# PLOS Genetics

## Mutation of SLC35D3 Causes Metabolic Syndrome by Impairing Dopamine Signaling in Striatal D1 Neurons

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## Abstract:

Obesity is one of the largest health problems facing the world today. Although twin and family studies suggest about two-thirds of obesity is caused by genetic factors, only a small fraction of this variance has been unraveled. There are still large numbers of genes to be identified that cause variations in body fatness and the associated diseases encompassed in the metabolic syndrome (MetS). A locus near a sequence tagged site (STS) marker D6S1009 has been linked to obesity or body mass index (BMI). However, its genetic entity is unknown. D6S1009 is located in the intergenic region between SLC35D3 and NHEG1. Here we report that the ros mutant mice harboring a recessive mutation in the Slc35d3 gene show obesity and MetS and...
reduced membrane dopamine receptor D1 (D1R) with impaired dopamine signaling in striatal neurons. SLC35D3 is localized to both endoplasmic reticulum (ER) and early endosomes and interacts with D1R. In ros striatal D1 neurons, lack of SLC35D3 causes the accumulation of D1R on the ER to impair its ER exit. The MetS phenotype is reversible by the administration of D1R agonist to the ros mutant. In addition, we identified two mutations in the SLC35D3 gene in patients with MetS, which alter the subcellular localization of SLC35D3. Our results suggest that the SLC35D3 gene, close to the D6S1009 locus, is a candidate gene for MetS, which is involved in metabolic control in the central nervous system by regulating dopamine signaling.

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Following your recommendations in your decision letter regarding our manuscript # PGENETICS-D-13-02352 dated on November 5, 2013, I am resubmitting here a revision of our manuscript entitled “Mutation of SLC35D3 causes metabolic syndrome by impairing dopamine signaling in striatal neurons” by Zhang et al. I hope this revised version will now be acceptable for publication in PLoS Genetics. I confirm that the data in this manuscript is original and the manuscript is not under consideration elsewhere.

Following the suggestions from the reviewers, we have included details of some additional experiments and added two figures into the Supplementary materials of the revised manuscript. Listed below are the major aspects of the revised manuscript.

1) Supplementary Figure 1 (Fig. S1): We have included details of additional experiments to show the presence of SLC35D3 in the striatum of younger mice, which was a concern raised by Reviewer #2.

2) Supplementary Figure 2 (Fig. S2): As suggested by Reviewer #2, we have moved Figures 3A-3D to the Supplementary materials, as these figures explain the data analysis in the Methods. We combined Figures 3E-3G with Figure 2. Thus the previous numbering of Figures 4-8 is changed to Figures 3-7 in the revision.

3) Supplementary Figure 3 (Fig. S3): We added this figure to demonstrate no effect of SLC35D3 on striatal D2R distribution in the ros mutant, which was also a concern of Reviewer #2.

4) We have re-organized the structure of the manuscript and moved some parts of Materials and Methods into the Supplementary materials. The revised parts in the manuscript text are highlighted in BLUE.
Finally, we provide point-to-point replies to the comments of the reviewers #2 and #3 below. I sincerely hope that the above improvements satisfy you and the reviewers and that the revised manuscript will now be suitable for inclusion in a future issue of *PLoS Genetics*.

Thanks in advance for your reconsideration of this manuscript.

Sincerely,

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Reviewer #2:

Zhang et al show that the ros mutant mouse exhibits a mutation in a genomic region associated with Slc35d3 resulting in Slc35d3 abnormalities and adult onset obesity and signs of metabolic syndrome. This phenotype is characterized by normal feeding but decreased physical activity, decreased D1R signaling, and abnormalities in D1R trafficking, presumably mediated via interaction of D1R with Slc35d3. Not surprisingly, increasing D1R signaling was sufficient to augment physical activity and to thus rescue the adult-onset metabolic abnormalities in ros mutant mice. Human subjects with genetic abnormalities in the Slc35d3 locus exhibited moderate obesity and metabolic syndrome but this was with very low prevalence.

Major comments

If the ros mutation results in Slc35d3 deletion, and if Slc35d3 is directly involved in D1R trafficking, which underlies obesity and metabolic syndrome, then how does one explain the adult onset of obesity and metabolic abnormalities? If the Slc35d3 deletion is present to begin with in the ros mice, and if this mutation is causally linked to obesity and metabolic syndrome, then why does the latter occur in adulthood and not earlier? Have the authors showed that the associations they observe between D1R and Slc35d3 are not present in young mice, prior to obesity onset? If they are not, then how does one relate this mechanism to obesity and metabolic syndrome?
**Reply:** The referee raises a very interesting point. In Figure 1B, we have shown that the body weight gain starts from 2 months of age and proceeds progressively with age. This suggests that the effects of loss of SLC35D3 are most significant in older mice. We performed additional experiments and detected the presence of SLC35D3 in the striatum of younger mice (Supplementary Figure 1 (Fig. S1)). Such a late onset phenotype is not unusual. For example, late onset phenotypes have been described in other obese mouse models including mice with deletion of *SH2B1* (Rebuttal Ref. 1) or *FTO* overexpression (Rebuttal Ref. 2). Compared to the early onset of obesity in the *ob/ob* mice which is initiated at the age of weaning (21 days postnatally) (Rebuttal Ref. 3), the weight gain in *ros* mice with late onset obesity is still a mystery given that SLC35D3 was present after birth (Fig. S1). The *FTO* gene functions in a dose-dependent manner with copy number variation (Rebuttal Ref. 2). Whether the expression level of the *FTO* gene in the patients increases with age is unknown. In the *ros* striatum, the expression of *Slc35d3* at different ages did not appear to change (Fig. S1). On the other hand, many obese people, especially those with central obesity, only gain weight during adulthood, which may be associated with lifestyle risk factors (Rebuttal Ref. 4). The two patients with *SLC35D3* mutations were diagnosed with adult central obesity, which also supports the late onset obesity in humans. We have added an additional Figure S1 showing the presence of SLC35D3 in the striatum of young mice and discussed the above issue in the revised manuscript.
The discussion seems heavily focused on using D1R agonists for adult onset obesity treatment. The striatonigral pathway is critical for movement and is involved in initiating and maintaining locomotor activity. Since the ros mice exhibit no change in food intake, yet decreases in physical activity, it seems plausible that the adult-onset metabolic syndrome is primarily due to a decrease in physical activity brought upon by impaired functioning of striatonigral neurons. If so, why not have patients increase their physical activity rather than undergo treatment with D1R compounds which may have considerable side effects.

**Reply:** We agree with the reviewer's viewpoint. As we discussed in the last paragraph of our manuscript, we have suggested that increases in physical activity in the obese population may be a useful strategy. However, some obese patients may be reluctant or not motivated to increase physical activity or eat less to reduce caloric intake, particularly if a genetic problem is a component of their condition (Rebuttal Ref. 5). In these cases, taking pharmaceuticals to alleviate their obesity could be an option. In our manuscript, the administration of D1R agonist is mainly suggested for a proof-of-concept that obesity could be caused by impaired D1R signaling and the intervention of D1R signaling could be effective for weight loss. We agree this is likely to have additional side effects. We have revised the manuscript by eliminating the statements that emphasize the use of D1R agonists in the treatment of obesity.
It is difficult to understand why figures 3A-3D are included. These are not mentioned in the text at all and do not show comparisons between the wt and mutant mice. If anything they should be listed at supplementary information.

Reply: Figures 3A-3D were referred to in the Materials and Methods. These figures are for the method description without regard to genotypes. Following this reviewer’s suggestion, we have moved these figures into the Supplementary Figure 2 in the revised manuscript.

Page 13 line 203: "this supports its specific role in striatal D1R-mediated signaling for metabolic control". I don't think there is evidence here for a specific role of D1R in metabolic control. Rather, the specific role seems to be on physical activity, which over time can lead to impaired metabolism. Therefore this statement should be revised.

Reply: We added a Supplementary Figure 3 in the revised manuscript to demonstrate that total D2R and the fraction of D2R on the plasma membrane were unchanged in the striatum of ros mutant mice. We revised this statement as "This indicates that SLC35D3 plays a role in D1R trafficking in the striatal D1 neurons, but does not affect D2R trafficking in the striatal D2 neurons".

Page 13 line 206: This conclusion is not necessarily correct. cAMP function may not be
due to a reduction of functional D1R on the plasma membrane per se but certainly reduced D1R function.

**Reply:** We agree with the reviewer that the cAMP reduction is an indirect link to the reduction of functional D1R on the plasma. We revised this statement as “Taken together, our results suggest that loss of SLC35D3 in ros striatum causes intracellular accumulation of D1R and reduces D1 receptors on the plasma membrane”.

Page 16 line 270: It is difficult to follow the significance of the human findings. The allele frequency and incidence rate were very low (too low to be significant for adult-onset metabolic syndrome, which in developed countries is observed in over 50% of individuals). In fact, in the discussion, the authors compare the prevalence to that of LEPR mutations, which are extremely rare. Moreover, if the human findings were specific to the heterozygous state, why is this also significant if the effects in heterozygous ros mice are negligible (1.5g or 0.5% total body weight)?

**Reply:** We are not claiming that this mutation is the cause of all cases of metabolic syndrome (MetS). Clearly MetS is a problem with a complex genetic and environmental etiology to which this gene may contribute in a subset of patients. Even at this low rate it would still include millions of individuals. We made the comparison to LEPR only to emphasize that this is a much more common mutation with much greater significance. Moreover 1.5g is 5% of the body weight of a mouse not 0.5% which is not a negligible
effect. We agree that the prevalence of the \textit{SLC35D3} mutation in obese population requires more screening on a large population. We revised accordingly to avoid overemphasis on the human findings.

Page 17 line 274: the authors state that they used a D1R agonist "to elucidate whether the pathological phenotype in obese ros mice is reversible by the recovery of D1R on plasma membrane". This manipulation, namely pharmacological activation of D1 receptors, does not enable one to elucidate this as it does not enable recovery of D1R on plasma membrane.

\textbf{Reply:} We agree that there is no direct evidence to show the recovery of D1R on plasma membrane after the treatment of D1R agonist. We have revised the text accordingly.

Page 14: it is also unclear why the authors chose a non-specific way to augment D1R signaling. D1R are located in many other brain areas (and in the periphery) and therefore using a systemic D1R agonist is not ideal. Perhaps this was to see if this effect was promising as a treatment intervention but it is important to specifically manipulate striatal D1 signaling locally in striatum.

\textbf{Reply:} We agree with the reviewer’s viewpoint. A systemic D1R agonist may have other effects on the non-striatal D1R. A better solution to the rescue experiment would be to use a SLC35D3-transgene applied locally in the striatum. In our \textit{in vitro} assays, we knew
that SLC35D3 has a dose-dependent effect on D1R trafficking. Co-expression of SLC35D3 and D1R in HEK293T cells leads to D1R retention in the ER (unpublished data), suggesting overexpression of SLC35D3 in striatum may also affect the ER exit of D1R to the plasma membrane, which is similar to the overexpression of DRiP78, another D1R trafficking protein, in which the D1R membrane trafficking is impaired (Rebuttal Ref. 6). However, how to control the copy number of the SLC35D3-transgene in vivo is a technical challenge. Therefore, we chose the pharmacological model for the proof-of-concept that intervention of D1R signaling is effective in the ros mice for rescuing the phenotypes.

With the exception of the ITT and GTT, it is not clear if the blood measures were done after fasting or controlled feeding. It is imperative that food intake controlled in these situations as differences between animals may lead to variable results.

Reply: In the revised Materials and Methods section, we have described that all the blood measurements were done after overnight fasting except that insulin was measured on non-fasted mice. For the insulin tolerance test (ITT) and glucose tolerance test (GTT), fasting plasma glucose levels were measured after fasting for 16 hours. The method for food intake was described in the Materials and Methods section. We measured 14 mice in the control group and 15 ros mice to control the variance from individual animals. We revised the figure legends accordingly.
Minor comments

Abbreviations should be followed throughout the text and be spelled out at first use.

Reply: We thank the reviewer for very careful reading of our manuscript and pointing out the errors and typos. We have gone through the whole manuscript and added the full names to the abbreviations at first use.

Page 7 line 80, this statement is unclear and should be rephrased: "Obesity and associated MetS or body mass index (BMI, MIM 606641) are regarded as complex traits on which additive genetic effects and environment influence"

Reply: We have revised the text accordingly.

Page 7 line 81: the word "addictive" should be "additive"

Reply: It has been corrected.

Page 9 line 114: "who were associated"

Reply: We have revised the sentence as follows "We found two MetS patients with mutations of the SLC35D3 gene".

Page 9 line 124: mimicking" is a strong choice of word. this statement should be rephrased to be more cautionary as I don't think its known how close 8 weeks in male mice "mimics" adult obesity onset age in humans.
Reply: We have replaced “mimicking” by “similar to”, and “adult obesity” by “late onset obesity” in the revised manuscript.

Page 10 line 138: should be "glucose intolerant"

Reply: It has been corrected.

Page 11 line 159: this needs to be rephrased: "suggesting the impact of the mutation is on the total amount of activity as well as the energy costs of locomotion"

Reply: Text now revised

Page 12 line 154: "we" should be "We"

Reply: Now corrected

Page 12 line 176: should be "but no signal was detectable…"

Reply: This sentence is now eliminated in the revision

Page 12 line 184: "therefore, we investigated whether…"

Reply: Now revised.

Page 14 line 215: should be "co-residing”… "were significantly higher"

Reply: Now corrected.

Page 14 line 218: "may account"

Reply: Now revised.

Page 16 line 263: "dose-dependent" is not correct as a choice of words here

Reply: We have revised as “gene dosage effect”.

Page 18 line 302: "compared"
Reply: Now corrected.

The discussion is in need of serious editing.

Reply: A co-author of the manuscript from UK, Prof. John Speakman, has edited the discussion as requested.

Reviewer #3:

In this manuscript, Zhang and colleagues study a mouse model with disrupted SCL35D3 that results in impaired dopamine signalling and 'late onset' obesity. Based on this phenotype and the fact that SNPs in the vicinity have been associated with increased BMI in humans, the authors argue that it should be considered a new candidate gene in the study of the metabolic syndrome.

This is an interesting study and disruption of SCL35D3 in mice undoubtedly results in altered energy homeostasis, due in large part to a reduction in locomotor activity. What I am less convinced of is the potential link to the human condition. Here are a few issues for the authors to consider and address:

1) Over what period of time was food intake measured? I am curious if a difference (if it exists) could be better measured over long term measurements.
**Reply:** The data shown in the manuscript was from a measurement of 7 consecutive days as described in the Materials and Methods section. We performed these experiments using the standard housing cages and also independently in the metabolic cages.

2) Are the insulin and glucose measurements made on fasted mice? This is not mentioned in the manuscript, as far as I can tell.

**Reply:** We apologize for this omission. In the revised Materials and Methods section, we have described that all the blood measurements were done after overnight fasting except that insulin was measured on non-fasted mice. For the insulin tolerance test (ITT) and glucose tolerance test (GTT), fasting plasma glucose levels were measured after 16 hours fast.

3) At line 159, with regards to the reduced locomotor phenotype the authors say: ‘……suggesting the impact of the mutation is on not only the total amount of activity but also the energy costs of locomotion.’ Are the authors making a comment on the efficiency of the animals during locomotion? If so, where is the data to support such a statement?

**Reply:** In Figure 2C, the velocity of movement in *ros* mice is lower than that in WT mice (*P*<0.05). In the metabolic cage studies, we have shown that total energy expenditure is lower in *ros* mice (revised Fig. 2E), but there is no significant difference for the energy
expenditure at the resting state analyzed by either method 1 (revised Fig. 2F) or method 2 (revised Fig. 2G). This suggests that the lower energy expenditure in ros mice is from the movement state, which is in agreement with lower movement velocity (Fig. 2C) and duration (Fig. 2D).

4) I find figures 5B and 6E completely unintelligible, and cannot see any difference between the images from the WT or ROS mice. It may or may not be because I don't look at such figures on a daily basis, but are there better representative figures available?

**Reply:** In the revision, we changed the original Figure 5 to Figure 4, and the original Figure 6 to Figure 5. In revised Fig. 4B, the representative EM pictures are for the subcellular localization of D1R on both plasma membrane and endomembrane structures in each genotype without much difference in the pictures between genotypes. The difference in distribution is the quantification result shown in revised Fig. 4C. In revised Fig. 5E, the difference in ros mice is that the larger particle (SEC61B-labeled) colocalizes with the smaller particles (D1R-labeled), while in WT mice, they do not colocalize. We quantified the percentage of colocalized D1R particles in total D1R particles which is shown in the right-hand side. We revised the figure legends to include a more detailed description to hopefully avoid the potential confusion.

5) I accept that it is interesting that in a screen on a limited number of humans, that two
'loss of function' mutations are found in individuals with metabolic syndrome. It is interesting that both are not morbidly obese (BMI<28). However, due to the small numbers (~300 cases; ~ 200 controls), it is absolutely impossible to conclude a) frequency of mutations in cases or in the general population for that matter, and thus inappropriate to comment on the fact that healthy individuals do not have mutations in SCL35D3.

Reply: We agree that the frequency of SLC35D3 mutation in the obese population requires more data for a large screen. We have revised this part to avoid overemphasis on the human findings.

6) Have any SNPs near SCL35D3 been found in the multiple GWAS studies that have been performed on obesity and BMI?

Reply: We have not found in the literature that SLC35D3 appears in any of the GWAS studies except that the D6S1009 locus near the SLC35D3 gene was shown in whole genome linkage studies from the Framingham Heart Study.

Rebuttal References


Mutation of SLC35D3 Causes Metabolic Syndrome by Impairing Dopamine Signaling in Striatal D1 Neurons

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Running Title: SLC35D3 in D1R Trafficking and Obesity.

Keywords: SLC35D3; striatum; D1 dopamine receptor; obesity; metabolic syndrome

Author Contributions


Word count: 5199 (excluding title page, abstract, references and figure legends)

Figure numbers: 7 (color figures: 3)

Table numbers: 0

Supporting information files: 1
Abstract

Obesity is one of the largest health problems facing the world today. Although twin and family studies suggest about two-thirds of obesity is caused by genetic factors, only a small fraction of this variance has been unraveled. There are still large numbers of genes to be identified that cause variations in body fatness and the associated diseases encompassed in the metabolic syndrome (MetS). A locus near a sequence tagged site (STS) marker D6S1009 has been linked to obesity or body mass index (BMI). However, its genetic entity is unknown. D6S1009 is located in the intergenic region between SLC35D3 and NHEG1. Here we report that the ros mutant mice harboring a recessive mutation in the Slc35d3 gene show obesity and MetS and reduced membrane dopamine receptor D1 (D1R) with impaired dopamine signaling in striatal neurons. SLC35D3 is localized to both endoplasmic reticulum (ER) and early endosomes and interacts with D1R. In ros striatal D1 neurons, lack of SLC35D3 causes the accumulation of D1R on the ER to impair its ER exit. The MetS phenotype is reversible by the administration of D1R agonist to the ros mutant. In addition, we identified two mutations in the SLC35D3 gene in patients with MetS, which alter the subcellular localization of SLC35D3. Our results suggest that the SLC35D3 gene, close to the D6S1009 locus, is a candidate gene for MetS, which is involved in metabolic control in the central nervous system by regulating dopamine signaling.
Author Summary

Genome-wide linkage analyses have revealed that an STS marker D6S1009 (about 55 kb from the SLC35D3 gene) is linked to obesity or BMI in the Framingham Heart Study, but its genetic entity is unknown. Here we characterized the features of obesity and metabolic syndrome with reduced physical activity levels in a previously identified ros mouse mutant, carrying a homozygous Slc35d3 mutation. These ros phenotypes are caused by the intracellular accumulation of D1R mostly on ER in the striatal neurons, impairing D1R signaling and reducing energy expenditure. In addition, we identified two mutations of SLC35D3 in patients with metabolic syndrome which are subcellularly mislocalized. We propose that the SLC35D3 gene is likely a novel candidate gene for MetS and obesity.

Introduction

The worldwide prevalence of obesity (MIM 601665) is increasing (data from the International Obesity Taskforce (IOTF) website). This has resulted in a significant increase in morbidity and mortality associated with the metabolic syndrome (MetS, MIM 605552). Obesity and associated MetS or body mass index (BMI, MIM 606641) are regarded as complex traits influenced by both additive genetic effects and environmental factors [1]. It has been estimated that genetic factors explain 67% of the variance in human obesity [2]. Currently, more than 150 loci have been implicated in the development of monogenic obesity, syndromic obesity and polygenic obesity. However,
only about 2% of the variance in this trait has been explained [3,4]. About 200 cases of severe obesity have been reported to be associated with a single gene mutations in a cohort of 11 genes [5]. Studies on extremely obese children have been successful in the characterization of the causative genes for monogenic obesity. However, progress with this approach has been very slow, and is expected to be faster in the era of whole exome sequencing. On the other hand, the identification of the FTO gene as an obesity gene is an example of loci uncovered by genome-wide association or linkage studies [6]. It remains a challenge to uncover genes responsible for mild or moderate obese phenotypes, especially those which develop in adulthood.

Genome-wide linkage analyses have revealed that a locus on chromosome 6q23-25 is linked to obesity in the Framingham Heart Study, with a major locus near the sequence tagged site (STS) marker D6S1009 [7,8,9]. D6S1009 is located within the intergenic region between SLC35D3 (55,419 bp apart at the centromere side) and NHEG1 (867 bp apart at the telomere side) in the NCBI Map Viewer. NHEG1 (neuroblastoma highly expressed 1) is a predicted gene with unknown function. No association with obesity of this gene has been documented. SLC35D3 (solute carrier family 35, member D3) is predicted as an orphan nucleotide sugar transporter or a fringe connection-like protein with 10 transmembrane domains in the database. Previous studies have characterized the recessively inherited \textit{ros}-/- mutant mouse (\textit{ros} hereafter), which has a spontaneous intracisternal A particle (IAP) insertion at the first exon of the \textit{Slc35d3} gene to disrupt its function [10]. Platelet dense granules are absent in the \textit{ros} mutant, suggesting that
SLC35D3 is involved in the biogenesis of platelet dense granules [10,11]. This function seems not to do with the solute carrier, and requires further investigation. Mouse Slc35d3 is specifically expressed in the brain as determined by multiple tissue Northern blots [10], suggesting it has specific roles in the central nervous system. In addition, Slc35d3 expression was restricted to the striatonigral medium spiny neurons (MSNs) expressing dopamine receptor D1 (D1R) rather than the striatopallidal MSNs expressing dopamine receptor D2 (D2R) [12]. Interestingly, during the breeding of this mouse, we observed that the adult ros mice gained weight progressively. Here we have characterized the ros mutant as a mouse model of MetS and obesity. In addition, we found two MetS patients with mutations of the SLC35D3 gene. Our results suggest that SLC35D3 is a candidate gene for obesity-related MetS, which is involved in metabolic control in the central nervous system by regulating dopamine signaling.

**Results**

**Ros mutant exhibits metabolic syndrome and lowered energy expenditure**

During the breeding of the ros mutant mice, we observed that adult ros mice became obese compared to sex and age-matched wild-type (WT) mice (Fig. 1A). Growth curves showed progressive and significant weight gain of ros mice relative to WT controls, starting at 8 weeks in males, which is similar to the features of late onset obesity in humans. However, the expression levels of SLC35D3 in the striatum did not change at
different postnatal stages (Fig. S1). Based on this observation, we chose mice at 12 weeks of age for behavior and molecular tests, mice at 24 weeks of age for phenotypic analyses of MetS. At 24 weeks of age, *ros* males were 31.5% heavier than age-matched WT males (Fig. 1B), while the naso-to-anal body length was increased by 6.2% in *ros* mice relative to WT controls (Fig. 1C).

To determine whether the increased weight of *ros* mice reflects body composition changes, we dissected and weighed two distinct fat pads, epididymal and perirenal white adipose tissue (WAT). Both epididymal and perirenal fat mass were enormously increased in *ros* mice (Fig. 1D). Serum cholesterol and triglycerides levels were increased about 19% and 61% respectively compared with the WT controls (Figs. 1E and 1F). Blood glucose levels in *ros* mice were likewise increased about 43% (Fig. 1G). In addition, serum insulin was increased about 4.2-fold in *ros* mice (Fig. 1H), while the insulin tolerance test (ITT) and glucose tolerance test (GTT) showed *ros* mice were insulin resistant and glucose intolerant (Figs. 1I and 1J). Taken together, the *ros* mice exhibited multiple features of the MetS with late onset obesity, hyperlipidemia, hyperglycemia and hyperinsulinemia.

The development of obese *ros* mice may result from elevated energy intake and/or decreased energy expenditure. To assess whether *ros* mice were hyperphagic, daily food intake was monitored in animals fed a standard chow diet *ad libitum* for 7 consecutive days at the age of 24 weeks when *ros* mice were already obese. There was no significant difference in daily food intake between WT and *ros* mice (Fig. 2A). This suggests that
energy intake is unaffected in *ros* mice. On the other hand, *ros* mice had significantly decreased physical activity including decreased movement distance, average velocity and movement duration (Figs. 2B-D).

The decreased physical activity in *ros* mice prompted us to measure daily energy expenditure over 5 day periods using an Oxymax system (details in the Method and Fig. S2). Total energy expenditure (resting plus activity metabolism) in the *ros* mice at age 2-3 months, prior to development of obesity, was significantly lower than in the WT mice using analysis of covariance (ANCOVA) [13] (Fig. 2E). However, there was no difference between the genotypes in their resting metabolic rates, independent of how the resting metabolism was evaluated (Figs. 2F and 2G). This indicated that the difference in energy expenditure between the genotypes was contributed to only by the differences in physical activity expenditure. When we included distance traveled in the respirometry chambers as a covariate in the analysis of total metabolic rate, this only marginally reduced the effect of genotype ($F_{1,12} = 11.48, P = 0.05$), suggesting the impact of the mutation is on the total amount of activity as well as the energy costs of locomotion.

Plasma membrane D1R is reduced in *ros* striatonigral neurons which impairs dopamine signaling

The brain-specific expression pattern in the multiple Northern blots [10] indicated a restricted expression and specific function of SLC35D3 in the brain. We detected no mutant SLC35D3 protein in striatum, substantia nigra and olfactory bulb of the *ros*
mutant with an antibody to mouse SLC35D3 (Fig. 3A), although transcription was upregulated in mutant tissues [10]. SLC35D3 protein was readily detectable in these tissues of WT mice (Fig. 3A). Although the expression of SLC35D3 in the Allen Brain Atlas shows a wider distribution, in either WT or mutant mice, we did not detect the SLC35D3 protein in other brain sub-regions and especially in the obesity-related brain tissues such as thalamus and hypothalamus (Fig. 3A), as well as in several organs involved in energy homeostasis such as adipose tissue, pancreas, liver and skeletal muscle (Fig. 3B). In addition, no apparent morphological changes or fat accumulation was observed in these organs (Figs. 3C-3F). Considering the enlargement of WAT pads (Fig. 1D), the adipocytes in adult ros mice exhibited hyperplasia (increase in number) rather than hypertrophy (increase in size), to contribute mainly to the weight gain. The presence of SLC35D3 in non-neuronal tissues is only known so far in platelets and it plays a role in the biogenesis of platelet dense granules [10,11]. These results suggest that SLC35D3 is selectively expressed in certain types of neurons with particular enrichment in the basal ganglia, and that ros mice allow us to investigate the phenotypes related to its dysfunction in these neurons.

It has been reported that Slc35d3 is specifically expressed in the striatonigral MSNs expressing D1R rather than the striatopallidal MSNs expressing D2R [12], therefore, we investigated whether SLC35D3 regulates the function of D1 neurons. Immunohistochemical analysis using an antibody to D1R showed that numerous intensely immunoreactive cell bodies were present in ros striatum (Fig. 4A), which is
similar to the intracellular accumulation or internalization of D1R after D1R agonist treatment [14]. To confirm this, in immuno-electronic microscopic (IEM) pictures labeled by anti-D1R, we quantified the gold-labeled particles located on the plasma membrane (PM) and endomembrane structures (EnM) (Fig. 4B). The proportion of D1R in EnM was significantly higher (66%) in ros striatum than that (48.3%) in wild-type (Fig. 4C).

We then tested cyclic AMP (cAMP) production to detect functional D1R activation at the cell surface. Stimulation of D1R by the specific agonist SKF82958 (10 µM) produced an accumulation of cAMP in both groups. Consistently, cAMP production in ros striatum was reduced about 36% compared with the WT controls (Fig. 4D), which may be attributable to the reduction of plasma membrane D1R. Western blotting of striatum lysates showed that total D1 receptor expression levels were similar between wild-type and ros samples (Fig. 4E), indicating the total number of striatal D1 neurons in ros mice is not changed. In comparison, total D2R and the fraction of D2R on the plasma membrane were unchanged in the striatum of ros mutant mice (Fig. S2), consistent with the observation that SLC35D3 is not expressed in the D2 neurons [12]. This indicates that SLC35D3 plays a role in D1R trafficking in the striatal D1 neurons, but does not affect D2R trafficking in the striatal D2 neurons. Taken together, our results suggest that loss of SLC35D3 in ros striatum causes intracellular accumulation of D1R and reduces D1 receptors on the plasma membrane.

SLC35D3 is localized to the ER and early endosomes and it interacts with D1R
To ascertain the underlying mechanism of the accumulation of D1R within the *ros* neurons, we first examined the subcellular localization of mouse SLC35D3. Consistent with a recent report [11], the EGFP-SLC35D3 protein was selectively localized to the ER and early endosomes, but not to the Golgi complex or late endosomes/lysosomes (Figs. 5A-5D).

We then investigated the intracellular location of accumulated D1R in *ros* striatal neurons. We performed immuno-EM by double-labeling with anti-D1R and anti-SEC61B (an ER marker) and found that the D1R particles co-residing with SEC61B in *ros* striatal neurons (19.6%) were significantly higher than that in wild-type (6.8%) (Fig. 5E). This indicates that the increased proportion of ER-retained D1R (12.8%, Fig. 5E) may mostly account for the reduction of plasma membrane D1R (17.7%, Fig. 4B) in *ros* mice. Our OptiPrep gradient assays further confirmed the shift of D1R from plasma membrane (fractions 16-25) to intracellular fractions 2-10 mainly corresponding to ER in *ros* striatum compared with the wild-type (Fig. 5F).

We then tested whether there is a physical interaction between SLC35D3 and D1R by co-immunoprecipitation. Indeed, we observed that Myc-SLC35D3 co-precipitated with Flag-D1R (Fig. 6A). Reciprocally, Myc-D1R co-precipitated with Flag-SLC35D3 (Fig. 6B). In addition, we found that the N-terminal portion of SLC35D3 (1-241aa) interacted with the C-terminal region of D1R (217-446aa) (Figs. 6A, 6B).

Taken together, our results indicate that SLC35D3 is likely involved in the membrane trafficking of D1R on its ER exit, and that loss of SLC35D3 leads to the intracellular
D1R retention mainly on ER, thus reducing the amount of plasma membrane D1R receptors and their signaling.

**Mutational screen of human SLC35D3 in patients with metabolic syndrome**

The above findings in *ros* mice prompted us to investigate whether there are mutations in the orthologous human *SLC35D3* gene in patients with MetS. We screened 363 Chinese Han patients with MetS and 217 unaffected individuals by sequencing the two exons and adjacent exon/intron boundaries together with 1 kb untranslated sequence upstream of the start codon of the *SLC35D3* gene.

Two variants of SLC35D3 leading to the frame-shift of the coding sequence were found in two unrelated patients. These variants were absent in the control group or the NCBI SNP database for the *SLC35D3* gene (Locus ID 340146). In patient #1 (Male, Age: 55, BMI: 26.1, waist circumference: 109cm, blood pressure: 135/85 mmHg, TG: 4.23 mmol/L, Chol: 5.28mmol/L, Gluc: 4.4 mmol/L), a heterozygous ΔK404 was identified (Fig. 6C). The mutated SLC35D3 showed the mislocalization to LAMP3-positive late endosomes/lysosomes in transfected cells compared with WT protein (Fig. 6D), suggesting its subcellular localization has been altered. In patient #2 (Male, Age: 51, BMI: 27.1, waist circumference: 100cm, blood pressure: 120/80 mmHg, TG: 2.52 mmol/L, Chol: 5.94 mmol/L, Gluc: 5.2 mmol/L), a heterozygous insL201 was identified (Fig. 6C). Similarly, the mutant insL201 colocalized with LAMP3, but not EEA1 or SEC61B (Fig. 6D), also suggesting that these mutations alter the subcellular localization of SLC35D3.
The residues around L201 are conserved in human, chimpanzee, dog, mouse and rat. However, the residues around K404 are less conserved in these species. The mislocalization of these two variants (insL201 and ΔK404) implicates localization or sorting signals may lie on these mutational sites.

We did not find a second mutation in the SLC35D3 gene in these two patients after excluding possible large deletions, suggesting that both patients are likely affected in the heterozygous state. Both patients were diagnosed as having MetS with central obesity according to the guidelines of International Diabetes Federation (IDF) [15] and central obesity in China [16]. Similarly, we observed moderate weight gain in heterozygous ros+/− mice at 5 months of age compared with WT littermates (WT: 29.4±0.38, n=7; ros+/−: 30.9±0.24, n=8; \( P<0.01 \)). The more severe weight gain in homozygous ros−/− mice at the same age is suggestive that the SLC35D3 mutation may have a gene dosage effect on D1R trafficking. It is unknown whether patients with homozygous or compound mutations may have more severe phenotypes. Unfortunately, we were not able to get access to the blood samples of the family members of these two patients, which precluded us to explore the penetrance of the mutations. This study suggests that mutant human SLC35D3 does not function properly in the ER exit of D1R, thus likely impairing the membrane trafficking of D1R and D1 signaling in the patients in a similar mechanism as revealed in ros mice.

**D1R agonist reduces body weight and reverses hyperlipidemia in ros mice**
To test whether the pathological phenotype in obese *ros* mice is reversible by the treatment with a D1R agonist, adult male mice received a daily intraperitoneal injection of D1 receptor agonist SKF38393. Following the 12-day treatment period, we observed that body weight loss of *ros* mice (13%) was significantly higher than that of the wild-type (7%). In contrast, body weight changes of saline-treated wild-type mice or *ros* mice were not significant (Fig. 7A). Treatment with SKF38393 did not change the levels of serum lipids and glucose compared with saline-treatment in wild-type mice. Strikingly, serum cholesterol and triglycerides levels were significantly decreased for SKF38393-treated *ros* mice to levels that were similar to those of wild-type mice. Blood glucose levels in *ros* mice were significantly reduced after the treatment of SKF38393 (Figs. 7B-7D). Thus, administration of SKF38393 caused body weight loss and rescued the hyperlipidemia in *ros* mice. In addition, physical activity was increased significantly after SKF38393 treatment in *ros* mice compared with WT (Fig. 7E). These results suggest that impaired D1R signaling could be reversible by D1R agonist in patients with MetS who carry SLC35D3 mutations.

**Discussion**

Obesity is caused by perturbations of the balance between energy intake and energy expenditure, which in turn is regulated by a complex physiological system that requires the coordination of several peripheral and central signals in the brain [17,18,19].
Dopaminergic signaling pathways are involved in the regulation of food intake and energy expenditure, including the mesolimbic pathway in food reward, the mesohypothalamic pathway in satiety and the nigrostriatal pathway in energy expenditure [20,21,22]. Both the D1 and D2 dopamine (DA) receptors act synergistically in the regulation of the basal ganglia function in the striatal MSNs [23,24]. Dysregulation of DA signaling has been previously implicated in the development of obesity [25]. However, the precise mechanism by which DA receptors regulate energy balance is still unclear [26]. Positron emission tomography revealed that striatal D2 receptor availability is lower in obese humans compared to lean individuals [25,27], but to date no human imaging studies have assessed the involvement of D1 receptors in obesity.

Reduction of DA receptors on the cell surface could result from 1) increased internalization, 2) reduced reinsertion to the plasma membrane due to increased degradation, and 3) reduced trafficking or expression ab initio. Previous extensive studies have focused on understanding the internalization of DA receptors following agonist occupancy, including agonist-elicited receptor desensitization, endocytosis, and resensitization or degradation [28,29,30,31]. Unlike D2R, which is generally trafficked to the lysosomal pathway for degradation [32], endocytosed D1R is recycled back to the plasma membrane [14,33]. However, mechanistic studies of the trafficking of D1R to the cell surface are limited, and its relevance of this trafficking to metabolic disorders is unclear [34].

Transit out of the ER has been shown to be a critical control point and rate-limiting
step in the expression of D1 receptors at the cell surface [35,36]. A number of DA receptor-interacting proteins have been identified [37]. One ER protein, DRiP78, acts as a chaperon for D1 receptor trafficking [35]. Similarly, our data have shown that SLC35D3 is localized to the ER and endosomes, where it interacts with D1R. Loss of SLC35D3 in *ros* mice blocks the ER exit of D1R, thus leading to retention in the ER and reduced D1R distribution on the cell surface, thereby impairing D1R signaling. We have not completely excluded the possibility that D1R trafficking from early endosomes to plasma membrane is also blocked. Given that the ER-retained fraction of D1R accounts for the greatest proportion of reduced plasma membrane D1R (Figs. 4B and 5E), we speculate that SLC35D3 plays a major role in the ER exit of D1R. Likewise, in human patients, the mutant SLC35D3 (insL201 or Δ404K) is mistargeted to late endosomes/lysosomes and therefore is likely unable to function properly in the ER exit of D1R. Therefore, SLC35D3 is identified as a novel regulator of D1R membrane trafficking from ER.

The restricted expression of SLC35D3 in the brain and the absence of expression in other peripheral organs (except for the platelets) laid the foundation of our hypothesis that the MetS phenotype in *ros* mice is attributable to lesions in the central nervous system. Since D2R distribution (Fig. S2) is unaffected, and SLC35D3 was not expressed in D2 neurons [12], the impaired D1R signaling perturbs the D1R/D2R balance, which likely led to the reduced movement and energy expenditure due to the dysfunction of the basal ganglion DA loop.

No apparent obesity phenotypes have been documented in several *D1r*-knockout (KO)
mouse lines as listed in the MGI database (MGI:99578). However, reduced spontaneous locomotor activity was reported in a line of D1r-KO mutants [38]. Although the number of plasma membrane D1R in ros mice is about 65% (34% / 51.7%) of the WT mice (Fig. 4C), the ros mutant does not mimic the D1r+/- mice as the total number of D1R is unchanged but redistributed mainly from the plasma membrane to ER. The other significant difference is that SLC35D3 is selectively expressed in striatal D1R-expressing neurons which may manifest specific effects related to D1R reduction. The D1r-KO mice in contrast may have additional defects given the wider expression of D1R in both neuronal and non-neuronal tissues. In fact the D1r-KO mice showed postnatal growth retardation [39]. Thus, complex multiple interacting effects may preclude the development of obesity in the D1r+/- or D1r-/- mice. In other words, the ros mutant mouse mimics a D1-neuron specific knockdown of plasma membrane D1R, rather than mimicking the conventional D1r-/- or D1r+/- mouse.

In contrast to the ob/ob mice which develop obesity from the age of weaning [40], the ros mice exhibited progressive weight gain starting from 2 months of age. The delayed weight gain in ros mice with late onset obesity is still a mystery given that the indicated protein is present in early stages after birth (Fig. S1). In addition, the two patients with SLC35D3 mutations were diagnosed with adult central obesity, which also suggests a late onset obesity phenotype in humans.

Our studies have elucidated the underlying genetic entity of a long-standing unresolved linked locus near marker D6S1009. Both mouse and human mutations of the SLC35D3
gene are associated with MetS, suggesting that SLC35D3 is a novel candidate gene for MetS. Considering that obesity affects 10–25% of the European population and nearly one third of the US population [41], a mutational screen of SLC35D3 in the obese population would be cost-effective as a precursor to potential D1R agonist treatment. Obese children receiving D1R agonist treatment reverse weight gain [42]. Similarly, administration of D1R agonist reversed most of the phenotype of MetS in ros mice. This effect may be caused by the stimulation of the residual D1R on the plasma membrane of ros striatum, or redirection of the ER-retained D1R to plasma membrane for its signaling. In addition, the reversible phenotypes upon D1R agonist treatment suggest that the reduced D1R numbers on the plasma membrane could be the primary cause of MetS in the ros mutant mice, although we have not excluded the effects on the substantia nigra and olfactory bulb where SLC35D3 are also expressed (Fig. 3A). SLC35D3 deficiency caused obesity primarily via effects on physical activity levels, suggesting genetic factors could be a component of low physical activity [43]. As reduced physical activity is the primary consequence of impaired D1R signaling, encouraging elevations in physical activity in these patients might be an alternative way to prevent or alleviate their symptoms of obesity [44].

Materials and Methods

Mice
The *ros* mutant (*ros-/-*) [10] and control C3H/HeSnJ mice (wild-type, WT) were originally obtained from Dr. Richard T. Swank’s laboratory and bred in the animal facility of the Institute of Genetics and Developmental Biology (IGDB), Chinese Academy of Sciences. All procedures were approved by the Institutional Animal Care and Use Committee of IGDB (mouse protocol KYD2006-002). To ensure the genotypes of *ros-/-* and wild-type, we developed a PCR method of genotyping (primer sequences are available upon request) based on the nature of the insertional mutation in the *Slc35d3* gene [10]. For the locomotion tests, only male mice of each genotype at 12 weeks old were selected to control for potentially confounding hormonal effects during the estrous cycle in females. Mice were housed in a room with a 12-hr light/dark cycle (lights on at 7:30 a.m. and off at 7:30 p.m.) with access to food and water *ad libitum*. For other phenotypic analyses, males at 24 weeks of age were used except for those specified in figure legends or methods.

**Study subjects**

We recruited 363 unrelated Chinese Han patients with MetS from The Affiliated Hospital of Qingdao University Medical College and The Affiliated Children’s Hospital of Nanjing Medical University, and 217 unaffected individuals from Beijing Tongren Hospital of Capital Medical University. The patients were diagnosed as MetS according to the guidelines of International Diabetes Federation (IDF) [15] and central obesity in China [16]. In brief, MetS is diagnosed as abdominal obesity (or central obesity) with any two of the following parameters, 1) TG>1.7mmol/L, 2) HDL<1.03mmol/L (male) or
For the diagnosis of abdominal obesity in Chinese population, we choose either 1) BMI > 28 as general obesity or 2) BMI between 24 to 28 as overweight, and waist circumference > 90 cm (male) or >85 cm (female). This study was approved by the Bioethic Committee of IGDB, Chinese Academy of Sciences (IRB approval number, IGDB-2011-IRB-002). The study was conducted according to the Declaration of Helsinki Principles. Written informed consents were obtained and 8 ml peripheral blood samples were collected from all subjects participating in this study. Primers for amplifying the two exons and about 1kb upstream of the human SLC35D3 gene are available upon request. Amplified PCR products were subjected to direct sequencing by an ABI PRISM 3700 automated sequencer (Applied Biosystems, Foster City, CA).

**Measurements of obesity features and metabolites in mice**

Growth curves for males were obtained by measuring body weight once a month from 4 to 24 weeks of age. For determination of body length, mice were anesthetized and fully extended to measure the naso-anal distance. Epididymal and perirenal fat pads were harvested from male mice and weighed. Blood was collected by cardiac puncture after an overnight fast for measuring blood glucose, serum cholesterol and triglycerides by colorimetric kit assays (Leadman, Beijing, China) and analyzed using an automatic biochemical analyzer (Hitachi, Tokyo, Japan). Insulin was measured by a rat/mouse insulin enzyme-linked immunoassay (ELISA) Kit on non-fasted mice (Millipore, Bedford, MA, USA). For the insulin tolerance test (ITT) and glucose tolerance test (GTT), fasting
plasma glucose levels were measured (16 hours fast, blood taken from the tail vein) using a glucosimeter (Teromo, Japan). Then insulin (Roche Diagnostics, Switzerland) was injected intraperitoneally (1U/kg) and blood glucose was measured again at 15, 30, 60, 90 and 120 min post injection. Alternatively, D-glucose (Sigma-Aldrich, St. Louis, Missouri, USA) was injected intraperitoneally (2g/kg body weight) and blood glucose was measured again at 15, 30, 60 and 120 min post injection.

**Spontaneous locomotor activity**
Mice were pre-exposed to the chamber before testing to allow environmental habituation, and activity was monitored under indirect dim light and sound-attenuated conditions. A single mouse was placed in a chamber (40cm length × 40cm width × 45cm height) for 30 min. Total distance traveled, average velocity and total movement duration measured spontaneous activity. All these parameters were measured by JLBehv software (JLGY, Shanghai, China). Behavioral testing was performed between 8:00 and 12:00 a.m.

**Food intake**
Before measurement of daily food intake, mice fed *ad libitum* were individually housed for 3 days to allow environmental habituation. Food was measured at 3:00 p.m. each day for 7 consecutive days.

**VO₂ and VCO₂ measurements**
Mice aged 8 to 12 weeks (prior to development of obesity) were measured using an indirect calorimetry system (Oxymax, Ohio, USA). Oxygen consumption, CO₂ production and physical activity (beam breaks) were recorded at 30-min intervals for 5
consecutive days (48 times a day). Volume of oxygen consumption \( (\text{VO}_2) \) and carbon dioxide production \( (\text{VCO}_2) \) were measured using electrochemical and spectrophotometric sensors respectively. Oxygen consumption data were converted to energy expenditure (Watts) using the measured RQ values using procedures outlined in Arch et al [45]. To report the total energy expenditure we averaged the 48 measurements collected each day across days 2 to 5, allowing the animals to acclimate during the first 24h in the chambers [13]. We recorded simultaneously the physical activity levels of the mice. Typical temporal patterns of oxygen consumption and physical activity are shown in Figs. S2A and S2B respectively. To establish the resting metabolic rate we used two different strategies. First we summarized all the half hourly oxygen consumption data in a histogram and then calculated the mean of the lowest 5% of values. Typical histogram for the data shown in Fig. S2A is shown in Fig. S2C. A second method was to use the regression approach outlined by Nonogaki et al [46]. This involved plotting the time matched data for \( \text{VO}_2 \) and physical activity levels (Fig. S2D) and then evaluating the resting metabolic rate from the intercept of a fitted linear regression model. The relationship between total metabolic rate and body weight for each genotype is shown in Fig. 2E, and that for the two resting metabolic rate approaches in Figs. 2F and 2G respectively. Following derivation of the total and resting rates of metabolism we corrected for the potentially confounding effects of body weight (Figs. 2E-2G) as recommended in Arch et al [45] and Tschop et al [13].

**Treatment of D1 receptor agonists on mice**
Twenty-four to 25-week-old male mice of each genotype received a daily intraperitoneal injection of dopamine D1 receptor SKF38393 (20 mg/kg) (Sigma-Aldrich) for a period of 12 days. Saline-treated mice served as controls. Body weight in all mice was measured on the fourteenth day. Blood glucose, serum cholesterol and triglycerides, and locomotor activities were measured as described above.

**cAMP ELISA measurement**

The dorsal striatum of mice was dissected as above and homogenized in Buffer A (10 mM Tris pH 7.4, 1 mM EDTA, 30 μM leupeptin, 1 μM pepstatin A) with 10% sucrose. Membranes were isolated by centrifugation (65 min at 100,000 g) onto a cushion of Buffer A with 44.5% (w/v) sucrose. The membranes at the interface were transferred to a new tube and washed twice with Buffer A and collected by centrifugation (30 min at 100,000 g). Protein concentrations were determined with Protein Assay (Bio-Rad, Hercules, CA, USA). Adenylyl cyclase activity was determined by incubating membrane protein (20 μg) at 30 °C for 10 min in 0.1 ml of buffer containing 10 mM imidazole (pH 7.4), 0.2 mM EGTA, 0.5 mM MgCl₂, 0.5 mM DTT, 0.1 mM ATP, 0.5 mM IBMX, and 10 μM D1 receptor agonists SKF82958 (Sigma-Aldrich). Reactions were terminated by placing the tubes into boiling water for 2 min. The cAMP concentrations were measured using the Direct cAMP Enzyme-linked Immunoassay Kit (Sigma-Aldrich) following the manufacturer’s instructions. Optical density was measured at 405 nm by a microplate reader (Bio-Rad).

**Data collection and analyses**
cAMP ELISA was performed in duplicate and was repeated three or four times. The standard curves were generated using non-linear regression curve fitting. The specific protein bands on Western blots were scanned and analyzed using the software program NIH Image J. All data were obtained from at least three independent experiments. Data were expressed as mean ± SEM and statistical significance was tested by Student’s t-test. Data from the calorimetry system was tested by ANCOVA. Distribution of gold-labeled particles in immuno-EM pictures were counted and statistical significance was tested by Chi-square test. Intensities of immunofluorescence in cultured cells were analyzed using NIH Image J.

**Supporting Information**

Additional materials and methods, three supplementary figures (Figs. S1-S3) and figure legends are included in the Supporting Information File, which is accessible online.

**Acknowledgements**

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Web Resources

International Obesity Taskforce (IOTF), http://www.iaso.org/iotf/obesity/


Mouse Genome Informatics (MGI) database, http://www.informatics.jax.org/


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Figure Legends

**Figure 1. Ros mice exhibit features of metabolic syndrome.** (A) Comparison of *ros* mouse and wild-type (WT) C3H/HeSnJ mouse at 24 weeks of age. (B) Growth curves of male WT and *ros* mice. Body weights of *ros* mice are significantly higher than those of WT starting at 8 weeks. (C) *ros* mice show significantly increased naso-to-anal body length (10.06±0.09 cm, n=10) compared with the WT controls (9.47±0.06 cm, n=10) at 24 weeks of age. (D) Perirenal and epididymal fat pad weight in *ros* mice (n=10) are significantly higher than those of the WT (n=10) respectively at 24 weeks of age. (E-G) Serum levels of cholesterol (Chol), triglycerides (TG) and blood glucose (Gluc) in fasted *ros* mice (n=14) are significantly higher than those in WT controls (n=14) at 24 weeks of age. In *ros*, mean values of Chol, TG, Gluc are 3.82, 1.0, 11.47 mmol/L, respectively; In WT, mean values of Chol, TG, Gluc are 3.21, 0.62, 8.03 mmol/L, respectively. (H) Plasma insulin levels were increased in non-fasted *ros* mice (3.62± 0.20 ng/ml, n=3) than in WT (0.87± 0.08 ng/ml, n=3) at 24 weeks of age. (I) Insulin tolerance tests (ITT) showed a tendency of impaired tolerance in *ros* mice compared with WT mice at 24 weeks of age (n=3). (J) Glucose tolerance tests (ITT) showed a tendency of impaired tolerance in *ros* mice compared with WT mice at 24 weeks of age (n=3). *P<0.05, **P<0.01, ***P<0.001.
Figure 2. Food intake, spontaneous locomotor activity and total and resting energy expenditure. Mice at 12 weeks of age were used in this study. (A) There was no significant difference in daily food intake between wild-type (WT) (n=14) and \textit{ros} mice (n=15). $P=0.066$. (B) The distance traveled during 30 min for \textit{ros} mice (n=25) was lower than that for the WT (n=20). (C) The average movement velocity during 30 min for \textit{ros} mice (n=25) was shorter than the WT (n=20). (D) The total movement duration during 30 min for \textit{ros} mice (n=25) was shorter than the WT (n=20). (E-G) Total energy expenditure (Watts) (E), resting energy expenditure (Watts) by Method 1 (F) and resting energy expenditure (Watts) by Method 2 (G) for WT and \textit{ros} mice measured at age 8 to 12 weeks (n = 8 per group). WT mice are black symbols and \textit{ros} mice are white symbols. Fitted linear regressions for each data set are shown. There was a significant effect of body weight and genotype but no interaction in total energy expenditure (ANCOVA: Body weight effect, $F_{1,13} = 44.85$, $p < 0.001$); genotype effect, $F_{1,13} = 10.47$, $p = 0.007$; interaction, not significant (ns.). There was a significant effect of body weight but no genotype or interaction effect in resting energy expenditure by Method 1 or 2 (Method 1 ANCOVA: Body weight effect, $F_{1,13} = 8.78$, $p = 0.011$; genotype effect, $F_{1,13} = 0.35$, $p = 0.566$; interaction: ns. Method 2 ANCOVA: Body weight effect, $F_{1,13} = 22.29$, $p < 0.001$; genotype effect, $F_{1,13} = 0.03$, $p = 0.873$; interaction, ns.). *$P$<0.05, **$P$<0.01.

Figure 3. SLC35D3 expression in brain sub-regions and non-neuronal tissues. Mice at 24 weeks of age were used in this study. (A) Immunoblotting of SLC35D3 protein in
nine brain sub-regions of wild-type (WT) and ros mice. OB: olfactory bulb, CC: cerebral cortex, HIP: hippocampus, STR: striatum, THA: thalamus, HYP: hypothalamus, SN: substantia nigra, CB: cerebellum, BS: brain stem. (B) Immunoblotting assay showed no expression of SLC35D3 in adipose tissue, pancreas, liver, or skeletal muscle compared with striatum or brain in WT mice. There was no expression of SLC35D3 in any of these tissues in ros mice. β-actin serves as a loading control. (C) The size of adipose from epididymal fat pad of ros mice was normal. (D-F) No apparent fat accumulation was observed in pancreas, liver, or skeletal muscle in ros mice. 200X magnification was applied in the above sections with H-E staining.

Figure 4. Plasma membrane D1R and its signaling are reduced in ros striatonigral neurons. Mice at 12 weeks of age were used in this study. (A) Immunohistochemical detection of D1R on the coronal brain sections of the striatum. In ros mice, visible immunoreactive cell bodies are present (arrows). Scale bar: 100 µm. (B, C) Representative immuno-EM pictures of D1R-labeled particles on plasma membrane (PM, upper panels) and endomembrane structures (EnM, lower panels) in wild-type (WT) and ros striatal neurons are shown without much difference (B). However, the quantification test revealed that the particles on PM in ros mice (34.0%, n=103) is significantly lower than that in WT (51.7%, n=87), **P<0.01. Scale bar: 200 nm. (D) SKF82958–induced cAMP accumulation in striatal membranes prepared from WT and ros mice. As compared with the WT (11.09 ± 0.61 pmol/ml, n=6), cAMP activity in ros membranes (7.11 ± 0.45
pmol/ml, n=6) was reduced about 36%. *P<0.001. (E) Immunoblot analysis of D1 receptor. left panel, Striatum tissues (20µg) from WT and ros mice were probed with the monoclonal D1R antibody. β-actin was used as a loading control. The immunoblots shown are representative of three independent experiments. right panel, Normalized percentages of the band intensities shown in left panel are means ± SEM (n=3). There is no significant difference of total D1R levels between WT and ros mice (P>0.05).

Figure 5. Subcellular localization of SLC35D3 protein and the effect of SLC35D3 on trafficking of D1R. (A-D) In HEK293T cells, EGFP-SLC35D3 co-localized with the ER marker SEC61B and the endosome marker EEA1, but not with the Golgi marker GM130 or the lysosome/late endosome marker LAMP3. (E) Representative pictures to show that the smaller gold-labeled D1R particles (10 nm, arrowheads) and the larger gold-labeled SEC61B particles (ER marker) (15 nm, arrows) are colocalized in ros striatum compared with the particles on different structures in wild-type (WT) under immuno-EM at 12 weeks of age. Scale bar: 200 nm. The percentage of D1R colocalized with SEC61B in ros mutant (19.6%, n=97) is significantly higher than that in wild-type (WT) (6.8%, n=74), *P<0.05. (F) Immunoblotting of Optiprep (Biocomp, USA) gradient (5-20%) fractions. One microgram tissue lysates of striatum at 12 weeks of age were separated into 26 fractions. D1R was shifted from plasma membrane (PM: GluR1 as a marker) in fractions 16-25 to intracellular fractions 2-10 mainly in endoplasmic reticulum (ER: BIP as a marker) in ros mice compared with WT mice. IN: lysate input; M: molecular marker.
Figure 6. SLC35D3 interacts with D1R and mutations of SLC35D3 alter its subcellular distribution. (A) C-terminal D1R interacted with SLC35D3. Cell lysates with the co-expression of Myc-SLC35D3 and Flag-D1R (or Flag-D1R-NT (1-216aa), or Flag-D1R-CT (217-446aa)) were immunoprecipitated. Both the full-length D1R and the D1R-CT bound with Myc-SLC35D3. (B) N-terminal SLC35D3 interacted with D1R. Cell lysates with the co-expression of Myc-D1R and Flag-SLC35D3 (or Flag-SLC35D3-NT (1-301aa), or Flag-SLC35D3-CT (242-422aa)) were immunoprecipitated. Both the full-length SLC35D3 and the SLC35D3-NT bound with Myc-D1R. (C) Two heterozygous mutations were identified in two patients with MetS. WT: wild-type SLC35D3; MU: mutant SLC35D3. In patient #1, a 3-bp deletion (c.1209_1211delGAA) as underlined led to an in-frame deletion of K404. In patient #2, a 3-bp insertion (c.601_602insCTG) as the arrow indicated led to an in-frame insertion of L201. (D) In HEK293T cells, wild-type SLC35D3 was partially colocalized with EEA1 (early endosome) and SEC61B (ER), without colocalization with LAMP3 (late endosome/lysosome). The ΔK404 and insL201 mutations were mislocalized mainly on late endosomes/lysosomes.

Figure 7. Effect of SKF38393 treatment on body weight loss, serum lipids and glucose, and activity of wild-type or ros mice. Mice at 24 weeks of age were used in this study. (A) Wild-type (WT) or ros mice (n=9 per group) received a daily
intraperitoneal injection of 20mg/kg SKF38393 for 12 days and weighed on day 14. Body
weights of saline-treated WT or *ros* mice had no significant changes, whereas body
weights of SKF38393-treated WT mice were reduced approximately 7% (from 29.61 ±
0.76 g to 27.5 ± 0.6 g), and body weights of SKF38393-treated *ros* mice were reduced
approximately 13% (from 38.56 ± 0.79 g to 33.39 ± 0.58 g). ***P<0.001. (B-D)

Compared with saline-treated WT mice (n=8), SKF38393-treated WT mice (n=9) exhibit
no significant changes of serum levels of triglycerides (TG), cholesterol (Chol) and blood
glucose (Gluc). In SKF38393-treated *ros* mice, serum levels of Chol, TG and Gluc were
significantly reduced compared to saline-treated *ros* littermates. SKF38393 vs. saline,
Chol: 3.43 ± 0.08 mmol/L (n=9) vs. 3.86 ± 0.05 mmol/L (n=7); TG: 1.29 ± 0.06 mmol/L
(n=8) vs. 1.63 ± 0.01 mmol/L (n=7); Gluc: 11.7 ± 0.57 mmol/L (n=9) vs. 14.67 ± 0.46
mmol/L (n=7). **P<0.01. (E) Total distances traveled during the period of 30 min to 60
min after SKF38393 treatment were increased in both WT and *ros* mice. However, the
distance traveled in *ros* mice was greater than that in WT (***P<0.001).
Figure 3

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Figure 4

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Figure 6
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