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Type 2 diabetes risk gene Dusp8 regulates hypothalamic Jnk signaling and insulin sensitivity

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Recent genome-wide association studies (GWAS) identified DUSP8, encoding a dual-specificity phosphatase targeting mitogen-activated protein kinases, as a type 2 diabetes (T2D) risk gene. Here, we reveal that Dusp8 is a gatekeeper in the hypothalamic control of glucose homeostasis in mice and humans. Male, but not female, Dusp8 loss-of-function mice, either with global or corticortopin-releasing hormone neuron–specific deletion, had impaired systemic glucose tolerance and insulin sensitivity when exposed to high-fat diet (HFD). Mechanistically, we found impaired hypothalamic-pituitary-adrenal axis feedback, blunted sympathetic responsiveness, and chronically elevated corticosterone levels driven by hypothalamic hyperactivation of Jnk signaling. Accordingly, global Jnk1 ablation, AAV-mediated Dusp8 overexpression in the mediobasal hypothalamus, or metyrapone-induced chemical adrenalectomy rescued the impaired glucose homeostasis of obese male Dusp8-KO mice, respectively. The sex-specific role of murine Dusp8 in governing hypothalamic Jnk signaling, insulin sensitivity, and systemic glucose tolerance was consistent with functional MRI data in human volunteers that revealed an association of the DUSP8 rs2334499 risk variant with hypothalamic insulin resistance in men. Further, expression of DUSP8 was increased in the infundibular nucleus of T2D humans. In summary, our findings suggest the GWAS-identified gene DUSP8 as a novel hypothalamic factor that plays a functional role in the etiology of T2D.

Conflict of Interest: MHT is a member of the scientific advisory board of ERX Pharmaceuticals, Inc. He was a member of the Research Cluster Advisory Panel (ReCAP) of the Novo Nordisk Foundation between 2017 and 2019. He attended a scientific advisory board meeting of the Novo Nordisk Foundation Center for Basic Metabolic Research in 2016. He received funding for his research projects from Novo Nordisk (2016–2020) and Sanofi-Aventis (2012–2019). He was a consultant for Bionorica SE (2013–2017), Menarini Ricerche S.p.A. (2016), and Bayer Pharma AG Berlin (2016). As former Director of the Helmholtz Diabetes Center and the Institute for Diabetes and Obesity at Helmholtz Zentrum München (2011–2018) and since 2018 as CEO of Helmholtz Zentrum München he has been responsible for collaborations with a multitude of companies and institutions, including, but not limited to, Boehringer Ingelheim, Eli Lilly, Novo Nordisk, Medigene, Arbormed, and BioSyngen. In this role, he was/is further responsible for commercial technology transfer activities of his institute(s), including diabetes-related patent portfolios of Helmholtz Zentrum München, e.g., WO/2016/188932 A2 or WO/2017/194499 A1.

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Introduction

Obesity-induced insulin resistance is associated with an increase in circulating levels of cytokines and free fatty acids (I) that activate proinflammatory signaling pathways. Mitogen-activated protein kinase (MAPK) signaling plays a major role in this inflammatory process via sequential, dual phosphorylation and thereby activation of the serine/threonine kinases c-Jun N-terminal kinase (Jnk), p38, and extracellular signal–regulated kinase (ERK) (2). The magnitude and duration of MAPK signaling dictates the specificity of signal transduction and the extent of biological signaling response (3). MAPKs are thus tightly controlled by MAPK phosphatases (MKPs) that form the structurally distinct family of dual-specificity phosphatases (DUPS) (4). The DUPS show high homology in an amino-terminal noncatalytic domain and in a carboxyl-terminal catalytic domain, but differ in their substrate specificities, tissue distribution, subcellular localization, and inducibility by extracellular stimuli (reviewed in refs. 3, 5). Recent work using genetic models has revealed potentially important roles of DUPS in the regulation of pathophysiological signaling with relevance to human diseases including cancer, diabetes, inflammatory, and neurodegenerative disorders (4). Here, the cytoplasmic, ERK-specific Dusp6 and Dusp9 and the inducible nuclear and promiscuous, i.e., ERK-, JNK-, and p38-dephosphorylating Dusp1 were reported to modulate glucose homeostasis. Less is known about MKPs specific for JNK and/or p38, namely Dusp8, Dusp10, and Dusp16 given their expression in both the cytoplasm and cell nucleus and their relatively selective substrate specificity (4, 6, 7). Dusp8 appears to be of particular importance for the control of glucose homeostasis based on recent genome-wide association studies (GWAS) that linked minor allele carriers of the SNP rs2334499 on chromosome 11p15, a region containing DUSP8 among a number of other genes, with a moderately increased risk for type 2 diabetes (T2D) (8, 9).

Dusp8 was originally identified as an immediate early gene, and its expression can be stimulated in vitro by nerve growth factor and insulin (10). Expression of DUSP8 was found to be strong in adult human brain and weak in heart and skeletal muscle (10). Dusp8 overexpression studies revealed greater phosphatase activity against Jnk than p38 or ERK (11). Here, we aimed to delineate the functional importance of Dusp8 for the etiology of T2D. We show that DUSP8 polymorphism rs2334499 is associated with hypothalamic insulin resistance in men and that DUSP8 expression is increased in the infundibular nucleus of humans with T2D. Phenotypic studies in global Dusp8-KO mice and in Dusp8-KO mice with the hypothalamic rescue of Dusp8 expression expose a specific role of Dusp8 as gatekeeper for systemic glucose tolerance and insulin sensitivity in an obesogenic environment. Mechanistic studies in Jnk1-Dusp8 double-KO mice and mice with conditional deletion of Dusp8 in corticotropin-releasing hormone (CRH) neurons further revealed that Dusp8 as Jnk-specific phosphatase acts as a regulator for hypothalamic Jnk activation and hypothalamic-pituitary-adrenal (HPA) axis feedback inhibition that is essential for systemic glucose homeostasis.

Results

Expression levels of Dusp8 are upregulated in the hypothalamus of mice by body adiposity. We first aimed to investigate the murine expression pattern of Dusp8, the product of a gene recently linked with an increased risk for T2D in humans (8, 9). Focusing on tissues relevant for glucose tolerance in mice, we largely confirmed earlier reports showing high expression levels of DUSP8 in adult human brain, heart, and skeletal muscle (10). Specifically, we showed high expression levels of Dusp8 in several brain areas including the hypothalamus, and considerable expression in selected types of skeletal muscle of mice (Figure 1A). We next assessed the impact of nutrient overload on Dusp8 gene expression in vivo in HFD-induced obese (DIO) mice. Dusp8 expression was not altered in muscle (Figure 1B) but significantly increased in hypothalami of DIO mice (Figure 1C). In contrast, prolonged fasting, which is known to mobilize massive amounts of free fatty acids into circulation, and refeeding by fat-free diet (FFD) or HFD had no impact on hypothalamic Dusp8 gene expression (Figure 1D). Moreover, hypothalamic Dusp8 expression was also increased in genetically obese, chow-fed Lepob mice (Figure 1E) but unaffected by lepin replacement therapy (data not shown). These data indicate that the transcriptional regulation of Dusp8 in mice is independent from leptin signaling or dietary fatty acids. In situ hybridization revealed that Dusp8 expression is increased in the mediobasal hypothalamus (MBH), a region known for the regulation of glucose and energy homeostasis, of Lepob mice compared with WT controls (Figure 1, F–H). Overall, we show that hypothalamic Dusp8 expression appears to be regulated by body adiposity, a metabolic state that often correlates with impaired glucose homeostasis.

Global ablation of Dusp8 exacerabets HFD-induced glucose intolerance specifically in male mice. To explore whether Dusp8 has indeed a gluco- and energy-regulatory function in vivo we studied mice with global ablation of Dusp8. Male and female Dusp8-KO mice that were fed a standard chow diet neither showed a difference in body weight (BW) or body composition nor displayed glucose or insulin intolerance compared with their respective WT controls (Supplemental Figure 1); supplemental material available online with this article; https://doi.org/10.1172/JCI136363DS1). After inducing nutrient overload and obesity by 12 weeks of HFD feeding, we found comparable changes in BW and body composition (Figure 2, A and B) as well as unperturbed food intake, energy expenditure, respiratory exchange ratios, locomotor activity, and leptin sensitivity in male Dusp8-WT and -KO mice (Supplemental Figure 2). However, despite similar propensities for DIO, male Dusp8-KO mice exposed to HFD showed an impairment of glucose homeostasis in a glucose tolerance test (GTT) (Figure 2, C and D). In a pyruvate tolerance test (PTT), we found increased baseline glucose levels after overnight fasting and impaired glucose clearance in HFD-fed Dusp8-KO males (Figure 2, E and F). An insulin tolerance test (ITT) revealed a decrease in insulin sensitivity in obese Dusp8-KO males (Figure 2, G and H). Male mice heterozygous for the Dusp8 deletion showed similar glucose tolerance and insulin sensitivity as WT littermates after HFD exposure (data not shown). In male HFD-fed Dusp8-KO mice fasting plasma insulin levels (WT, 36.5 ± 12.0 μU/mL; Dusp8-KO, 67.0 ± 10.4 μU/mL) and homeostatic model assessment of insulin resistance (HOMA-IR) (Figure 2I) indices were significantly higher compared with their WT counterparts. Plasma markers for lipid metabolism (Supplemental Figure 3, A–C) and hepatic triglyceride stores (Supplemental Figure 3D) were comparable between HFD-fed
overexpressing adeno-associated virus (AAV) into the MBH of WT mice, resulting in a 4-fold higher hypothalamic Dusp8 expression, compared with controls injected with green fluorescent protein–AAV (GFP-AAV) (Figure 2, L and M). AAV-mediated overexpression of Dusp8 in the MBH had no effect on glucose tolerance in HFD-fed WT male mice compared with GFP controls (Figure 2, N and O). However, overexpression of Dusp8 in the MBH of global Dusp8-KO males significantly improved their impaired glucose tolerance and normalized their glucose levels to those of HFD-fed WT males (Figure 2, N and O). None of the groups displayed changes in BW or body composition in response to AAV treatment (data not shown). The rescue of glucose intolerance by AAV-mediated Dusp8 overexpression in the MBH points toward a central hypothalamic role of Dusp8 in regulating glucose homeostasis in an obesogenic environment.

**Impaired hypothalamic insulin sensitivity, elevated glucocorticoid signaling, and reduced sympathetic responsiveness in HFD-fed Dusp8-KO males.** The impaired glucose tolerance in Dusp8-KO males was reflected by aberrant expression levels of key enzymes...
involved in glucose uptake, glycolysis, glycogen synthesis, and glycogen breakdown in liver and skeletal muscle of HFD-fed Dusp8-KO mice, compared with WT controls (Figure 3, A and B). In an acute insulin challenge test, we moreover observed reduced hypothalamic Akt phosphorylation in HFD-fed Dusp8-KO males relative to WT controls, which indicates impaired hypothalamic insulin responsiveness (Figure 3, C and D). In the hypothalamus, we further observed increased expression levels of arginine vasopressin (Avp), oxytocin (Oxt), and Crh. Elevated Crh mRNA levels in the hypothalamus were reflected by increased adrenocorticotropic hormone (Acth) and CRH receptor 1 (Crhr1) expression in the pituitary as well as steroid synthesis enzymes in the adrenals (Figure 3E). Expression levels of glucocorticoid signaling components in liver, muscle, and hypothalamus were also
increased (Figure 3, F–H), thus indicating elevated glucocorticoid action in HFD-fed Dusp8-KO males.

Increased glucocorticoid action in humans has recently been associated with an impaired sympathetic outflow (12) and lower norepinephrine (NE) levels (13), thereby impeding systemic glucose control to systemically drive glucose intolerance (14). Consistent with these human data, we observed lower baseline NE levels in livers of Dusp8-KO males compared with WT mice (Figure 3I; 0 hours). In contrast, NE baseline levels were unaltered in soleus muscle and pancreas of Dusp8-KO mice (Figure 3, J and K; 0 hours).
However, the application of a single dose of the tyrosine hydroxylase inhibitor α-MPT (15) revealed decreased NE turnover rates in liver, muscle, and pancreas of Dusp8-KO mice (Figure 3, L–N). In contrast to the HFD-fed Dusp8-KO males, female Dusp8-KO mice did not display deregulated glucose metabolism or elevated glucocorticoid action when exposed to HFD (Supplemental Figure 4), which is in agreement with their unaffected glucose tolerance and insulin sensitivity. Overall, the data point toward blunted sympathetic responsiveness as a consequence of increased glucocorticoid action in HFD-fed male Dusp8-KO mice.

Blunted HPA axis feedback control in Dusp8-deficient male mice. Consistent with the elevated expression of glucocorticoid receptor (GR) target genes, we found increased basal corticosterone levels measured 2 hours after the onset of the light phase (9 am) in HFD-fed Dusp8-KO males compared with WT controls (Figure 4A). Increased glycogen storage in livers of HFD-fed Dusp8-KO mice (Figure 4B) and our recent finding of decreased hippocampal mass in Dusp8-KO males (16) were further indicators for chronic hypercorticosteronemia driven by Dusp8 deficiency. The absence of a marked adrenal hypertrophy in HFD-fed Dusp8-KO mice compared with WT controls (Figure 4C) suggested a dysfunctional HPA axis feedback at the level of the hypothalamus or the pituitary as cause for the observed increased basal corticosterone levels. A combined dexamethasone suppression/CRH stimulation test (Dex/CRH) (17) revealed diminished suppression of corticosterone production after low-dose dexamethasone administration in HFD-fed Dusp8-KO mice (Figure 4D). Peripheral injection of CRH induced a robust increase in corticosterone concentrations, which was significantly more pronounced in Dusp8-KO mice compared with WT controls. Taken together, these data indicate compromised GR-mediated negative feedback at the level of the hypothalamus and at the pituitary, possibly due to a hypothalamic CRH overdrive.

Chemical adrenalectomy normalizes glucose tolerance in HFD-fed Dusp8-KO mice. To determine whether hypercorticosteronemia is causally driving the observed glucose intolerance in HFD-fed Dusp8-KO mice, we blocked endogenous corticosterone production by metyrapone treatment (18). Following this widely used method of chemical adrenalectomy, we found a normalization of the glucose intolerance phenotype, with comparable glucose excursions and AUC levels for male WT and Dusp8-KO mice after 14 days of metyrapone treatment (Figure 4, E and F). Metyrapone treatment was further sufficient to normalize gene expression of key genes involved in T2D pathophysiology and multiple glucocorticoid target genes (Supplemental Figure 5, A–G). Prior to metyrapone treatment, we confirmed glucose intolerance in our cohort of HFD-fed Dusp8-KO mice compared with WT controls (Supplemental Figure 5, H and I). We furthermore found no effect of metyrapone treatment on BW (Supplemental Figure 5J). The normalization of glucose tolerance and peripheral glucoregulatory gene profiles by metyrapone treatment suggests that the elevated basal cortisol concentrations are causal for the dysregulation of glucose homeostasis in Dusp8-KO mice.

Hypercorticosteronemia and glucose intolerance in CRH neuron-specific Dusp8-KO mice. Given the increased hypothalamic expression of CRH and the blunted HPA axis feedback control indicating CRH overdrive from the hypothalamus in HFD-fed global Dusp8-KO mice, we next investigated whether the conditional ablation of Dusp8 in CRH-producing neurons (Figure 4G) can recapitulate the glucose intolerance and hypercorticosteronemia phenotype of the global Dusp8-KO mice. Male Dusp8CRH-Cre WT and KO mice had similar BW and body composition after exposure to HFD for 12 weeks (Figure 4, H and I). As hypothesized, ablation of Dusp8 specifically in CRH neurons indeed impaired glucose tolerance (Figure 4, J and K) and insulin sensitivity (Figure 4, L and M) in HFD-fed mice. Plasma insulin levels (WT, 267.7 ± 85.5 μU/mL; Dusp8-KO, 325.6 ± 56.0 μU/mL; *p = 0.0277) and HOMA-IR (Figure 4N) indices were significantly higher in male Dusp8CRH-Cre KO mice on HFD compared with their WT counterparts. Further, we found increased basal corticosterone levels in Dusp8CRH-Cre KO mice compared with WT controls (Figure 4O).

Given that the impairments in glucose tolerance and insulin sensitivity appeared milder in the Dusp8CRH-Cre KO mice compared with global Dusp8-KO mice, we hypothesized a glucoregulatory role of Dusp8 also in other neuronal subpopulations in the hypothalamus. We thus generated mice with a conditional ablation of Dusp8 in Agouti-related peptide (AgRP) or pro-opiomelanocortin (POMC) neurons. Both hypothalamic neuronal populations are essential for metabolic homeostasis (19), and interconnections of AgRP neurons with CRH neurons are moreover linked with stress regulation and energy homeostasis (20). However, we did not reveal any alterations in the susceptibilities to diet-induced weight or fat mass gain, glucose or insulin tolerance, or corticosterone levels between HFD-fed male Dusp8AgRP-Cre WT versus KO males (Supplemental Figure 6, A–G) or Dusp8POMC-Cre WT versus KO males (Supplemental Figure 6, H–N). Overall, these results suggest a prominent role of Dusp8 in CRH neurons in the regulation of HPA axis reactivity, plasma corticosterone levels, and glucose homeostasis.

Dusp8 is a gatekeeper for Jnk activity and Jnk-dependent regulation of GR activity. To identify the signaling pathway mediating the impaired glucose tolerance and HPA axis feedback inhibition in our Dusp8 loss-of-function mice, we initially assessed the phosphatase activity of Dusp8 toward the MAPKs Jnk, p38, and ERK using an in vitro model of Dusp8 overexpression. Exposure of cells to the general MAPK activator and ER stressor anisomycin showed that overexpression of Dusp8 abolished the phosphorylation of Jnk and partially that of p38 (Figure 5, A-D). Notably and despite recent evidence suggesting a role for Dusp8 in ventricular remodeling via altering ERK1/2 activity (21), we found unaltered ERK phosphorylation in HEK293 cells stimulated with anisomycin (Figure 5, A and E). Overall, these data indicate that Dusp8 shows highest phosphatase activity toward Jnk, weaker activity toward p38, and no activity toward ERK in cells stimulated by anisomycin.

In the past, Jnk hyperactivation was shown to impair GR activity via phosphorylation at the inhibitory-site Ser226 (22). First, we corroborated that anisomycin treatment increases Jnk activity as well as GR phosphorylation at the inhibitory Ser226 residue (Figure 5F). We then found that overexpression of Dusp8 abolishes both Jnk activation as well as GRSer226 phosphorylation (Figure 5, F and G). In a GR luciferase reporter assay, we further revealed functional relevance for the protective role of Dusp8 in Jnk-driven GRSer226 phosphorylation. Overexpression of Dusp8 had no effect on basal, dexamethasone-induced stimulation of GR.
Figure 4. Chronic hypercorticosteronemia drives impaired glucose intolerance in HFD-fed male Dusp8-KO mice in a CRH neuron–specific manner. (A) Plasma corticosterone levels in male mice sacrificed under basal conditions at 9 am (n = 11 WT, n = 12 Dusp8-KO; 17 weeks HFD). (B) Liver glycogen levels and (C) adrenal weight normalized to body weight were determined after exposure to HFD for 17 weeks (n = 15 WT, n = 14 Dusp8-KO). (D) Plasma corticosterone levels of HFD-fed Dusp8-KO (n = 11) and WT (n = 11) males that were subjected to a combined Dex/CRH test with pharmacological suppression of adrenal corticosteroid activity by dexamethasone (0.05 mg/kg BW) and a subsequent stimulation with CRH (0.15 mg/kg BW). (E and F) Glucose tolerance test (GTT) was carried out after 14 days of daily metyrapone injections (i.p., 100 mg/kg BW) in male, HFD-fed Dusp8-WT (n = 5) and -KO (n = 6) mice. (G) Schematic for the generation of conditional, CRH neuron–specific Dusp8-KO mice. (H) Body weight gain over 12 weeks, and (I) fat mass and lean mass of male Dusp8CRH-Cre WT (n = 11) and KO (n = 11) littermates after 12 weeks of HFD feeding. (J–M) GTTs and insulin tolerance tests (ITTs) were carried out after 12 weeks or 13 weeks of HFD exposure, respectively (n = 7 WT, n = 9 Dusp8CRH-Cre KO). (N) HOMA-IR of male Dusp8CRH-Cre WT (n = 11) and KO mice (n = 11) after 14 weeks of HFD exposure. (O) Plasma corticosterone levels of male Dusp8CRH-Cre WT (n = 14) and KO mice (n = 20) after 14 weeks of HFD exposure. Data are shown as box-and-whisker plots (A–D, F, I, K, and M–O) or as means ± SEM (E, H, J, and L). *P < 0.05; **P < 0.01 by Student’s t test (A–D, F, I, K, and M–O) or 2-way ANOVA (E, H, J, and L).
Figure 5. The Jnk-specific phosphatase Dusp8 ameliorates the inhibitory effect of Jnk signaling on glucocorticoid action. (A) Representative Western blot of an acute stimulation with the MAPK activator anisomycin (30 minutes) in HEK293 cells with Dusp8 overexpression (OE, confirmed by presence of Myc), compared with pCMV control vector. Densitometric analysis of anisomycin-induced phosphorylation of (B) Jnk. (C) c-Jun. (D) p38, and (E) ERK relative to β-actin (n = 3). (F) Representative Western blot and (G) densitometric analysis of HEK293 cells with hGR OE and/or Dusp8 OE that were stimulated with and without anisomycin (30 minutes) and then analyzed for phosphorylated GR at Ser226 relative to total GR (n = 3). (H) GR luciferase reporter assay activity in HEK293 cells overexpressing Dusp8 or an empty control plasmid stimulated with dexamethasone (5 hours, n = 3 in biological triplicates). EtOH, ethanol. (I) Dexamethasone-induced GR luciferase reporter assay activity in HEK293 cells with Dusp8 OE that were pretreated with anisomycin (overnight, n = 3 in biological triplicates). (J) Western blot of hypothalami of HFD-fed (16 weeks) male Dusp8-KO and WT mice (n = 4 each). Genotypes were confirmed by PCR followed by agarose gel electrophoresis (WT = 370 bp, KO = 430 bp). (K–P) Densitometric analysis of phosphorylated Jnk, c-Jun, p38, and ERK as well as total protein levels of Jnk and c-Jun relative to GAPDH (n = 4 WT, n = 4 KO; 16 weeks HFD). (Q) Western blot and (R) densitometric analysis of phosphorylated GR at Ser226 relative to total GR in hypothalami of male Dusp8-KO (n = 4) and WT mice (n = 4). Data are shown as scatter dot plots. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 by 1-way ANOVA (B–E and G) or Student’s t test (H, I, K–P, and R).

Activity (Figure 5H). Addition of the stressor anisomycin dramatically repressed the transcriptional activity of GR (Figure 5I), but the Jnk-mediated downregulation of GR activity was ameliorated by Dusp8 overexpression (Figure 5I). These data indicate that a reduction in Jnk activity by Dusp8 signaling can augment GR-dependent transcription.

Next, we assessed the effect of Dusp8 ablation on MAPK phosphorylation in our Dusp8-KO mouse model (Figure 5, F–P). Due to the unavailability of a specific antibody against Dusp8 (Supplemental Figure 7), genotypes were confirmed by PCR analysis. Phosphorylation of the Jnk downstream target c-Jun was upregulated in the hypothalamus of HFD-fed Dusp8-KO males compared with WT controls (Figure 5, J and L) along with elevated total protein levels of Jnk and c-Jun (Figure 5, J, O, and P). In muscle of HFD-fed mice the phosphorylation of MAPK was barely detectable (Supplemental Figure 8, A and B). In livers of Dusp8-KO males we found increased phosphorylation of Jnk and c-Jun (Supplemental Figure 8, C and D), whereas no differences were detected for epididymal white adipose tissue (eWAT) (Supplemental Figure 8, E and F). In line with the in vitro results in cells, we found a trend toward increased GR phosphorylation at the inhibitory Ser226 residue in the hypothalamus of Dusp8-KO mice (Figure 5, Q and R). GR Ser226 phosphorylation was significantly increased in muscle, but decreased in liver and unaltered in eWAT of Dusp8-KO mice (Supplemental Figure 8, G–L). Hypothalamic phospho-proteome analyses revealed an overall increase in the number of phosphorylated peptides in Dusp8-KO males compared with WT controls (Supplemental Figure 8M), but due to a relatively low abundance we could not detect any significantly deregulated MAPK phosphorylation sites in the hypothalamus of Dusp8-KO males (Supplemental Figure 8N and Supplemental Table 1). Combined, these results confirm substrate preference of Dusp8 for Jnk, but also indicate a putative crosstalk between MAPK. Overall, our data suggest an important protective role of Dusp8 as gatekeeper for hypothalamic Jnk signaling, the Jnk-dependent negative feedback inhibition of hypothalamic GR signaling, and glucocorticoid action.

Jnk1 codeletion normalizes glucose tolerance and hypercorticosteronemia in HFD-fed Dusp8-KO mice. Hypothalamic phosphatase activity of Dusp8 toward Jnk was especially high in the arcuate nucleus (ARC), a major hypothalamic center governing energy and glucose homeostasis, measured by increased phosphorylation of c-Jun in HFD-fed mice with global ablation of Dusp8 compared with WT controls (Figure 6, A–C). The number of nuclei with phosphorylated c-Jun was also increased in the ARC of HFD-fed male Dusp8 KO mice compared with WT controls (Supplemental Figure 9). Microarray analyses of laser-capture-microdissected ARC from HFD-fed global Dusp8 WT and KO mice confirmed alterations in MAPK and GR signaling in Dusp8-KO males relative to WT controls (Figure 6, D and E, and Supplemental Table 2). We further observed an enrichment of deregulated genes involved in neuroinflammation, insulin action, and AMPK, LXR/RXR, PI3K, PKA, and STAT3 signaling. Single-cell expression data suggested a mostly neuronal role of Dusp8 (23), which was in line with the absence of microgliosis or astrocytosis in global Dusp8-KO males on HFD (Supplemental Figure 10).

To corroborate that glucose intolerance of Dusp8-KO males is driven by altered Jnk activity, we generated global Jnk1-Dusp8 double-knockout (dKO) mice. When fed chow diet, WT, Dusp8-KO, Jnk1-KO, and Jnk1-Dusp8-dKO mice displayed comparable BWs and body composition, as well as unchanged glucose tolerance and insulin sensitivity in all genotypes (Supplemental Figure 11). Upon exposure to HFD, Jnk1-KO mice were protected from DIO (Figure 6, F–H), as published previously (24). BWs and body composition of Jnk1-Dusp8-dKO mice on HFD were unaltered compared with Jnk1-KO mice but showed a tendency to be reduced when compared with Dusp8-KO mice and WT controls (Figure 6, F and G). Consistent with the high hypothalamic Jnk activity in Dusp8-KO males on HFD, the hypothalamic expression of inflammatory markers Emr1, Ikkb, Il1b, and Nfkbia was increased in Dusp8-KO males on HFD but reversed when Jnk1 was codeleted in Dusp8-KO mice (Figure 6I). In line with the known role of hypothalamic Jnk as regulator of systemic glucose homeostasis (25), codeletion of Jnk1 in Dusp8-KO mice on HFD improved glucose tolerance (Figure 6, J and K) and insulin sensitivity (Figure 6, L and M) compared with Dusp8-KO mice. The improvements in glucose homeostasis in Jnk1-Dusp8-dKO mice were reflected by improved fasting insulin (Figure 6N) and HOMA-IR values (Figure 6O) relative to Dusp8-KO mice. We further revealed that the increased Jnk activity in the global Dusp8-KO mice was causally driving the observed hypercorticosteronemia, as Jnk1-Dusp8-dKO mice on HFD showed normalized plasma corticosterone levels (Figure 6P).

Finally, as p38 MAPK is another potential target of Dusp8 (11), we coinserted a dominant-negative p38 allele into global Dusp8-KO mice, which led to hypomorphic p38 activity (26), but had no effect on impaired glucose tolerance in HFD-fed Dusp8-KO mice (Supplemental Figure 12). Overall, our results demonstrate that codeletion of Jnk1 in Dusp8-KO mice reverses the hypothalamic inflammation and impaired glucose tolerance and insulin sensitivity observed in HFD-fed Dusp8-KO mice. This finding is con-
sistent with several reports showing that decreasing Jnk activity sensitizes insulin metabolic actions (24, 25, 27, 28).

Hypothalamic insulin resistance in humans carrying the DUSP8 diabetes-risk allele. After revealing a regulatory role of Dusp8 in the control of glucose homeostasis in mice, we next aimed to translate our findings to humans by first assessing the hypothalamic DUSP8 expression in human brain tissue collected by the Netherlands Brain Bank (29). In the infundibular nucleus of T2D patients the expression of DUSP8 was significantly increased compared with non-diabetic control subjects (Figure 7A).

Next, we assessed the hypothalamic insulin sensitivity of human carriers of the minor T allele in the intergenic T2D risk variant rs2334499. Built on our recent work that established a reduced cerebral blood flow (CBF) after nasal insulin as a marker of high hypothalamic insulin sensitivity (30), we used MRI to quantify CBF in the hypothalamus (Figure 7B) before and 15 minutes after application of intranasal insulin in 47 volunteers (Supplemental Table 3). The insulin-induced change in hypothalamic blood flow was significantly associated with the DUSP8 SNP rs2334499, with a stronger insulin-dependent suppression of CBF in major C allele carriers (C/C) (P = 0.0345, adjusted for sex; P = 0.0334, adjusted for sex, age, BMI) shown by the lower values of change in hypothalamic CBF levels compared with heterozygous (C/T) or minor T allele carriers (T/T) (Figure 7C). Stratification by sex then revealed that this association between impaired hypothalamic insulin sensitivity and the DUSP8 variant was driven by a reduced insulin-induced suppression of hypothalamic CBF levels in men (Figure 7D; P = 0.0131; P = 0.0054, adjusted for age, BMI) but not women (Figure 7E; P = 0.9006; P = 0.9737, adjusted for age, BMI) carrying the DUSP8 SNP rs2334499 minor allele (T/T).

CBF was further assessed in the hippocampus (Figure 7F) as a second insulin-sensitive and glucoregulatory brain region (31). We chose the hippocampus because of our recent work that revealed a smaller hippocampal size in Dusp8-KO mice, and reduced volumes of the hippocampal subregions substriculum and CA4 in humans carrying the risk variant rs2334499:C>T (16). We did not observe any differences in hippocampal CBF in response to intranasal insulin between the major, heterozygous or minor allele carriers regardless of their sex (Figure 7, G–I). Finally, whole-body insulin sensitivity was assessed by an oral glucose tolerance test (OGTT), but no correlation of the OGTT-derived insulin sensitivity index with the DUSP8 SNP rs2334499 in these volunteers was found (P = 0.7, adjusted for sex; P = 0.9, adjusted for sex, age, BMI) (Supplemental Table 3). Similarly, plasma cortisol levels were assessed but no difference between rs2334499 genotypes regardless of sex was found (Supplemental Table 3). Taken together, these findings from murine and human studies indicate that Dusp8 plays a role specifically in hypothalamic insulin sensitivity.

Discussion
We here describe a functional role for the GWAS target DUSP8 in the hypothalamic regulation of glucose homeostasis. DUSP8 was identified as a potentially novel T2D risk gene in GWAS (9) and meta-analyses coalescing GWAS cohorts (32). In a small but well-characterized cohort, we were able to show that homozygous male carriers of the minor frequency risk allele for DUSP8 have impaired hypothalamic insulin sensitivity. We further showed increased DUSP8 mRNA levels in the infundibular nucleus of individuals with T2D. Murine studies further revealed systemic glucose intolerance and insulin resistance in male Dusp8 loss-of-function models exposed to HFD. Mechanistically, loss of the phosphatase Dusp8 was linked with Jnk hyperactivation in the MBH, leading to diminished GR activity, an impairment of HPA axis feedback, and aggravation of systemic glucose tolerance due to basal hypercorticosteronemia driven by CRH neurons. Overall, our findings demonstrate a regulatory role of Dusp8 in hypothalamic inflammation, HPA axis reactivity, and insulin sensitivity.

Collectively, our experiments revealed sex-specific effects on insulin sensitivity in mice and humans that were only present in male but not female subjects. This is in line with a previous report (9) where the association of the analyzed DUSP8 SNP rs2334499 with T2D was also limited to male subjects. Reasons for the sex specificity remain elusive, but the absence of effects on glucocorticoid action or hypothalamic inflammation in female Dusp8-KO mice may entail direct effects of sex hormones such as estradiol (33) on HPA axis circuitry or peripheral glucoregulatory organs. Overall, our findings resonate with the emerging consensus that sex as a biological variable has to be a special focus in murine models and human studies. Another sex-related but entirely different phenomenon is the observation of the initial GWAS showing that the T2D risk associated with rs2334499 was only inherited with the paternal allele (8). Such parent-of-origin information for the alleles was unfortunately not available in our cohort of volunteers. However, not including the family data in our analyses most likely led us to underestimate the effect sizes of the detected association, as we pooled the paternally inherited allele carrying the increased diabetes risk with the maternally inherited allele. The DUSP8 SNP rs2334499 itself falls within close proximity of imprinted genes, but the region harboring it was reported to not be imprinted in human tissues or in mice, which was confirmed by clear biallelic expression for DUSP8 in humans (8). Consistent with this, we did not observe a phenotype in HFD-fed heterozygous Dusp8-
KO males regardless of whether the mutated allele was inherited from the sire or dam.

Hypothalamic inflammation has been linked to impaired glucose metabolism and insulin sensitivity in obesity experimental models (34, 35, 36) and in humans (37). Acute hypothalamic inflammation is induced by long-chain saturated fatty acids that accumulate in the hypothalamus after crossing the blood-brain barrier (34), where they activate pathways such as IKKβ/NF-κB (38) to ultimately enhance the expression of proinflammatory genes in the hypothalamus (28, 34), as observed in our Dusp8-KO model. Our finding of exacerbated hypothalamic Jnk activation, hypothalamic inflammation, and an impairment of systemic glucose homeostasis in HFD-fed Dusp8-KO mice is moreover consistent with earlier reports that linked HFD-induced activation of Jnk signaling with both hypothalamic inflammation (28) and impaired glucose metabolism (25). Our hypothesis of a Jnk-driven phenotype is further supported by a recent report on the restoration of hypothalamic insulin action after the pharmacological inhibition of Jnk (39).

Mechanistically, our data suggest that hypothalamic Jnk hyperactivation due to Dusp8 ablation causes an impairment of GR activity (22) and aberrant negative HPA axis feedback, which ultimately drives excessive glucocorticoid secretion. The latter is a well-known risk factor for T2D (14, 40) that perturbs hepatic gluconeogenesis and glycogen storage as well as glucose uptake in skeletal muscle and WAT (41). We observed impaired glucose homeostasis due to elevated basal corticosterone levels and aberrant hepatic expression profiles of glucoregulatory enzymes in global HFD-fed Dusp8-KO males. Moreover, we found hyperactivation of Jnk signaling in the ARC of both global Dusp8-KO and Dusp8cre+/- KO male mice. This finding appears paradoxical, as it suggests that a Jnk-mediated impairment of the negative HPA axis feedback control in Dusp8-KO mice involves CRH neurons located (with their cell bodies) in the paraventricular nucleus (PVN), but originates in the ARC. Our data are nonetheless consistent with an earlier report that assessed the GR-mediated feedback inhibition of corticosterone on CRH release from PVN neurons (42). Specifically, when a GR antagonist was injected into the PVN where the CRH neurons were located, unperturbed feedback inhibition on adrenal corticosterone production was observed. In contrast, injection of the GR antagonist into the ARC disrupted the negative feedback, indicated by persistently increased circulating corticosterone levels (42). At present, we can only speculate on the nature of this effect. Multiple studies have established dense communication between both areas, and ARC-specific AgRP neurons were shown to require hormonal input from CRH neurons (via CRFR1 receptor activation) to adapt to environmental challenges (20). Ablation of Dusp8 from AgRP neurons was, however, without effect on energy and glucose metabolism. Alternatively, Dusp8-positive CRH neuronal processes located within the ARC and close to the median eminence may be susceptible to the effects of circulating corticosterone, a process perturbed locally in the ARC by hypothalamic inflammation. The phenomenon, i.e., impaired negative feedback of corticosterone on CRH release that is driven by the ARC, remains unresolved and is a limitation of our study that warrants future investigation.

Impaired HPA axis feedback inhibition in Dusp8-KO mice subjected to a Dex/CRH test, and the rescue of glucose intolerance
in Dusp8-KO mice subjected to AAV-mediated Dusp8 overexpression in the MBH, nonetheless corroborates an important role for hypothalamic Dusp8 as a regulator of Jnk1-driven feedback inhibition of the HPA axis. Our finding of reduced sympathetic outflow in HFD-fed Dusp8-KO mice was further in accordance with studies in humans showing glucocorticoid-induced sympathoinhibition (12, 13). Similarly, when patients with metabolic syndrome were stratified for insulin resistance and challenged with a glucose bolus, they showed blunted sympathetic activity and reduced NE clearance from plasma compared with insulin-sensitive patients with metabolic syndrome (43). Taken together, these data indicate that impaired glucocorticoid feedback in the hypothalamus and the reduction in sympathetic nervous system tone contribute to the insulin resistance observed in our Dusp8-KO model.

In line with the central role for Jnk1 in the regulation of glucose metabolism (24, 25), codeletion of Jnk1 normalized glucose tolerance and hypercorticosteronemia in HFD-fed Dusp8-KO mice. However, significant MAPK crosstalk (2) and Dusp8 phosphatase activity toward other Jnk isoforms (44) or p38 (11) have been reported. Accordingly, our data cannot fully exclude that MAPKs other than Jnk1 play a role in the etiology of the glucose intolerance phenotype of HFD-fed Dusp8-KO males. Similarly, our findings do not exclude a role for Dusp8 in hypothalamic subpopulations distinct from CRH neurons, in other brain areas, or in peripheral tissues. Moreover, the ablation of Dusp8 may exert distinct effects in each of these subpopulations, areas, or tissues, and future studies are thus warranted to address these current limitations. Based on our data on Dusp8-KO, p38AF dominant negative (DN) double mutants, we can already exclude a potential role for p38. Similarly, our data do not point toward ERK as a potential mediator of impaired glucose metabolism in HFD-fed Dusp8-KO males, as hypothalamic ERK was shown to be prominently involved in the control of food intake, BW, and thermogenic sympathetic outflow, but not in the regulation of glucose homeostasis (45). Collectively, our data support a model whereby hypothalamic Dusp8 becomes activated upon DIO to control and change the magnitude of hypothalamic Jnk activity. Dusp8 thus serves as gatekeeper against the deleterious effects that chronically increased Jnk signaling has on the regulation of energy and glucose homeostasis (2, 24).

Hypothalamic insulin resistance was present in HFD-fed Dusp8-KO mice and human carriers of the DUSP8 T2D risk allele. Stimulating brain insulin action by intranasal insulin improves whole-body insulin sensitivity in healthy human men, but not in obese patients with hypothalamic insulin resistance (46, 47). We have previously linked hypothalamic insulin responsiveness with pancreatic insulin secretion (48) and revealed an elevated second-phase insulin secretion in humans upon hypothalamic insulin action (49). Similarly, we showed that hypothalamic insulin action was linked with suppressed endogenous glucose production and elevated glucose uptake (47) as well as alterations in parasympathetic output (50). Studies in young men further linked intranasal insulin administration with attenuated HPA axis activity (51), but molecular underpinnings for this central action of insulin remained unresolved (52). Future studies are thus warranted to clarify the impact of central insulin, and rs2334499 polymorphisms, on the HPA axis of human subjects with or without T2D.

Overall, our results indicate a multisystemic process whereby hypothalamic insulin resistance, combined with deregulated HPA axis reactivity, autonomous nervous system activity, and corticosterone release, exacerbates the detrimental effects of chronic HFD feeding on systemic glucose homeostasis in male Dusp8-deficient mice via increased hypothalamic Jnk activity. Importantly, these effects appear to translate into the human situation, where individuals with T2D had increased DUSP8 expression in the infundibular nucleus and male carriers of T2D risk variant rs2334499 displayed impaired hypothalamic insulin sensitivity. Replication of our human finding would be central, but our findings already extend previous GWAS that suggest Dusp8 as a T2D risk variant. In sum, we reveal that hypothalamic Dusp8 is a crucial gatekeeper for the Jnk-dependent control of systemic glucose homeostasis.

Methods

Animals. C57BL/6J mice were obtained from Janvier and Lepob (stock 000632), Jnk1-KO (stock 004319), and p38AF DN (stock 012736) mice were obtained from The Jackson Laboratory. Dusp8-KO mice were derived from breeding of Dusp8 heterozygous mice (21) with a pure C57BL/6J background. Jnk1-Dusp8-dKO mice were derived from breedings of Dusp8 heterozygous mice with Jnk1 heterozygous mice that had been crossed back to the C57BL/6J background. Dusp8-KO, p38AF DN mice were generated by breeding mice heterozygous for the DN allele of p38 (p38afs) with Dusp8 heterozygous mice.

The Dusp8 conditional KO line was generated from the Dusp8fl/fl (European Mammalian Mutant Cell Repository) as detailed in the Supplemental Methods. Conditional Dusp8 (Dusp8afs) mice were crossed to CRH-Cre (stock 012704), AgRP-Cre (stock 012899), or POMC-Cre (stock 005965) mice (all Jackson Laboratory).

Mice at an age of 8 to 10 weeks were either maintained on chow (5.6% fat, LM-485, Harlan Teklad or Altromin 1314) or switched to HFD (45% kcal fat; Research Diets Inc.) for up to 30 weeks. All WT and KO mice used in our studies were littermates. Mice were group housed on a 12-hour light/dark cycle at 22°C with free access to food and water, unless indicated otherwise. Mice were distributed into experimental groups based on their BWs to assure an equal distribution of BWs at the beginning of the study. In vivo experiments were performed without blinding of the investigators. All studies were based on power analyses to assure adequate sample sizes.

Body composition analysis. Fat mass and lean mass were measured via nuclear magnetic resonance technology (EchoMRI).

GTT, ITT, and PTT. For the GTT and ITT, mice were subjected to 6 hours of fasting 1 hour after the onset of the light phase. For the PTT, mice were fasted overnight for 16 hours. Subsequently, HFD-fed mice were i.p. injected with 1.5 g glucose/kg BW for the GTT, 0.75 or 1.0 U insulin/kg BW (0.09 U/ml; Humalog Pen, Eli Lilly) for the ITT, and 0.75 g pyruvate/kg BW for the PTT. Chow-fed mice received 2.0 g glucose/kg BW for the GTT and 0.75 U insulin/kg BW for the ITT. Tail blood glucose levels (mg/dL) were measured with a handheld glucometer (TheraSense Freestyle) before (0 minutes) and at 15, 30, 60, and 120 minutes after injection. For the glucose-stimulated insulin secretion test (GSIST), 1.5 mg glucose/kg BW was injected intravenously in 6-hour-fasted HFD-fed mice. Tail blood for insulin measurements was collected at 0, 1, 5, 10, and 60 minutes after injection.
AAV infusion in mice. Custom-made AAVs (serotype AAV5) with full-length murine Dusp8 cDNA under the control of the CMV promoter or an AAV with CMV-GFP as control (pAAV5-CMV-Dusp8 vs. pAAV5-CMV-EGFP; 2 × 10^10 viral genome particles/mL; Sirion Biotechnology) were injected bilaterally into the MBH of single-housed, DIO WT and Dusp8-KO littermates (6 months old) using a motorized stereotaxic system from Neurostar as detailed in the Supplemental Methods.

Acute insulin challenge. To assess biochemical responses to insulin stimulation, insulin was i.p. administered at a dose of 3 U/kg BW in age-matched DIO WT and Dusp8-KO littermates. Control mice received vehicle instead. Mice were sacrificed 8 minutes after insulin administration by cervical dislocation. Hypothalami were collected to measure signal transduction markers by Western blotting.

Catecholamine turnover rate determination. Catecholamine turnover was measured on the basis of the decline in tissue NE content after the inhibition of catecholamine biosynthesis with α-methyl-DL-tyrosine methyl ester hydrochloride (α-MPT, M3281, MilliporeSigma) as described previously (53) and in the Supplemental Methods.

Dex/CRH test. In the combined Dex/CRH test (17), the corticosterone secretion of Dusp8-KO and WT mice was monitored in response to a pharmacological suppression of adrenocortical activity with dexamethasone (0.05 mg/kg BW) and a subsequent stimulation with CRH (0.15 mg/kg BW) as detailed in the Supplemental Methods.

Metyrapone injections. Age-matched DIO WT and Dusp8-KO littermates received daily i.p. injections of 100 mg/kg BW metyrapone (catalog M2696, MilliPoreSigma) for 14 days that were shown to reduce endogenous corticosterone production (18).

Immunohistochemistry and immunofluorescence. Paraformaldehyde-fixed brains were processed for cryosectioning followed by immunohistochemical and immunofluorescent staining in the free-floating approach as detailed in the Supplemental Methods.

In situ hybridization. Coronal hypothalamic sections (16 μm) were cut on a cryostat and immediately stored at -80°C until hybridization. Sense and antisense probes were generated from the Dusp8 cDNA sequence using a digoxigenin (DIG) RNA labeling kit (Roche). The hybridization procedure is described in detail in the Supplemental Methods.

Blood chemistry. Blood was collected in EDTA-containing centrifuge tubes and centrifuged at 4°C and 2000g for 10 minutes. Plasma triglycerides, cholesterol, and nonesterified fatty acids were measured by commercial enzymatic assay kits (Wako Chemicals). Insulin and leptin were measured by ultrasensitive murine insulin and murine leptin ELISA kits (Merck Millipore). Plasma corticosterone levels were measured by radioimmunoassay (MP Biomedicals; sensitivity 6.25 ng/mL) or corticosterone ELISA kit (Arbor Assays). All samples were run in triplicate. Luciferase activity was measured using a microplate luminometer (PHERAstar, BMG Labtech) and the Dual-Glo Luciferase Assay System (Promega), according to the instructions from the manufacturers.

Protein extraction and Western blotting analysis. HEK293 cells and murine tissues were processed for protein extraction, concentration determination, and Western blot analysis as detailed in the Supplemental Methods.

Microarray analysis of laser-capture-microdissected ARC. Perfused and sucrose-equilibrated brains of global Dusp8-WT and -KO males were cut coronally followed by laser capture microdissection of the ARC. Subsequently, RNA was isolated from the ARC and gene expression was analyzed by microarray. Details are given in the Supplemental Methods.

RNA isolation and qPCR analysis. RNA was isolated from flash-frozen murine tissues and postmortem formalin-fixed, paraffin-embedded (FFPE) human brain tissue with commercially available kits and reverse transcribed into cDNA followed by gene expression analysis as detailed in the Supplemental Methods.

Human data. DUSP8 expression in the infundibular nucleus was assessed in postmortem hypothalamic tissues of 11 T2D and 12 non-T2D control subjects obtained from the Netherlands Brain Bank (29). Regional brain insulin sensitivity was assessed in a group of 47 healthy participants of the ongoing Tübingen Family (TUF) study (ref. 55 and Supplemental Table 3), using an established protocol that combines the measurement of CBF by functional MRI with the delivery of 160 U of insulin as nasal spray as described previously (30). Blood flow for the hypothalamic and hippocampal region of interest was extracted and associations with the DUSP8 SNP rs2334499 (major allele [C/C], heterozygous [C/T], minor allele [T/T]) were tested by multiple linear regression analyses with adjustment for age.

Data availability. The microarray data have been submitted to the Gene Expression Omnibus (GEO) database at the NCBI (GSE112688: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112688). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (56) partner repository with the data set identifier PXD019451. All other data generated or analyzed during this study are available within the paper and its supplementary information file. Antibodies for the detection of proteins are given in Supplemental Table 4, and Taqman probes and primer sequences for gene expression analyses are given in Supplemental Tables 5 and 6. Last, we provide images of uncut gels for all Western blot data in the Supplemental Materials.

Limitations of human data. A replication of our human finding of reduced CBF in rs2334499 minor allele carriers would be central. As the effect size for the association with hypothalamic insulin sensitivity is much larger than the effect size of the association with diabetes risk, a considerably smaller cohort than the initial discovery cohorts for dia-


