and Anna-Maria Veljanovska-Ramsay, Lund University Diabetes Center.

### **Funding:**

We are thankful for support from the Novo Nordisk Foundation, The Danish Research Council, The Danish Diabetes Academy, The European Foundation for the Study of Diabetes, The A.P. Møller Foundation, The Swedish Research Council, The Region Skåne (ALF), Albert Pålsson foundation and The Swedish Diabetes foundation. LE is a senior Researcher at the Swedish Research Council. GLC hold a Postdoc grant from the Danish Diabetes Academy.

### **Duality of interest:**

MLBJ, CB and KSF are employees of Novo Nordisk A/S.

### **Contribution statement:**

NB, GLC, MLBJ, CB and LE designed the study. GLC, MLBJ, AW, IM, JF, KSF, MM, CB, NB and LE participated in acquisition, analysis and interpretation of data. GLC, NB and LE



drafted the manuscript. GLC, MLBJ, AW, IM, JF, KSF, MM, CB, NB and LE revised the manuscript critically for important intellectual content and approved the final version to be published. NB is the guarantor of this work.

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## Figure legends.

### Figure 1 BMP4 inhibits glucose stimulated insulin secretion without affecting total insulin content.

a) Neonatal rat islets were pre-exposed to 50 ng/ml BMP4 for 0-96 hours and GSIS was performed as described. b) Insulin content of islets post-assay. DNA content was used for normalization in a) and b). c) 8000 intact neonatal rat islets were exposed to 50 ng/ml BMP4 for 0-96 hours. *InsI* mRNA expression is shown as relative values normalized against *Ppia* expression. All data are shown as mean +SEM. Statistical significance was evaluated using Anova followed by Dunnet's t-test. \* indicates  $p < 0.05$

### Figure 2 BMP4 diminish capacitance and $\text{Ca}^{2+}$ influx through voltage dependent $\text{Ca}^{2+}$ channels

a) Example trace of depolarization-induced exocytosis, measured as changes in cell membrane capacitance ( $\Delta C_m$ ) in a single mouse beta cell. b) Mean increase in membrane capacitance evoked by a train of ten depolarizations, c) the first depolarization and d) depolarizations 2 to 10. e) Mean capacitance increase evoked by a second train applied 2 minutes later. f) Example trace from a VDCC in a beta cell incubated in the absence (grey) and presence of BMP4 (black). g) The measured charge (Q) as a function of the membrane voltage ( $V_m$ ) during a 50 ms depolarization. Open squares; Ctrl cells, Black squares; BMP4 treated cells. h) The  $\text{Ca}^{2+}$ -sensitivity of the exocytotic response measured as the capacitance increase during the first depolarization of the train ( $\Delta C_m$ ) in c) divided by the  $\text{Ca}^{2+}$ -influx (Charge; Q) during the same 500 ms-depolarization. Data presented in b-e and g-h are mean  $\pm$  SEM of  $n=9$  to 16 experiments in each group. \* $p < 0.05$ , \*\* $p < 0.01$ . i) Representative example of a calcium trace obtained from one islet pre-treated for 3 days with vehicle (control) or j) BMP4.  $C_0$  - calcium dip occurring when 16.7 mM glucose reach the islets;  $D_0$  - time delay between 16.7 mM glucose exposure and the first calcium peak;  $C_1$  - amplitude of first phase peak;  $D_1$  - time delay in response to low glucose;  $C_K$  - amplitude of high potassium peak. The

staircase indicates the glucose concentration: 2.8 mmol/L glucose (2.8) and 16.7 mmol/L glucose (16.7). The line with K<sup>+</sup> indicate the addition of 70 mmol/L KCl. Summary statistics of calcium imaging traces shown in k) C<sub>0</sub>, l) D<sub>0</sub>, m) C<sub>1</sub>, n) D<sub>1</sub> o) C<sub>K</sub>

### **Figure 3 BMP4 stimulate the expression of Calbindin1**

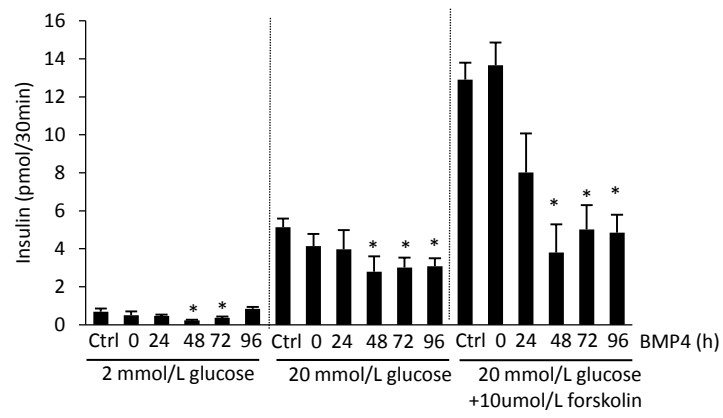
Islets were stimulated with 50 ng/ml BMP4 for 0-96 hours. a) Calbindin1 mRNA expression in neonatal rat islets is shown in relative values and normalized against the expression on *Ppia*. b) Protein expression in neonatal rat islets was determined by western blotting using primary antibodies against Calbindin1 and  $\beta$ -actin. A representative western blot is shown and quantification of densitometry of 3 blots was performed in image J. c) Isolated mouse islets were stimulated with 50 ng/ml BMP4 for 0-96 hours. *Calbindin1* mRNA expression is shown in relative amounts and normalized against the expression of *Ppia*. All data are presented as mean + SEM. n=3-4

### **Figure 4 Calbindin1 expression affects islet glucose and BMP4 responsiveness.**

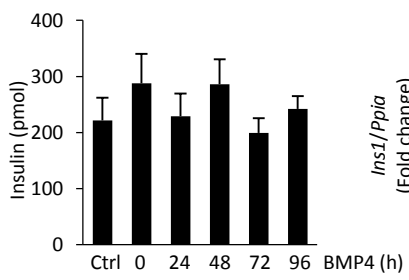
a) GSIS from dispersed neonatal rat islet cells overexpressing Calbindin1 or GFP. Insulin secretion is depicted as % of control cells exposed to high glucose. b) Western blot showing lentiviral overexpression of Calbindin1. c) Islets from Calbindin1 KO mice (KO) and littermate wild type controls (WT) were exposed to 50 ng/ml BMP4 for 96 hours and subsequently GSIS was determined, n=3. All data are presented as mean+SEM. d) Mean increase in membrane capacitance evoked by the full train, e) the first depolarization and f) 2-10th depolarizations in single mouse beta cells isolated from Calbindin1 KO mice (KO) and littermate wild type controls (WT).

Figure 1

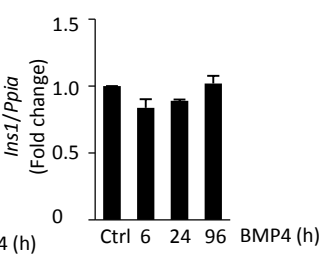
a



b



c



**Figure 2**

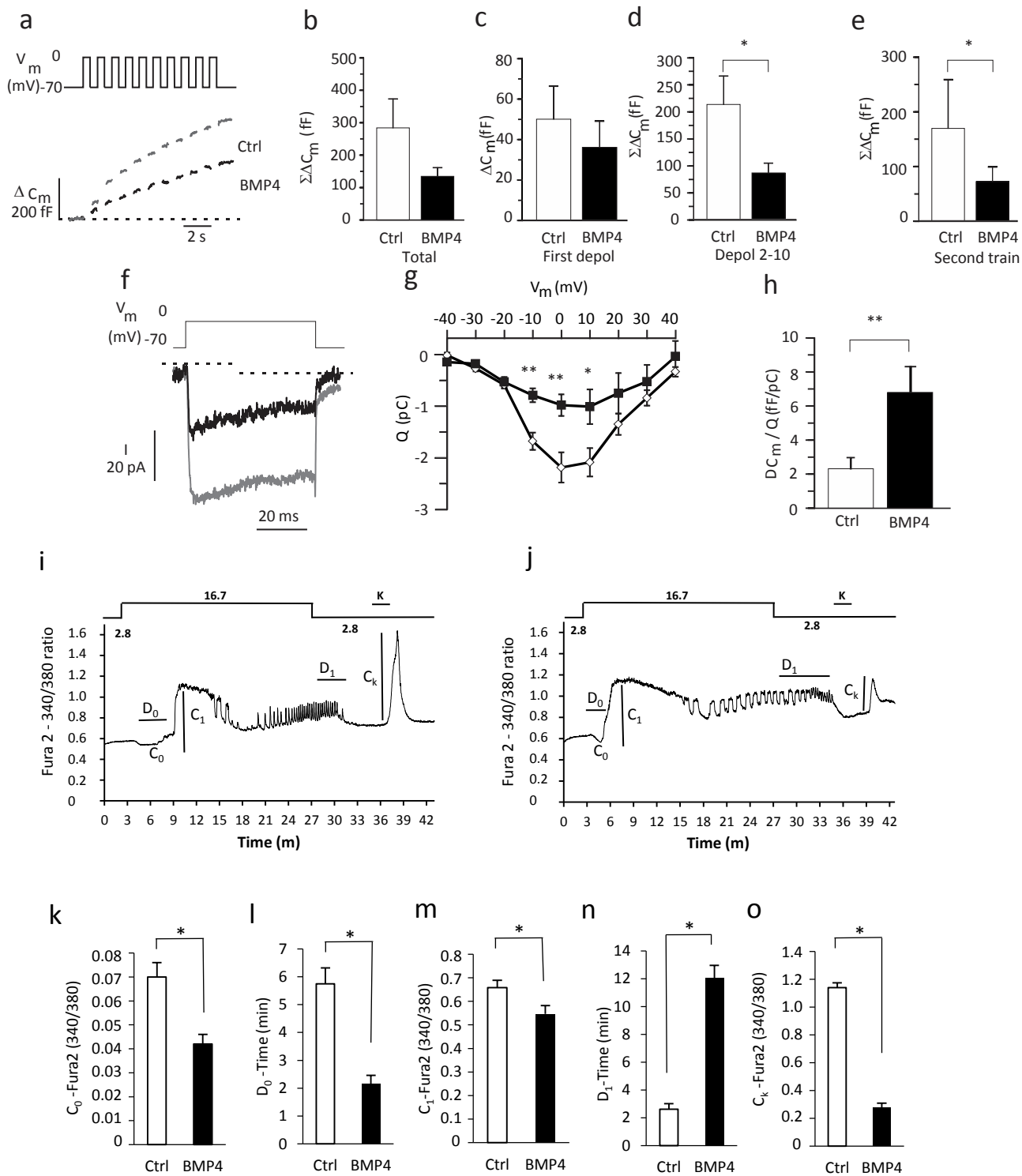
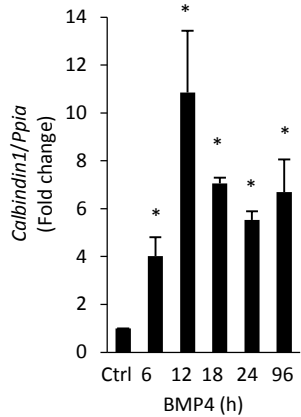
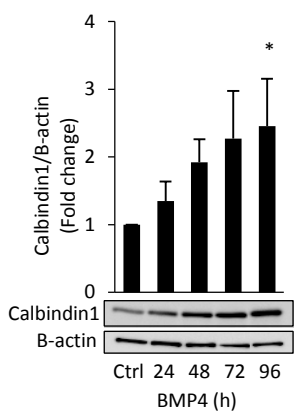


Figure 3

a



b



c

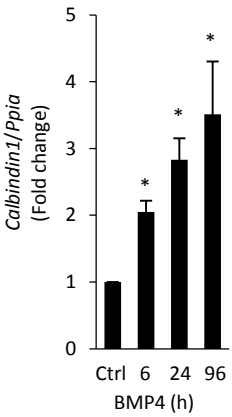
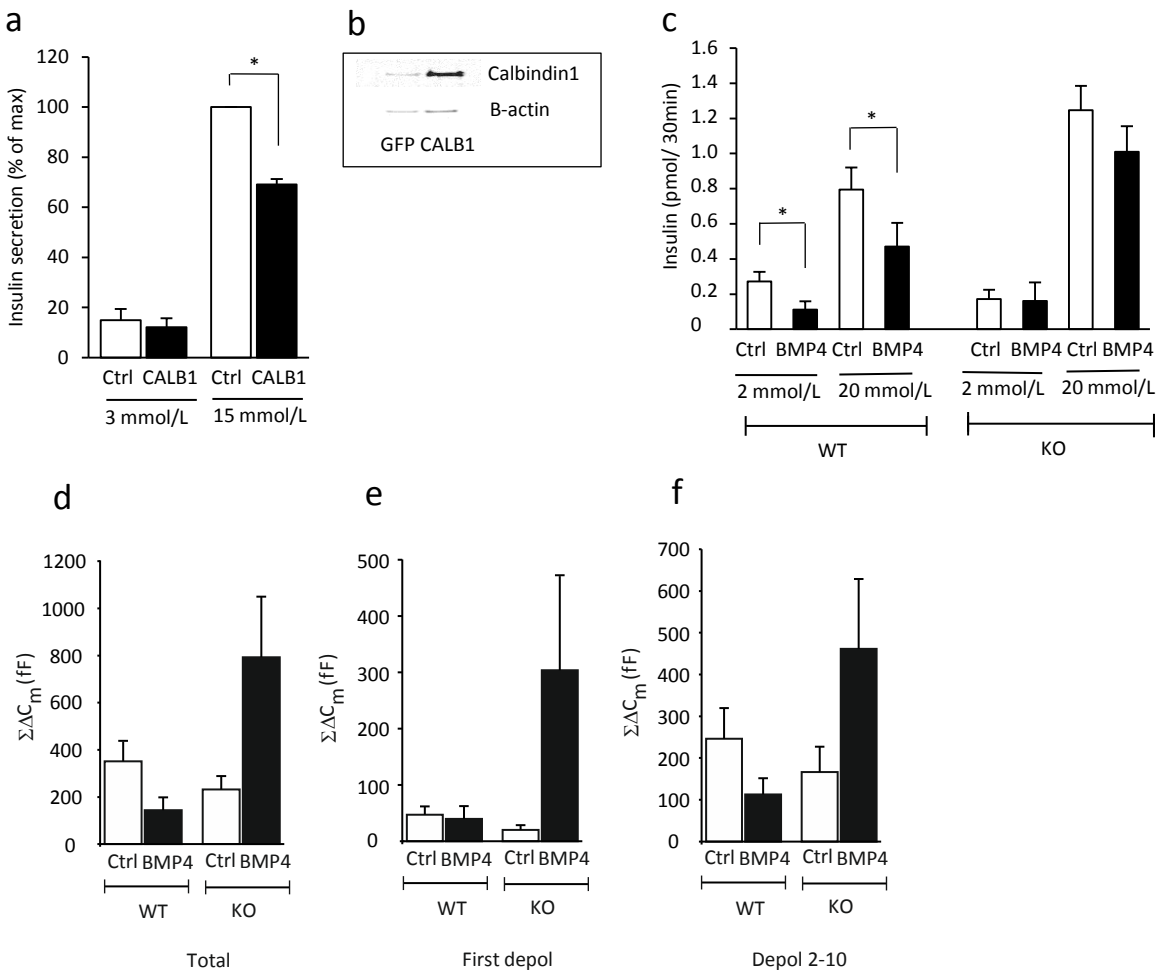


Figure 4





**Table 1**

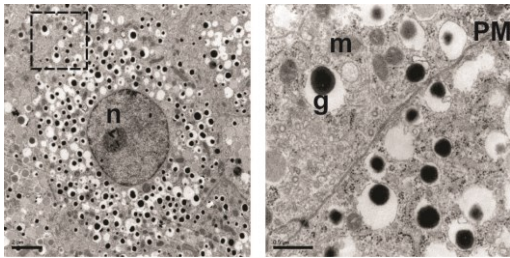
Genes regulated  $\geq 2$ -fold in neonatal rat islets exposed to 50 ng/ml BMP-4 for 96 hrs.

Gene	Protein name	Fold change
<i>Id3</i>	DNA-binding protein inhibitor ID-3	17.92*
<i>Id1</i>	DNA-binding protein inhibitor ID-1	16.52*
<i>Lypd8</i>	Ly6/PLAUR domain containing protein 8 precursor	9.66
<i>Irx-3</i>	Iroquois-class homeodomain protein IRX-3	5.40
<i>Calb1</i>	Calbindin1	4.62*
<i>Id2</i>	DNA-binding protein inhibitor ID-2	4.25*
<i>Micalcl</i>	MICAL C-terminal-like protein	4.06
<i>Fam101a</i>	Family with sequence similarity 101. member A (Fam101a)	3.85
<i>Lgals4</i>	Galectin-4	3.72
<i>Bambi</i>	BMP and activin membrane-bound inhibitor homolog	3.25*
<i>Atoh8</i>	Protein atonal homolog 8	3.13
<i>Arc</i>	Activity-regulated cytoskeleton-associated protein	3.08
<i>Mlph</i>	Melanophilin	2.90
<i>Camk1g</i>	Calcium/calmodulin-dependent protein kinase type 1G	2.82
<i>Chst10</i>	Carbohydrate sulfotransferase 10	2.81
<i>Dlk1</i>	Protein delta homolog 1	2.75
<i>Tppp3</i>	Tubulin polymerization-promoting protein family member 3	2.72
<i>Tmem100</i>	Transmembrane protein 100	2.68
<i>Ckb</i>	Creatine kinase B-type	2.63
<i>Ppp1r36</i>	Protein phosphatase 1 regulatory subunit 36	2.59
<i>Tmem100</i>	Transmembrane protein 100	2.51
<i>Rtn4rl1</i>	Reticulon-4 receptor-like 1	2.44
<i>St5</i>	Suppression of tumorigenicity 5 protein	2.34
<i>Akap12</i>	A-kinase anchor protein 12	2.30
<i>Vash2</i>	Vasohibin-2	2.20
<i>Ddx31</i>	Probable ATP-dependent RNA helicase DDX31	2.07
<i>Fads1</i>	Fatty acid desaturase 1	0.45
<i>Nod3l</i>	NOD3-like protein	0.44
<i>Bmp3</i>	Bone morphogenetic protein 3	0.43*
<i>Htr5b</i>	5-hydroxytryptamine (serotonin) receptor 5B	0.33
<i>Gpr6</i>	G protein-coupled receptor 6	0.28*

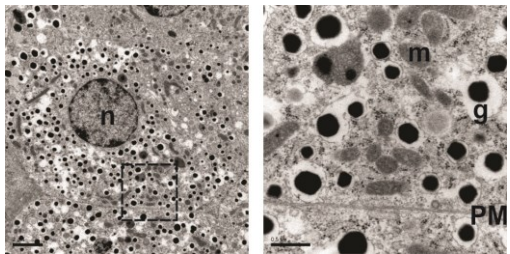
\* Regulation have been verified by q-pcr on independent neonatal rat islet samples

## Supplementary Figure 1

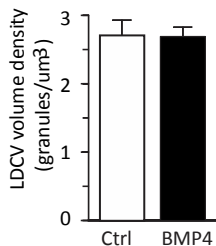
a



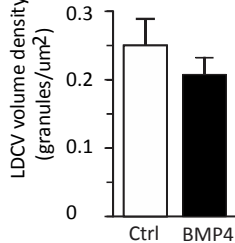
b



c



d



### Supplementary figure 1

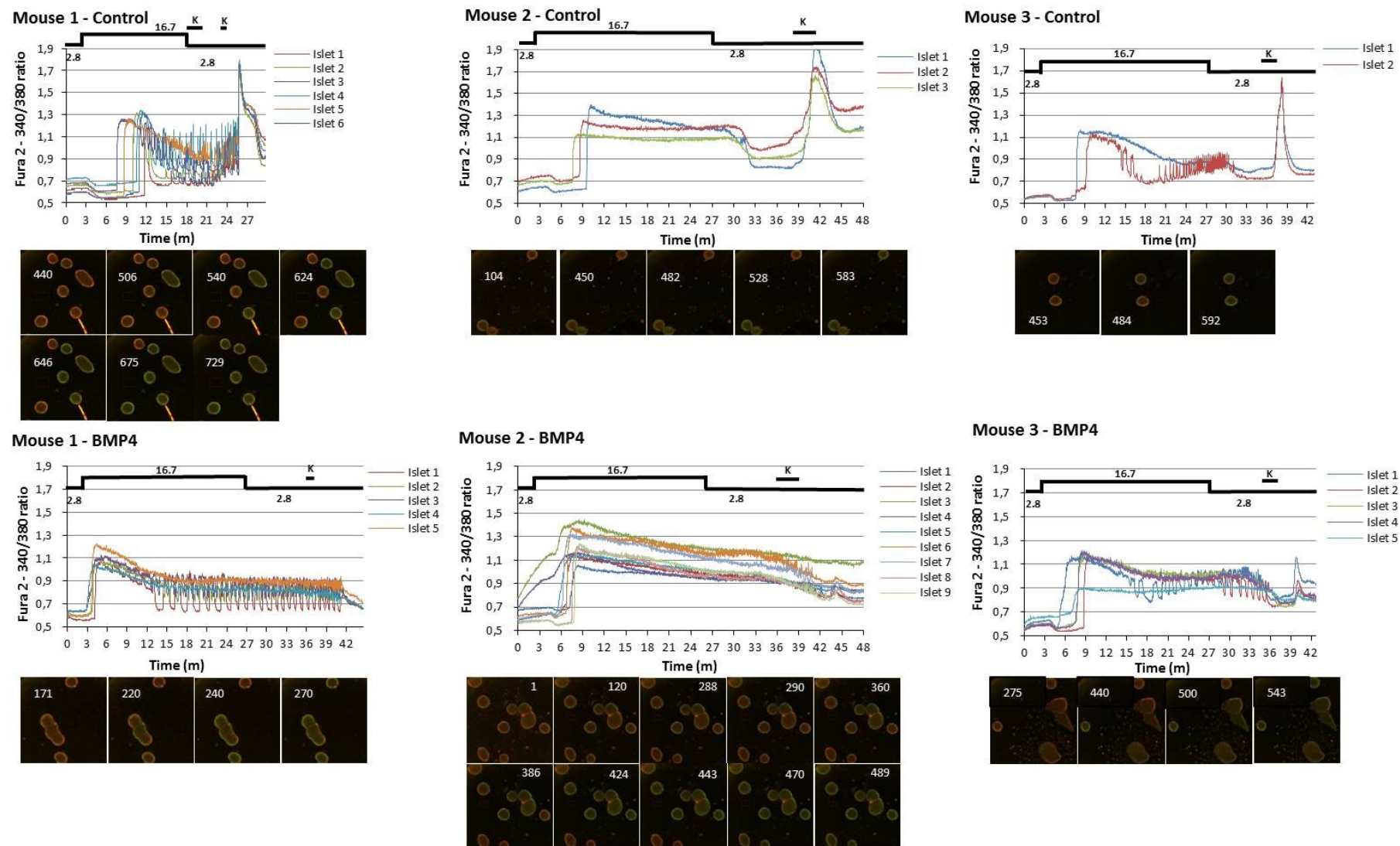
BMP4 does not affect the ultrastructure of mouse beta cells

a Transmission electron micrograph of a single beta cell within an islet that has been cultured in the absence of BMP4 (left; scale bar 2 μm). The area within the square is highlighted to the right (scale bar 0.5 μm). PM-plasma membrane; g-granule; m-mitochondria; n-nucleus. b As in a, but showing a single beta cell within an islet that have been cultured for 3 days in the presence of 50 ng/ml BMP4. c Histogram summarizing the total number of granules within beta cells after the different culture conditions presented as the volume density (granules/μm³). d Histogram summarizing the number of docked granules within beta cells after the different culture conditions presented as the surface density (granules/μm²). Granules were considered docked if the center of the granule was < 200 nm from the plasma membrane. Data in c and d is presented as mean + SEM of *n*= 30 (Ctrl) and 32 (BMP4) treated cells.

### Method: Transmission electron microscopy

Mouse islets were fixed in 2.5% glutaraldehyde in freshly prepared Millonig and post-fixed in 1% osmium tetroxide before being dehydrated and embedded in AGAR 100 (Oxford Instruments Nordiska AB) and cut into ultrathin sections (70-90 nm). The sections were put on Cu-grids and contrasted using uranyl acetate and lead citrate. The islet containing sections were examined in a JEM 1230 electron microscope (JEOL-USA, Inc.). Micrographs were analysed with respect to the intracellular distribution as described elsewhere in as described elsewhere in Olofsson CS, et al 2002: Fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic B-cells. *Pflügers Arch* 444:43-51"

## Supplementary Figure 2



**Figure 2**

Traces of all islets imaged for each NMRI mouse under control or BMP4 conditioning. Below each trace are selected snapshots showing the relative position of the islets and the moment at which the first response to 16.7mM glucose can be visualized at 340nm (frame identified by number on image: 1 frame per second; 60 frames = 1 min). The position of each change in buffer perfusion: 2.8mM glucose (2.8), 16.7mM glucose (16.7), and 2.8mM glucose in 70mM KCl buffer (2.8 + K) are indicated by arrows above each set of traces.

**Supplementary Table 1 – Summary statistics of calcium imaging traces**

Parameter	C <sub>0</sub> (Fura2 340/380 ratio)						D <sub>0</sub> (min)						C <sub>1</sub> (Fura2 340/380 ratio)					
Islet Class	All		Class 1		Class 2		All		Class 1		Class 2		All		Class 1		Class 2	
Condition	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4
Number of islets	11	19	7	5	4	14	11	19	7	5	4	14	11	19	7	5	4	14
Mean	0.070	0.042	0.077	0.059	0.058	0.037	5.745	2.149	6.552	1.560	4.333	2.360	0.658	0.547	0.692	0.570	0.598	0.539
SEM	0.006	0.004	0.008	0.008	0.005	0.004	0.571	0.315	0.706	0.801	0.447	0.321	0.031	0.035	0.023	0.028	0.072	0.047
p-value	5.63E-04		0.15		1.50E-02		1.78E-06		9.19E-04		8.05E-03		4.39E-02		7.04E-03		0.56	

Parameter	F <sub>H</sub> (peaks/min)		P <sub>M</sub> (Fura2 340/380 ratio)						P <sub>A</sub> (Fura2 340/380 ratio)			
Islet Class	Class 1		All		Class 1		Class 2		Class 1		Class 2	
Condition	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4
Number of islets	7	5	11	19	7	5	4	14	7	5	4	14
Mean	1.357	0.940	0.962	0.997	0.884	0.892	1.100	1.035	0.168	0.126	0.030	0.031
SEM	0.248	0.234	0.046	0.029	0.032	0.033	0.079	0.033	0.019	0.027	0.002	0.002
p-value	0.27		0.51		0.87		0.39		0.22		0.84	

Parameter	D <sub>1</sub> (min) #						C <sub>K</sub> (Fura2 340/380 ratio)					
Islet Class	All		Class 1		Class 2		All		Class 1		Class 2	
Condition	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4	Ctrl	BMP4
Number of islets	5	14	1	2	4	12	11	19	7	5	4	14
Mean	2.610	12.077	3.200	7.134	2.463	12.900	1.140	0.279	1.145	0.381	1.132	0.242
SEM	0.402	0.885	na	0.264	0.746	0.941	0.035	0.031	0.021	0.062	0.098	0.031
p-value	6.02E-05		na		5.90E-05		8.03E-17		1.09E-07		2.97E-09	

### **Supplementary Table 1 – Summary statistics of calcium imaging traces**

Temporal fluctuation of intracellular calcium response to 16.7mM glucose were organized into 2 classes: class 1 islets present separate 1<sup>st</sup> and 2<sup>nd</sup> phases with a clear 1<sup>st</sup> phase peak followed by a lowering of calcium and a 2<sup>nd</sup> phase with distinct regularly spaced calcium oscillations; class 2 islets present a rise in calcium with no clear first and second phases and no distinct oscillation in 2<sup>nd</sup> phase. Evaluation of calcium fluctuations was made by measuring differences in Fura-2 340/380 ration points.

Calcium was measured in a total of 30 islets from 2-3 mice: 11 control and 19 BMP4 treated islets. In control islets we observed 7/11 class 1 islets (64%) and in BMP4 treated islets we observed 5/19 class 1 islets (26%). This reduction in the number of class 1 islets may represent a loss of first phase insulin secretion in the BMP4 treated islets.

**C<sub>0</sub>** - calcium dip occurring when 16.7 mM glucose reach the islets and before 1<sup>st</sup> phase peak; **D<sub>0</sub>** - time delay between the moment 16.7 mM glucose reached the islet and the first calcium peak; **C<sub>1</sub>** - amplitude of first phase peak response to 16.7mM glucose; **F<sub>H</sub>** - frequency of 2<sup>nd</sup> phase oscillations (in category 1 islets); **P<sub>M</sub>** - mean ratio of oscillations during 2<sup>nd</sup> phase; **P<sub>A</sub>** - mean amplitude of oscillations during 2<sup>nd</sup> phase; **D<sub>1</sub>** - time delay in response to low glucose; **C<sub>K</sub>** - amplitude of high potassium peak.

Supplementary table 2. Genes regulated by 96h treatment with 50 ng/ml BMP4 (FDR&lt;5%)

id	gene	description	p-value	Fold change
ENSRNOG000000026124_at	Id3	DNA-binding protein inhibitor ID-3 [Source:UniProtKB/Swiss-Prot;Acc:P41138]	0,000114	17,92
ENSRNOG000000021750_at	Id1	DNA-binding protein inhibitor ID-1 [Source:UniProtKB/Swiss-Prot;Acc:P41135]	0,000025	16,52
ENSRNOG000000026844_at	LOC691259	RCG34567, isoform CRA_aUncharacterized protein [Source:UniProtKB/TrEMBL;Acc:D3ZP75]	0,003470	9,66
ENSRNOG000000011533_at	D3ZU03_RAT	iroquois-class homeodomain protein IRX-3 [Source:RefSeq peptide;Acc:NP_001100883]	0,000292	5,40
ENSRNOG000000007456_at	Calb1	Calbindin [Source:UniProtKB/Swiss-Prot;Acc:P07171]	0,000947	4,62
ENSRNOG000000007237_at	Id2	DNA-binding protein inhibitor ID-2 [Source:UniProtKB/Swiss-Prot;Acc:P41137]	0,000256	4,25
ENSRNOG000000016210_at	Micalcl	MICAL C-terminal-like protein [Source:UniProtKB/Swiss-Prot;Acc:Q4G091]	0,000225	4,06
ENSRNOG000000001011_at	Fam101a	family with sequence similarity 101, member A (Fam101a), mRNA [Source:RefSeq DNA;Acc:NM_001109547]	0,000003	3,85
ENSRNOG000000020338_at	Lgals4	Galectin-4 [Source:UniProtKB/Swiss-Prot;Acc:P38552]	0,003973	3,72
ENSRNOG000000016066_at	Bambi	BMP and activin membrane-bound inhibitor homolog [Source:UniProtKB/Swiss-Prot;Acc:Q91XN4]	0,000013	3,25
ENSRNOG000000010716_at	Atoh8	protein atonal homolog 8 [Source:RefSeq peptide;Acc:NP_001102711]	0,000457	3,13
ENSRNOG000000043465_at	Arc	Activity-regulated cytoskeleton-associated protein [Source:UniProtKB/Swiss-Prot;Acc:Q63053]	0,000800	3,08
ENSRNOG000000019763_at	MLph	melanophilin [Source:RefSeq peptide;Acc:NP_001012135]	0,000037	2,90
ENSRNOG000000006470_at	Camk1g	Calcium/calmodulin-dependent protein kinase type 1G [Source:UniProtKB/Swiss-Prot;Acc:Q7TNJ7]	0,000093	2,82
ENSRNOG000000012815_at	Chst10	Carbohydrate sulfotransferase 10 [Source:UniProtKB/Swiss-Prot;Acc:Q54702]	0,001042	2,81
ENSRNOG000000019584_at	DIk1	protein delta homolog 1 [Source:RefSeq peptide;Acc:NP_446196]	0,003163	2,75
ENSRNOG000000016890_at	Tppp3	Tubulin polymerization-promoting protein family member 3 [Source:UniProtKB/Swiss-Prot;Acc:Q5PPN5]	0,000056	2,72
ENSRNOG000000038480_at	CN050_RAT	Uncharacterized protein C14orf50 homolog [Source:UniProtKB/Swiss-Prot;Acc:Q68FW6]	0,000013	2,68
ENSRNOG000000010872_at	Ckb	Creatine kinase B-type [Source:UniProtKB/Swiss-Prot;Acc:P07335]	0,000487	2,63
ENSRNOG000000001720_at	D4ADT9_RAT	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:D4ADT9]	0,003472	2,59
ENSRNOG000000002434_at	Tmem100	Transmembrane protein 100 [Source:UniProtKB/Swiss-Prot;Acc:Q569C0]	0,002680	2,51
ENSRNOG000000003121_at	Rtn4r1	Reticulon-4 receptor-like 1 [Source:UniProtKB/Swiss-Prot;Acc:Q80WD0]	0,001370	2,44
ENSRNOG000000013934_at	St5	suppression of tumorigenicity 5 protein [Source:RefSeq peptide;Acc:NP_001101017]	0,001590	2,34
ENSRNOG000000019549_at	Akap12	A-kinase anchor protein 12 [Source:UniProtKB/Swiss-Prot;Acc:Q5QD51]	0,000319	2,30
ENSRNOG000000003832_at	Vash2	vasohibin-2 [Source:RefSeq peptide;Acc:NP_001102552]	0,001632	2,20
ENSRNOG000000013040_at	Ddx31	probable ATP-dependent RNA helicase DDX31 [Source:RefSeq peptide;Acc:NP_001101294]	0,000066	2,07
ENSRNOG000000002209_at	D3Z8P4_RAT	arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 2 [Source:RefSeq peptide;Acc:NP_001100686]	0,003196	2,00
ENSRNOG0000000049873	Ap1s3	Adaptor-related protein complex 1, sigma 3 subunit	0,000577	1,97
ENSRNOG000000034075_at	Ube2ql1	ubiquitin-conjugating enzyme E2Q-like protein 1 [Source:RefSeq peptide;Acc:NP_001138635]	0,001700	1,97
ENSRNOG000000017873_at	Sct	Secretin [Source:UniProtKB/Swiss-Prot;Acc:P11384]	0,002561	1,95
ENSRNOG000000011824_at	Trh	ProthyroliberinThyroliberin [Source:UniProtKB/Swiss-Prot;Acc:P01150]	0,002298	1,93
ENSRNOG0000000004828_at	Acrv1c	Activin receptor type-1C [Source:UniProtKB/Swiss-Prot;Acc:P70539]	0,003683	1,87
ENSRNOG000000001331_at	Rnf34	E3 ubiquitin-protein ligase RNF34 [Source:UniProtKB/Swiss-Prot;Acc:Q6AYH3]	0,000717	1,80
ENSRNOG0000000004362_at	Rps6ka5	ribosomal protein S6 kinase alpha-5 [Source:RefSeq peptide;Acc:NP_001101518]	0,000179	1,74
ENSRNOG000000022704	Esy13	Extended synaptotagmin-like protein 3	0,003161	1,73
ENSRNOG000000036693_at	Slc25a10	mitochondrial dicarboxylate carrier [Source:RefSeq peptide;Acc:NP_596909]	0,000314	1,67
ENSRNOG0000000009761_at	Tmod1	Tomopodulin-1 [Source:UniProtKB/Swiss-Prot;Acc:P70567]	0,003524	1,64
ENSRNOG000000027888_at	RGD1309437	Uncharacterized protein C3orf26 homolog [Source:UniProtKB/Swiss-Prot;Acc:Q5FVR6]	0,001291	1,62
ENSRNOG0000000002191_at	LOC498368	Uncharacterized protein C4orf19 homolog [Source:UniProtKB/Swiss-Prot;Acc:Q6AYA8]	0,003808	1,62
ENSRNOG0000000006519_at	Tmem107	transmembrane protein 107 [Source:RefSeq peptide;Acc:NP_001103118]	0,002142	1,62
ENSRNOG0000000036816_at	WLS_RAT	protein wntless homolog isoform 1 [Source:RefSeq peptide;Acc:NP_955440]	0,001280	1,61
ENSRNOG000000038686_at	Ap1s2	AP-1 complex subunit sigma-2 [Source:RefSeq peptide;Acc:NP_001121003]	0,000012	1,59
ENSRNOG000000012513_at	Pdk3	pyruvate dehydrogenase kinase, isozyme 3 [Source:RefSeq peptide;Acc:NP_001100051]	0,001113	1,56
ENSRNOG000000019466_at	Agpat2	1-acyl-sn-glycerol-3-phosphate acyltransferase beta [Source:RefSeq peptide;Acc:NP_001101291]	0,000605	1,56
ENSRNOG000000012787_at	Tmem164	transmembrane protein 164 [Source:RefSeq peptide;Acc:NP_001102484]	0,000684	1,55
ENSRNOG000000018106_at	Neu3	Sialidase-3 [Source:UniProtKB/Swiss-Prot;Acc:Q99PW5]	0,000168	1,55
ENSRNOG000000013286_at	Pdc13	Phosducin-like protein 3 [Source:UniProtKB/Swiss-Prot;Acc:Q4KLJ8]	0,004009	1,52
ENSRNOG000000036692_at	Gcgr	Glucagon receptor [Source:UniProtKB/Swiss-Prot;Acc:P30082]	0,002636	1,50
ENSRNOG000000004200_at	Golsyn	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:F1LUC0]	0,000378	1,50
ENSRNOG0000000049019	Tmem170a	Transmembrane protein 170A	0,001936	1,49
ENSRNOG000000007023_at	Galm	Aldose 1-epimerase [Source:UniProtKB/Swiss-Prot;Acc:Q66HG4]	0,001670	1,49
ENSRNOG000000018359_at	Smad7	Mothers against decapentaplegic homolog 7 [Source:UniProtKB/Swiss-Prot;Acc:Q88406]	0,000303	1,47
ENSRNOG000000001958_at	ift57	intraflagellar transport protein 57 homolog [Source:RefSeq peptide;Acc:NP_001100563]	0,001778	1,46
ENSRNOG000000020939_at	Ms4a8a	membrane-spanning 4-domains, subfamily A, member 8A [Source:RefSeq peptide;Acc:NP_001101989]	0,000367	1,45
ENSRNOG000000002866_at	Rassf6	Ras association domain-containing protein 6 [Source:UniProtKB/Swiss-Prot;Acc:Q4QR82]	0,002048	1,44
ENSRNOG000000012597_at	RGD1311805	similar to RIKEN cDNA 2400010D15 (RGD1311805), mRNA [Source:RefSeq DNA;Acc:NM_001009638]	0,000061	1,41
ENSRNOG0000000019346_at	Tmco3	transmembrane and coiled-coil domain-containing protein 3 [Source:RefSeq peptide;Acc:NP_001129329]	0,000804	1,41
ENSRNOG000000001090_at	Stard13	stAR-related lipid transfer protein 13 [Source:RefSeq peptide;Acc:NP_001102530]	0,004083	1,41
ENSRNOG000000016558_at	Plip	Plasmolipin [Source:UniProtKB/Swiss-Prot;Acc:P47987]	0,001675	1,39
ENSRNOG000000013557_at	Lanc1l	LanC-like protein 1 [Source:UniProtKB/Swiss-Prot;Acc:Q9QX69]	0,002269	1,38
ENSRNOG000000006302_at	Gclc	Glutamate-cysteine ligase catalytic subunit [Source:UniProtKB/Swiss-Prot;Acc:P19468]	0,000674	1,37
ENSRNOG0000000009892_at	Adamts15	A disintegrin and metalloproteinase with thrombospondin motifs 15 [Source:RefSeq peptide;Acc:NP_001100280]	0,001249	1,37
ENSRNOG000000016338_at	Fam92a1	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:D3ZP87]	0,000627	0,73
ENSRNOG000000028640_at	Siva1	Apoptosis regulatory protein Siva [Source:UniProtKB/Swiss-Prot;Acc:P59692]	0,001121	0,72
ENSRNOG000000027767_at	Slc38a5	Sodium-coupled neutral amino acid transporter 5 [Source:UniProtKB/Swiss-Prot;Acc:A2VCW5]	0,002558	0,71
ENSRNOG0000000009372_at	Tacr3	Neuromedin-K receptor [Source:UniProtKB/Swiss-Prot;Acc:P16177]	0,000105	0,71
ENSRNOG000000009779_at	Krt8	Keratin, type II cytoskeletal 8 [Source:UniProtKB/Swiss-Prot;Acc:Q10758]	0,002497	0,71
ENSRNOG000000002095_at	Arhgap24	Rho GTPase-activating protein 24 [Source:UniProtKB/Swiss-Prot;Acc:Q5U2Z7]	0,000905	0,71
ENSRNOG000000006200_at	Stt18	Suppression of tumorigenicity 18 protein [Source:UniProtKB/Swiss-Prot;Acc:Q9QXZ7]	0,000132	0,71
ENSRNOG000000014549	Arhgef26	Rho gJanine nucleotide exchange factor (GEF) 26	0,002788	0,71
ENSRNOG000000020456_at	Nucb2	Nucleobindin-2 [Source:UniProtKB/Swiss-Prot;Acc:Q9J185]	0,002516	0,70
ENSRNOG000000005589_at	RGD1565002	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:D4A0T8]	0,000693	0,70
ENSRNOG0000000017209_at	Tubb3	Tubulin beta-3 chain [Source:UniProtKB/Swiss-Prot;Acc:Q4QRB4]	0,003566	0,69
ENSRNOG000000003915_at	Tmem206	Transmembrane protein 206 [Source:UniProtKB/Swiss-Prot;Acc:Q6GH28]	0,000320	0,69
ENSRNOG000000000606_at	Pcdh15	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:F1LT00]	0,002055	0,68
ENSRNOG000000021870_at	Slco4a1	Solute carrier organic anion transporter family member 4A1 [Source:UniProtKB/Swiss-Prot;Acc:Q99N01]	0,000636	0,68
ENSRNOG000000011421_at	Smap2	stromal membrane-associated GTPase-activating protein 2 [Source:RefSeq peptide;Acc:NP_001094139]	0,003692	0,68
ENSRNOG000000017965_at	Afg3l2	AFG3-like protein 2 [Source:RefSeq peptide;Acc:NP_001128336]	0,001111	0,68
ENSRNOG000000007808_at	Nap115	Nucleosome assembly protein 1-like 5 [Source:UniProtKB/Swiss-Prot;Acc:Q5PPG6]	0,000447	0,68
ENSRNOG0000000008526_at	Pdzd3	Na(+)/H(+) exchange regulatory cofactor NHE-RF4 [Source:RefSeq peptide;Acc:NP_001178925]	0,000112	0,67
ENSRNOG000000016827_at	Slc38a3	Sodium-coupled neutral amino acid transporter 3 [Source:UniProtKB/Swiss-Prot;Acc:Q9JH29]	0,002477	0,67
ENSRNOG000000019587_at	Ptprn	Receptor-type tyrosine-protein phosphatase-like N [Source:UniProtKB/Swiss-Prot;Acc:Q63259]	0,000613	0,66
ENSRNOG000000002151_at	TAGL3_RAT	Transgelin-3 [Source:UniProtKB/Swiss-Prot;Acc:P37805]	0,002394	0,66
ENSRNOG000000013215_at	DCTD_RAT	Deoxycytidylate deaminase [Source:UniProtKB/Swiss-Prot;Acc:Q5M9G0]	0,000715	0,63
ENSRNOG000000001628_at	Pcp4	Purkinje cell protein 4 [Source:UniProtKB/Swiss-Prot;Acc:P63055]	0,000133	0,63
ENSRNOG000000010753_at	Alg1	androgen-induced gene 1 protein [Source:RefSeq peptide;Acc:NP_001127897]	0,000486	0,63
ENSRNOG000000002579_at	Pargm1	Prostate androgen-regulated mucin-like protein 1 homolog [Source:UniProtKB/Swiss-Prot;Acc:Q6P9X9]	0,000667	0,62
ENSRNOG000000012183_at	Glr1x	Glutaredoxin-1 [Source:UniProtKB/Swiss-Prot;Acc:Q9ESH6]	0,000256	0,61
ENSRNOG000000014819_at	Hap1	Huntingtin-associated protein 1 [Source:UniProtKB/Swiss-Prot;Acc:P54256]	0,000601	0,60
ENSRNOG000000024923_at	Nnat	Neuronatin [Source:UniProtKB/Swiss-Prot;Acc:Q62649]	0,000459	0,60
ENSRNOG000000020455_at	Cst6	cystatin-M [Source:RefSeq peptide;Acc:NP_598250]	0,000069	0,58
ENSRNOG000000032669_at	Serpina1	Alpha-1-antiproteinase [Source:UniProtKB/Swiss-Prot;Acc:P17475]	0,001816	0,56
ENSRNOG000000007600_at	Igsf1	Immunoglobulin superfamily member 1 [Source:UniProtKB/Swiss-Prot;Acc:Q925N6]	0,000485	0,54
ENSRNOG000000011201_at	Cmtm8	CKLF-like MARVEL transmembrane domain-containing protein 8 [Source:RefSeq peptide;Acc:NP_942049]	0,000689	0,54
ENSRNOG000000014264_at	Phactr1	Phosphatase and actin regulator 1 [Source:UniProtKB/Swiss-Prot;Acc:P62024]	0,003136	0,52
ENSRNOG000000005615_at	Gadd45a	Growth arrest and DNA damage-inducible protein GADD45 alpha [Source:UniProtKB/Swiss-Prot;Acc:P48317]	0,000084	0,51
ENSRNOG000000015971_at	Slc12a2	solute carrier family 12 member 2 [Source:RefSeq peptide;Acc:NP_113986]	0,003419	0,50
ENSRNOG000000020480_at	Fads1	Fatty acid desaturase 1 [Source:UniProtKB/Swiss-Prot;Acc:Q920R3]	0,001893	0,45
ENSRNOG000000039856_at	nod3l	Uncharacterized protein C6orf154 homolog [Source:UniProtKB/Swiss-Prot;Acc:Q587K4]	0,002516	0,44
ENSRNOG000000002381_at	Bmp3	Bone morphogenetic protein 3 [Source:UniProtKB/Swiss-Prot;Acc:P49002]	0,002547	0,43
ENSRNOG000000002549	HTR5B	5-hydroxytryptamine (serotonin) receptor 5B	0,003570	0,33
ENSRNOG000000049580	GPR6	G protein-coupled receptor 6	0,000178	0,29