Mechanical force is critical for the development and remodeling of bone. Here we report that mechanical force regulates the production of the metabolite asymmetric dimethylarginine (ADMA) via regulating the hydrolytic enzyme dimethylarginine dimethylaminohydrolase 1 (Ddah1) expression in osteoblasts. The presence of -394 4 N del/ins polymorphism of Ddah1 and higher serum ADMA concentration are negatively associated with bone mineral density. Global or osteoblast-specific deletion of Ddah1 leads to increased ADMA level but reduced bone formation. Further molecular study unveils that mechanical stimulation enhances TAZ/SMAD4-induced Ddah1 transcription. Deletion of Ddah1 in osteoblast-lineage cells fails to respond to mechanical stimulus-associated bone formation. Taken together, the study reveals mechanical force is capable of down-regulating ADMA to enhance bone formation.
During the post-translational methylation of arginine residues within proteins and proteolysis, asymmetric dimethylarginine (ADMA) is released into the cytoplasm. ADMA has been generally implicated as an important risk factor for atherosclerosis, cardiovascular diseases and renal diseases. ADMA is regarded as a competitive inhibitor of nitric oxide synthase (NOS) enzymes. Elevation of ADMA accelerates oxidative stress but reduces the production of NO. It is suggested that ADMA remains stable until it is hydrolyzed to D-citrulline and dimethylamines by the hydrolytic enzyme, dimethylarginine dimethylamine hydrolase (DDAH). To date, two distinct DDAH isoforms (DDAH1 and DDAH2) have been identified. DDAH1 has confirmed to mainly contribute to the overall DDAH activity in several tissues. Emerging evidences suggest that plasma ADMA levels are significantly associated with SNPs (Single Nucleotide Polymorphisms) in \textit{Ddah1}, which contributed to cardiovascular diseases and diabetes. A loss-of-function \textit{Ddah1} promoter polymorphism is associated with increased susceptibility to cardiovascular diseases and thrombosis stroke. Thus, these SNPs of \textit{Ddah1} are likely associated with metabolic syndrome including metabolic bone diseases. Therefore, our initial aim was to investigate if the loss-of-function \textit{Ddah1} promoter polymorphism is associated with osteoporosis.

Previous studies suggested that deletion of NOS enzymes led to reduced bone formation, decreased osteoblast number and mineralization rates. Additionally, it was suggested that NO regulated by NOS enzymes is responsive to mechanical force, which was important for bone homeostasis. To note, ADMA is a competitive inhibitor of NOS enzymes; therefore, our second aim was to investigate whether ADMA hydrolyzed by DDAH is involved in bone remodeling responsive to mechanical force. Here, we found that associations between ADMA level, the -394 4N del/ins polymorphism of \textit{Ddah1}, and bone mineral density were found in the Chinese population, suggesting the potential role of DDAH1 expression in bone health. We further explored the underlying mechanisms. Deletion of \textit{Ddah1} in osteoblasts led to increased ADMA in serum and bone samples, which finally suppressed osteoblast differentiation and thus reduced bone mass in mice. Subsequently, we intended to explore which signaling pathway was involved in the regulation of DDAH1 mediated by mechanical force. Previous studies provided evidence that expression of DDAH1 was controlled by Yap1/Taz, also knocking down of Lats2 reduced the expression of DDAH2. Not surprisingly, Yap1/Taz signaling pathway activated by mechanical force was confirmed by several groups, which implied that mechanical force might regulate DDAH1 expression via Yap1/Taz pathway.

**Results**

The -394 4N del/ins polymorphism of \textit{Ddah1} and increased level of ADMA is negatively associated with bone mineral density. Given that loss-of-function polymorphism of \textit{Ddah1} promoter was associated with increased susceptibility to metabolic syndrome (MS), we asked whether ADMA level and \textit{Ddah1} promoter polymorphism were associated with the bone mineral density (BMD) in humans. A Chinese population of 1404 participants were included, and 570 participants from the cohort were subjected to analyze ADMA concentrations. The characteristics of the participants are presented in Supplementary Table S1 and Supplementary Table S2. A novel -394 4N del/ins polymorphism of \textit{Ddah1} promoter region was previously reported to affect the mRNA levels of \textit{Ddah1} in patients. Consistent with the previous study, the mRNA level of \textit{Ddah1} was lower in participants with the del/ins and ins/ins polymorphisms (Fig. 1a). The median serum ADMA concentrations were significantly higher in individuals with one or both copies of the -394 4N ins allele (206.3 ± 26.2 ng/ml or 181.1 ± 8.6 ng/ml, respectively) compared to individuals with both copies of the -394 4N del allele.

![Fig. 1 Association of ADMA levels and the -394 4N del/ins polymorphism of Ddah1 with bone mineral density in a large Chinese population-based study.](image)
(158.1 ± 3.0 ng/ml; Fig. 1b). Furthermore, the plasma ADMA concentrations in 570 participants with normal BMD, osteopenia, or osteoporosis was examined. The serum ADMA concentrations were significantly higher in individuals with osteopenia and osteoporosis than in control subjects (Fig. 1c). The association of the -394 4N del/ins polymorphism with osteoporosis was also investigated based on 1404 participants. One or both copies of the -394 4N ins allele was significantly associated with increased risks of developing osteopenia and osteoporosis, both with or without adjustment for conventional risks, including age, sex, and the body mass index (Table 1). Compared with the control group, the group with osteopenia had an unadjusted odds ratio (OR) of 1.45 (P = 0.004) and an adjusted OR of 1.42 (P = 0.015). The group with osteoporosis had an unadjusted OR of 1.45 (P = 0.015) and an adjusted OR of 1.57 (P = 0.037). Taken together, these results provide evidence that the -394 4N del/ins polymorphism of Ddah1 was associated with bone health, as well as the serum concentration of ADMA.

**Table 1 Association between the -394 4N del/ins polymorphism of Ddah1 and osteoporosis, analyzed by logistic regression.**

| Samples (n = 1404) | Genotype, n (%) | Frequency | Unadjusted OR (95% CI) | Adjusted OR (95% CI) |
|-------------------|----------------|-----------|------------------------|---------------------|
| Control (n = 470) | del/del 378 (80.4) | 19.6 | Reference | |
| Osteopenia (n = 635) | del/ins 84 (17.9) | 26.9 | 1.45 (1.13-1.86) | 1.42 (1.07-1.88) |
| Osteoporosis (n = 239) | ins/ins 8 (1.7) | 26.8 | 1.45 (1.08-1.94) | 1.57 (1.03-2.40) |

Odds ratios (ORs) and 95% confidence intervals (CIs) were obtained by logistic regression, with and without adjustment for sex, age, and body mass index.

*p < 0.05. **p < 0.01. OR, an indicator of the degree of association between exposure and disease, and was analyzed by logistic regression.

loss of function polymorphism of Ddah1 was related to BMD in the population, we intended to study the effects of DDAH1 on bone metabolism. In this study, Ddah1 global knockout mice was used. As shown, 10-week old Ddah1−/− mice displayed decreased BV/TV ratios in the femur and also a decrease in the cortical bone thickness, compared with wild-type (WT) control mice (Fig. 2a, b). Consistently, the histomorphometric analysis showed that the MAR, BFR/BS and Ob.S/BS were all reduced in Ddah1−/− mice compared with WT mice (Fig. 2c, d). Furthermore, the results of the ELISA assay were demonstrated that Ddah1 deficiency led to a decrease of P1NP concentration in serum (Fig. 2e). Additionally, immunofluorescence staining of bone tissues showed that Ddah1−/− mice had fewer osteocalcin-positive (OCN+) osteoblasts, compared to Ddah1+/+ mice (Fig. 2f). Interestingly, knocking out of Ddah1 did not affect osteoclast number but slightly increased osteoclast surface of bone surface (Oc.S/BS) in Ddah1−/− mice (Fig. 2g, h). Finally, we also generated Ddah2−/− mice and found global knockout of Ddah2 had no effects on bone mass (Fig. 2i, j), which might be due to unchanged concentrations of ADMA (Supplementary Fig. 1). Taken together, these data supported that deletion of Ddah1 led to bone loss and weakened bone formation.

**Ddah1 deficiency but not Ddah2 resulted in bone loss and weakened bone formation.** According to the loss-of-function polymorphism of Ddah1 was related to BMD in the population, we intended to study the effects of DDAH1 on bone metabolism. In this study, Ddah1 global knockout mice was used. As shown, 10-week old Ddah1−/− mice displayed decreased BV/TV ratios in the femur and also a decrease in the cortical bone thickness, compared with wild-type (WT) control mice (Fig. 2a, b). Consistently, the histomorphometric analysis showed that the MAR, BFR/BS and Ob.S/BS were all reduced in Ddah1−/− mice compared with WT mice (Fig. 2c, d). Furthermore, the results of the ELISA assay were demonstrated that Ddah1 deficiency led to a decrease of P1NP concentration in serum (Fig. 2e). Additionally, immunofluorescence staining of bone tissues showed that Ddah1−/− mice had fewer osteocalcin-positive (OCN+) osteoblasts, compared to Ddah1+/+ mice (Fig. 2f). Interestingly, knocking out of Ddah1 did not affect osteoclast number but slightly increased osteoclast surface of bone surface (Oc.S/BS) in Ddah1−/− mice (Fig. 2g, h). Finally, we also generated Ddah2−/− mice and found global knockout of Ddah2 had no effects on bone mass (Fig. 2i, j), which might be due to unchanged concentrations of ADMA (Supplementary Fig. 1). Taken together, these data supported that deletion of Ddah1 led to bone loss and weakened bone formation.

**Loss of Ddah1 accumulated ADMA to induce bone loss in vivo.** To explore how deletion of Ddah1 led to bone loss in vivo, we first intended to analyze whether ADMA was involved in bone loss in vivo. Thus, we analyzed the concentrations of ADMA in serum and bone tissues, and we found that concentrations of ADMA were both dramatically increased in Ddah1−/− and Ddah1Prx1−/− mice compared with the littermate Ddah1+/+ and Ddah1f/f mice, respectively (Fig. 4a, b). Simultaneously, we performed an immunofluorescence assay to analyze the level of endothelial nitric oxide synthase (eNOS) in tibiae (Fig. 4c, d). The data showed that deletion of DDAH1 in osteoblasts caused elevated ADMA, which then caused impaired NO production in tibiae. To further confirm that accumulating ADMA led to bone loss and reduced bone formation, we bred mice with a high dose of ADMA, and serum concentrations of ADMA suggested that ADMA was accumulated in vivo (Fig. 4g). Indeed, mice treated with ADMA had decreased trabecular bone volume, trabecular bone number, and cortical bone thickness (Fig. 4e, f). While ADMA-treated mice also had decreased concentrations of P1NP in serum (Fig. 4h). Consistent with the previous data, ADMA-treated mice showed a weakened bone formation compared with vehicle group mice (Fig. 4i, j). As far as mechanical properties are concerned, treatment with ADMA impaired stiffness and the ultimate force of femurs compared with control group mice (Fig. 4k). Since knockout of Ddah1 resulted in impaired bone formation, we concluded that deletion of Ddah1 led to accumulated ADMA to inhibit NO production and thus impaired bone formation in mice.

**The expression of DDAH1 was a response to mechanical force via TAZ/SMAD4 signaling pathway.** NO synthesis is regulated by mechanical force, which is critical for the skeletal system.
Therefore, we proposed whether DDAH1 mediated ADMA hydrolysis to link mechanical force and NO synthesis. In an attempt to address the hypothesis, we firstly analyzed the expression of DDAH1 and DDAH2 in unloading and exercise mice. Intriguingly, we found that DDAH1 was increased in the bones of exercise mice but was decreased in the bones of unloading mice (Fig. 5a, b). Simultaneously, we performed metabonomics to explore the changed metabolites responsive to mechanical force. The data showed that ADMA was significantly decreased in serum samples of mice after exercise treatment (Fig. 5c, d). Further LC-MS assay confirmed that the concentration of ADMA was reduced in serum samples of exercise mice, but it was increased in serum samples of unloading mice (Fig. 5e). Interestingly, although the concentrations of ADMA and arginine were upregulated in exercise treatment mice, NO production was increased in the exercise mice (Supplementary Fig. 3). Next, we
used a Flexcell tension system to apply a tension force to the osteoblasts. We found that 5% tension rate force applied to pre-OBs induced DDAH1 expression and reduced the ADMA concentration in the cell supernatant (Supplementary Fig. 4a, b). We next investigated whether the potential mechanical force-response factors including YAP, TAZ, Piezo1, and β-catenin were involved in regulating DDAH1. We found that TAZ knockdown most dramatically reduced the Ddah1 expression in comparison with the control (Supplementary Fig. 4c, d). Actu-
al., the application of tension force activated YAP/TAZ pathway and increased the expression of ALP (Fig. 5f). Furthermore, the YAP/TAZ antagonist verteporfin (VP) suppressed DDAH1 expression in BMSCs and osteoblasts (Supplementary Fig. 5a). Conversely, the YAP/TAZ agonist LPA induced DDAH1 expression in BMSCs and osteoblasts (Supplementary Fig. 5b).

Using a bioinformatics method, we found that there are no TAZ-binding sites in the promoter region of Ddah1. However, the previous study suggested that TAZ cooperates with SMAD4 to regulate osteogenesis, and we found that the Ddah1 promoter region contains several potential SMAD4-binding sites. To confirm the potential underlying mechanism, we performed co-immunoprecipitation (Co-IP) assay and found that tension force promoted the interaction between TAZ and SMAD4 (Fig. 5g). Furthermore, immunofluorescence and nuclear-plasma separation assay demonstrated that tension force promoted the interaction between TAZ and SMAD4 and the nuclear translocation status of these two factors (Fig. 5h and Supplementary Fig. 6). In addition, chromatin immunoprecipitation (ChIP) assay demonstrated that tension force promoted SMAD4 binding to the promoter regions of Ddah1 (Fig. 5i, j). Accordingly, luciferase reporter assays provided evidence that silencing Smad4 or Taz reduced Ddah1 transcriptional activity in stimulation with tension force (Fig. 5k). Consistent with the results of tension force treatment, fluid shear stress (FSS) and hard matrix stiffness promoted osteogenesis by analyzing ALP staining and mRNA levels of osteoblast-specific genes (Supplementary Fig. 7a, b, d). Meanwhile, these two different forms of mechanical force also induced DDAH1 expression by activating TAZ/SMAD4 pathway (Supplementary Fig. 7c, e, f). Taken together, these findings indicate that mechanical force activates the TAZ/SMAD4 pathway to induce DDAH1 transcriptional expression.

**Ddah1 deficiency in osteoblast-lineage cells lacked the response to mechanical force.** To further elucidate whether Ddah1 mediated the mechanical force-responsive changes of ADMA in bone, we next subjected Ddah1−/− and Ddah1Prx1 mice to run on the treadmill. As shown in Figs. 4a, b, exercise increased the bone volume/total volume (BV/TV) of trabecular bone in the femurs of Ddah1Prx1 mice, but the favorable effects were partially impaired by deletion of Ddah1, like in Ddah1Prx1 mice. Meanwhile, the cortical bone thickness (Ct. Th) was neither increased after exercise in Ddah1Prx1 mice (Fig. 6a, b). Furthermore, exercise increased the number of OCN+ osteoblasts in the femurs of Ddah1Prx1 mice, but not in the Ddah1Prx1 mice (Fig. 6c, d). In addition, Ddah1Prx1 mice showed partially impaired MARs after being treated by exercise (Fig. 6e, f). However, ADMA was still in a higher concentration in Ddah1Prx1 mice after being treated by exercise (Fig. 6g). Collectively, these data suggest that deletion of Ddah1 in osteoblast-lineage cells leads to a partial loss of response to mechanical force and accordingly regulates bone mass by hydrolyzing ADMA.

**Inhibition of DDAH1 by PD404182 had no effects on preventing bone formation in Ddah1−/− mice, but administration of AAV-DDAH1 accelerated bone healing in vivo.** We next sought to understand whether inhibition of DDAH1 could prevent the bone formation in mice. We, therefore, treated the mice with PD404182 (PD), an inhibitor of DDAH1, which is reported in a previous study (Fig. 7a). We confirmed that the administration of PD promoted bone loss in Ddah1−/− mice, but the effects of inhibiting bone formation were not found in Ddah1−/− mice treated with PD (Fig. 7b). As shown in Fig. 7c–f, micro-CT analysis revealed that the values of BMD, BV/TV, Tb.N and Tb.Th of wild-type mice treatment with PD were markedly lower than Ddah1−/− mice treated with vehicle. Furthermore, administration of PD decreased the OCN positive cells of the bone parameter in tibiae of mice (Fig. 7g–k). Conversely, administration of PD had no effects on promoting the loss of OCN positive cells in hindlimbs of Ddah1−/− mice. Consistent with the data of immunofluorescence assay, the calcine labeling assay demonstrated that PD remarkably inhibited bone formation in hindlimbs of Ddah1−/− mice, but the values of MAR and BFR/BS of PD-treatment Ddah1−/− mice were not significantly different from those of vehicle-treatment Ddah1−/− mice (Fig. 7h–j). As expected, the concentration of ADMA in serum samples in Ddah1−/− mice was notably higher than that of Ddah1−/− mice, whereas PD dramatically increased the concentration of ADMA in Ddah1−/− mice but had no effects on the concentration of ADMA in Ddah1−/− mice (Fig. 7l). In addition, we administered AAV-DDAH1 to treat bone defects of Ddah1 conditional knock out mice. The data showed that administration of AAV-DDAH1 dramatically accelerated bone healing in vivo (Fig. 7m, n). Therefore, these results intimate that inhibition of DDAH1 by administering of PD 404182 promotes bone loss in wild-type mice, but it does not work in Ddah1 knock out mice. While administration of AAV-DDAH1 accelerates bone healing in Ddah1 conditional knock out mice.
In this study, we found that the -394 4N del/ins polymorphism of Ddah1 was closely associated with low BMD, as well as an increased concentration of ADMA in human serum samples. Deletion of Ddah1 but not Ddah2 led to bone loss in mice mainly via impairing bone formation. Meanwhile, an increase of ADMA regulated by Ddah1 deficiency was contributed to bone loss. In particular, high ADMA accumulation was observed and did not respond to exercise-induced bone formation in Ddah1 conditional knockout mice. Underlying mechanism study unveiled that mechanical force enhanced TAZ/SMAD4 mediated Ddah1 transcription, which in turn regulated ADMA level during bone formation.

**Fig. 3** Ddah1 deficiency in osteoblast-lineage cells leads to lower bone mass. a) Relative mRNA expression of Ddah1 in several different tissues of C57 mice. b) RT-PCR assay of Ddah1 in osteoblasts under osteogenic medium. c) Indicated protein levels of DDAH1 in osteoblasts under osteogenic medium. d) Immunofluorescence staining of DDAH1 expression in osteoblasts. Scale bar = 100 μm. Green, DDAH1. Red, Phalloidin. Blue, DAPI. e) Representative micro-CT images of trabecular bone and cortical bone in the distal femur (left) in littermate controls (Ddah1f/f, n = 6) and osteoblast-specific knock out mice (Ddah1prx1, n = 5) male mice at 4 weeks of age, and Ddah1f/f(n = 6) compared with Ddah1prx1(n = 6) at 12 weeks of age. f) Bone volume/total volume (BV/TV), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), trabecular bone separation (Tb.Sp), and cortical bone thickness (Ct.Th) of Ddah1f/f and Ddah1prx1. 4-week-old Ddah1f/f, n = 6, Ddah1prx1, n = 5. 12-week-old Ddah1f/f, n = 6, Ddah1prx1, n = 6. **p < 0.01. Data are represented as mean values ± SD. g) Representative histomorphometric images of the femur and calcein double staining. Scale bars, 100 μm. h) Quantification of histomorphometric parameters of femur in Ddah1f/f and Ddah1prx1 littermates at 12 weeks of age. Ddah1f/f, n = 6, Ddah1prx1, n = 6. **p < 0.01. Data are represented as mean values ± SD. i) Representative immunohistochemistry staining images of trabecular bone in Ddah1f/f and Ddah1prx1 mice. Red arrows for OCN positive cells. Scale bar = 100 μm. j) Quantification analysis of OCN+ cells in Ddah1f/f and Ddah1prx1 mice. Ddah1f/f, n = 5. Ddah1prx1, n = 5. **p < 0.01. Data are represented as mean values ± SD. k) Biomechanical analysis of the femurs of Ddah1f/f and Ddah1prx1 mice. Ddah1f/f, n = 8. Ddah1prx1, n = 5. *p < 0.05, **p < 0.01. Data are represented as mean values ± SD. The data were analyzed by an unpaired two-tailed Student’s t test in two groups compared. One-way analysis of variance (ANOVA) with post-hoc Tukey’s test was used for experiments with three or more groups.

**Discussion**

In this study, we found that the -394 4N del/ins polymorphism of Ddah1 was closely associated with low BMD, as well as an increased concentration of ADMA in human serum samples. Deletion of Ddah1 but not Ddah2 led to bone loss in mice mainly via impairing bone formation. Meanwhile, an increase of ADMA regulated by Ddah1 deficiency was contributed to bone loss. In particular, high ADMA accumulation was observed and did not respond to exercise-induced bone formation in Ddah1 conditional knockout mice. Underlying mechanism study unveiled that mechanical force enhanced TAZ/SMAD4 mediated Ddah1 transcription, which in turn regulated ADMA level during bone formation.
formation. Finally, inhibition of DDAH1 by PD404182 reduced bone formation in wild-type mice, but administration of AAV-DDAH1 accelerated bone healing in bone defect mice.

Recent discoveries have highlighted the SNPs of Ddah1 was associated with cardiovascular disease and diabetes 10–12. In particular, a novel loss-of-function Ddah1 promoter polymorphism was associated with increased susceptibility to cardiovascular diseases and thrombosis stroke12. Thus, these SNPs of Ddah1 were likely associated with metabolic syndrome (MS), which was regarded as an increased risk factor of low bone density. Finally, inhibition of DDAH1 by PD404182 reduced bone formation in wild-type mice, but administration of AAV-DDAH1 accelerated bone healing in bone defect mice.

**Fig. 4** Ddah1 deficiency increases concentrations of ADMA to induce bone loss in vivo. a Serum and bone samples of 10-week old Ddah1+/+ and Ddah1−/− mice were analyzed by LC-MS assay. Ddah1+/+, n = 5. Ddah1−/−, n = 5. **p < 0.01. ***p < 0.005. Data are represented as mean values ± SD. b Serum and bone samples of 12-week-old Ddah1+/+ and Ddah1+/− mice were analyzed by an ADMA specific ELISA assay. Ddah1+/+, n = 5. Ddah1+/−, n = 5. *p < 0.05. **p < 0.01. Data are represented as mean values ± SD. c Immunofluorescence staining of eNOS (green) in bones of Ddah1+/+ and Ddah1−/− mice. Scale bar = 100 μm. d Immunofluorescence staining of NOS enzymes in bones of Ddah1+/+ and Ddah1+/− mice. Scale bar = 100 μm. e Mice were treated with saline solution or ADMA (12.5 mg/ml), and typical micro-CT images from tibiae are shown. Vehicle-treated group, n = 5, ADMA-treated group, n = 5. f Bone-microstructure analysis by micro-CT. The bone volume of total volume (BV/TV), trabecular bone separation (Tb.Sp), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), and cortical bone thickness (Ct.Th) values are shown. *p < 0.05. Data are represented as mean values ± SD. g The serum ADMA concentrations were confirmed in ELISA assays. Vehicle-treated group, n = 5, ADMA-treated group, n = 5. **p < 0.01. Data are represented as mean values ± SD. h Serum P1NP concentrations were confirmed by performing ELISA assays. Vehicle-treated group, n = 5, ADMA-treated group, n = 5. **p < 0.01. Data are represented as mean values ± SD. i Bone biomechanics were analyzed by performing a three-point bending mechanics test. The bone stiffness and ultimate force are shown. Vehicle-treated group, n = 5, ADMA-treated group, n = 5. *p < 0.05. Data are represented as mean values ± SD. The data were analyzed by an unpaired two-tailed Student’s t test in two groups compared.
However, whether the loss-of-function of Ddah1 promoter polymorphism being contributed to bone mineral density was not clear. As we have shown, the -394 4N del/ins polymorphism was strongly associated with bone mineral density in a large Chinese population. Meanwhile, the plasma concentration of ADMA was correlated with the -394 4N del/ins polymorphism of Ddah1, which was contributed to the expression of Ddah1. Therefore, these data suggested that loss-of-function DDAH1 promoter polymorphism was a risk factor for bone loss in humans.

To further explore whether DDAH expression was contributed to bone metabolism or not, we firstly generated Ddah1−/− mice and Ddah2−/− mice. The results suggested that global knockout of Ddah1 but not Ddah2 resulted in bone loss in mice mainly via...
Fig. 5 The expression of DDAH1 is responsive to mechanical force via TAZ/SMAD4 signaling pathway. a The typical images of immunofluorescence staining of DDAH1 (green) and DDAH2 (red) in bones of exercise-treated or tail-suspension mice. Scale bar = 100 μm. b Quantification analysis of DDAH1 and DDAH2 expression of bone parameters, ns no significance. Ground group mice, n = 5. Unload group mice, n = 5. Control group, n = 5. Exercise group, n = 5. *p < 0.05. ***p < 0.005. Data are represented as mean values ± SD. c Metabonomics of serum samples of control mice and exercise mice. d Volcano plot of different metabolites responsive to exercise treatment. e Serum samples of exercise mice or tail-suspension mice were analyzed by LC-MS assay. Ground group mice, n = 5. Unload group mice, n = 5. Control group, n = 5. Exercise group, n = 5. ***p < 0.005. Data are represented as mean values ± SD. f Western blotting analysis of the association between SMAD4 and TAZ in simulation with tension force. Scale bars = 100 μm. g Co-IP assay of the association between SMAD4 and TAZ in simulation with tension force. Data shown in all panels were analyzed by an unpaired two-tailed Student’s t test. One-way analysis of variance (ANOVA) with post-hoc Tukey’s test was used for experiments with three or more groups.

Fig. 6 Ddah1 deficiency in osteoblast-lineage cells lacks of the response to mechanical stimulation. a Representative micro-CT images of trabecular bone and cortical bone in the distal femur (left) of Ddah1f/f and Ddah1prx1 male mice under exercise condition. b Bone volume/total volume (BV/TV) and cortical bone thickness (Ct. Th.) of Ddah1f/f and Ddah1prx1 mice. *p < 0.05. ns, no significance. Ddah1f/f control, n = 5. Ddah1f/f exercise, n = 5. Ddah1prx1 control, n = 4. Ddah1prx1 exercise, n = 4. Data are represented as mean values ± SD. c Representative immunofluorescence staining images of trabecular bone in Ddah1f/f and Ddah1prx1 mice treated with running or not. Red for DDAH1, Green for OCN. Scale bar =100 μm. d Quantification analysis of DDAH1 positive or OCN positive cells in bone parameters, ns, no significance. ns, no significance. Ddah1f/f control, n = 5. Ddah1f/f exercise, n = 5. Ddah1prx1 control, n = 5. Ddah1prx1 exercise, n = 5. *p < 0.05. **p < 0.01. ***p < 0.005. Data are represented as mean values ± SD. e Representative double-calcein staining images of trabecular bone in Ddah1f/f and Ddah1prx1 mice treated with running or not. Scale bars =100 μm. f MAR of double-calcein staining analysis. ns, no significance. Ddah1f/f control, n = 4. Ddah1f/f exercise, n = 4. Ddah1prx1 control, n = 4. Ddah1prx1 exercise, n = 4. *p < 0.05. Data are represented as mean values ± SD. g Results from LC-MS assay for ADMA concentrations of Ddah1prx1 and Ddah1f/f treated with running or not, ns, no significance. Ddah1f/f control, n = 4. Ddah1f/f exercise, n = 4. Ddah1prx1 control, n = 4. Ddah1prx1 exercise, n = 4. *p < 0.05. **p < 0.01. Data are represented as mean values ± SD. The data were analyzed by an unpaired two-tailed Student’s t test in two groups compared. One-way analysis of variance (ANOVA) with post-hoc Tukey’s test was used for experiments with three or more groups.
suppressing bone formation, which might be due to the unchanged concentrations of ADMA. We compared the DDAH1 expression in osteoblasts with that in osteoclasts. However, we confirmed that DDAH1 expression was relatively abundant in osteoblasts. Thus, we speculated that the function of osteoclasts was relatively insignificant compared with the function of osteoblasts in Ddah1<sup>−/−</sup> mice. Previous studies have focused on the effects of DDAH1 on brain, kidney and heart tissues because DDAH1 has beneficial effects on nitric oxide synthase (NOS) to regulate NO production by degrading ADMA. NO production was also important for bone homeostasis. NOS expression has been examined in osteoblast lineage cells both in vitro and in vivo, and eNOS is the most expressive isoform in bone tissue. It is suggested that deletion of eNOS gene leads to
impaired osteogenesis and bone formation, which implied that loss of NO production leads to a reduction of osteogenesis. As expected, we found that concentrations of ADMA in serum samples of Ddah1+/− mice and Ddah1Prx1 mice were increased, while concentrations of ADMA in bone samples of Ddah1+/− mice and Ddah1Prx1 mice were also increased. The results provided evidence that deletion of Ddah1 in osteoblast-lineage cells led to the accumulation of ADMA both in bone marrow niches and peripheral blood. Given the elevation of ADMA concentration contributed to reducing NO production and NO is generally beneficial for osteogenesis, we propose that this downstream mechanism is partially involved in the effects of ADMA on bone. Subsequently, the data suggested that NO production was indeed decreased both in the bones of Ddah1+/− mice and Ddah1Prx1 mice. Consistent with the previous data, mice bred with a high dose of ADMA reduced bone mass, due to weakened bone formation.

As the previous studies reported, mechanical force regulated NO synthesis to enhance the function of endothelial cells and osteoblasts, and the efficiency of NO production was important for MS and bone diseases17–19. While the exercise was confirmed to enhance bone mass and bone strength39. However, DDAH1-mediated ADMA hydrolyzation response to mechanical force was still unclear. To address the hypothesis, we treated mice by tail-suspension and running on a treadmill. Intriguingly, we found that only DDAH1 was response to different mechanical treatments with increased levels of ADMA in unloading mice or decreased levels of ADMA in exercise mice. To date, several proteins as key regulators that are responsive to mechanical forces, such as YAP/TAZ, Piezo1/2, and β-catenin. Laminar shear stress promoted β-catenin nuclear localization in MSCs, which upregulated downstream genes and enhanced osteogenesis40. YAP and TAZ are key molecules of the hippo signaling pathway, which are also regarded as mechanosensitive transcription regulators41. It has been suggested that the localization and transcriptional activity of YAP and TAZ are regulated by the extracellular matrix (ECM) stiffness and external mechanical force42. Other researchers demonstrated that YAP/TAZ signaling was mediated by LATS, which depended on the actin cytoskeleton33. Although YAP and TAZ are generally considered as cofactors to regulate cell fate, data from several studies have suggested that they also can function independently. Pan et al.44 revealed that YAP itself promoted osteogenesis and suppressed adipogenesis by regulating β-catenin signaling. While Byun et al.45 provided evidence suggesting that Wnt signaling stabilizes TAZ but not YAP to regulate osteogenesis. In addition, YAP nuclear localization is influenced by Piezo1 in osteoblasts. Piezo1 has been reported to coordinate osteoblast-osteoclast crosstalk through directly responding to mechanical forces in osteoblasts46. Meanwhile, Zhou et al.47 also suggested that Piezo1/2-mediated mechanotransduction was essential for bone formation. In this study, we found that neither YAP nor Piezo1 affects the expression of DDAH1 during stimulation with tension force. However, TAZ played a role in regulating DDAH1 transcription. Previously findings showed that the TAZ/SMAD4 axis played a reciprocal role in promoting osteogenesis, and direct binding to SMAD4 promotes the nuclear retention of TAZ33. Interestingly, our data suggest that TAZ recruits SMAD4 to promote nuclear localization of SMAD4 to enhance the transcriptional expression of Ddah1. Meanwhile, mechanical forces enhanced the binding capacity between TAZ and SMAD4, as well as transcription of Ddah1.

To further confirm whether exercise treatment enhanced bone mass and quality by regulating DDAH1/ADMA pathway, we forced Ddah1+/− and Ddah1Prx1 mice to run on the treadmill. Exercise dramatically enhanced trabecular bone volume of Ddah1+/− mice, but slightly enhanced that of Ddah1Prx1 mice. Notably, the cortical thickness of Ddah1+/− mice was increased by treatment with exercise, but it was not significantly changed in Ddah1Prx1 mice. Consistent with the micro-CT analysis, OCN positive cells and MAR were also not significantly increased in Ddah1Prx1 mice after treatment with running on a treadmill. Meanwhile, the serum level of ADMA was not eliminated by bone tissues in Ddah1Prx1 mice. Thus, our data suggest that deletion of Ddah1 in osteoblast-lineage cells impairs the ability to hydrolyze ADMA in vivo. On the other hand, tail-suspension treatment-induced bone loss and accumulation of ADMA in vivo. However, inhibition of DDAH1 by intraperitoneal injection with PD040182 promoted bone loss in vivo by inhibiting bone formation, but the effects were not found in Ddah1+/− mice. The data provided evidence that improving inhibition of DDAH1 could inhibit bone formation in vivo. In addition, administration with AAV-DDAH1 accelerated bone defect healing in Ddah1Prx1 mice compared with AAV-Zsgreen treated mice, which was consistent with the previous data.

To date, the reduction of bone mass due to osteoporosis has been treated by different approaches including bisphosphonates, PTH, denosumab, and romosozumab48–50 that still have the limitations of treating osteoporosis. Indeed, astronauts and prolonged bed-rest patients are typically bearing serious bone loss and decrease in bone strength51,52. Although the underlying mechanism may be complicated, our study at least demonstrated that ADMA and DDAH1 levels are associated with disuse-related
osteoarthritis, while deletion of Ddahi1, a mechanical response gene, is contributed to the reduction of bone formation. Our findings also inform that further studies can be focused on potential therapeutic approaches related to reducing ADMA or enhancing DDAH1 for preventing osteoporosis.

Methods

Mice and in vivo treatment. Generation of Ddah1m/mice were described previously from Prof. Yingjie Chen, University of Minnesota (Mn). Ddah1 conditionally knockout (Ddah1 cKO) in osteoblast lineage cells were generated by crossing male Ddah1+/y (a gift from Prof. Yingjie Chen, University of Minnesota) with female Prx1cre mice (Jackson Lab #008584). Male Ddah1+/y and Ddah1m/mice were sacrificed at 12 weeks of age. Male and female Ddah1f/f (C57BL/6N-Ddah1tm1cyagen) were offered by Cyagen Biology Technology. Female wild-type C57BL/6 (B6) mice were from the SLAC Laboratory Animal Company (Shanghai, China). All animal procedures were conducted in compliance with all applicable ethical regulations using procedures approved by the Sir Run Run Shaw Hospital Committee for Animal Resources. The mice were housed with conditions of 12 h dark/12 h light cycle, 22 °C ambient temperature, and 50% humidity. All mice were routinely genotyped using standard PCR protocols.

The compounds used in this study are as follows: NG,NG-dimethyl-L-Arginine (ADMA, APEX BIO, CS216), PD 404182 (Ddah1 inhibitor, R&D Systems, #5124). Dosages and time courses are noted in the corresponding text and figure legends.

Mouse exercise protocol. Exercise capacity was determined using a treadmill (Life Science, Woodland Hills, CA, USA) running tests as detailed below and previously described84. Briefly, exercise group mice were subjected to running on the treadmill at speed of 20 cm/s, 30 min/day for 21 consecutive days. All animal studies were performed according to approved guidelines for the use and care of live animals (Guideline on Administration of Laboratory Animals released in 1988, and 2006 Guideline on Humane Treatment of Laboratory Animals from China). All of the experimental procedures were approved by the Committees of Animal Ethics and Experimental Safety of Sir Run Run Shaw Hospital and Zhejiang Medical University.

Tail-suspension mouse model. Tail-suspension is attempted to achieve an unloaded status of hindlimbs. Briefly, the 12-week-old WT mice were individually caged or suspended by the tail with a strip of adhesive surgical tape that was attached to a chain hanging from a pulley. The mice were suspended at a 30° angle to the floor with only the forelimbs touching the floor, which allowed the mice to move and to access food and water freely. The mice were subjected to hindlimb unloading through tail suspension for 28 days. After euthanasia, the bone tissues were collected. All animal studies were performed according to approved guidelines for the use and care of live animals (Guideline on Administration of Laboratory Animals released in 1988, and 2006 Guideline on Humane Treatment of Laboratory Animals from China). All of the experimental procedures were approved by the Committees of Animal Ethics and Experimental Safety of Sir Run Run Shaw Hospital.

Adeno-associated virus administration model. When assessing the therapeutic effects of AAV-DDAHA1 or AAV-ZsGreen, the gelatin sponge was manually soaked with AAV-DDAHA1 or AAV-ZsGreen for 1 h on ice and immediately placed to the bone defect area of femurs in Ddah1+/y and Ddah1m/mice. All mice were euthanized by CO2 at time points indicated, the femurs were analyzed by micro-CT and biomechanical testing

Cell culture and in vitro treatment. Primary murine pre-osteoblasts were isolated from calvarial cells of fetal mice, while bone marrow mesenchymal stem cells (BMSCs) were extracted from femurs and tibiae of 6-week-old mice. The marrow plug was flushed by using syringe with a range of 1 mL. These plugs were then dispelled into single cell and were seeded in a 10-cm dish containing α-MEM (Corning, New York, USA) supplemented with 10% fetal bovine serum (FBS). For osteogenesis, mature osteoblasts were differentiated using the standard osteogenic medium in α-MEM with 10% FBS, 50 μg/ml l-ascorbic acid, and 1080 mg/ml β-glycerophosphate, 1% penicillin/streptomycin. The osteoblast differentiation test was performed as the osteoblastogenesis protocol, as the previous study described47. For quantitative analysis of ALP activity, osteoblasts were cultured with 0.1% Alizarin Red S solution, 1% formaldehyde for 48 h. Alizarin Red S staining, mice limbs were not decalcified, they were embedded into methacrylate and cut into 5-μm-thick sections.

Immunofluorescence assay. Immunofluorescence was mainly performed as described46. Briefly, freshly dissected bones were fixed in 4% paraformaldehyde for 48 h and incubated in 15% DEPC-EDEA (pH 7.8) for decalcification. Then, specimens were embedded in paraffin or OCT and sectioned at 8 μm. Sections were blocked in PBS with 10% horse serum for 1 h and then stained overnight with mouse-anti-Osteocalcin (Santa Cruz, 1:100, sc-376726), rabbit-anti-Ddah1 (SAB, 1:200, #37368), mouse-anti-Ddahi1 (Santa Cruz, 1:100, sc-271337), rabbit-anti-Ddah2 (SAB, 1:200, #38934), mouse-anti-TAZ (Abcam, 1:200, ab242313), rabbit-anti-YAP (Abcam, 1:200, ab52771), and eNOS (Santa Cruz, 1:200, sc-376751), Goat-anti-mouse FITC (1:1000; Jackson ImmunoResearch, 705-165-147) and donkey-anti-rabbit Alexa Fluor 488 (1:1000; Molecular Probes, A21206) were used as secondary antibodies. DAPI (Cell Signaling Technology, #4083) and DyLight™ 594 Phalloidin (Cell Signaling Technology, #12877) were used for counterstaining. All immunofluorescence experiments were confirmed by at least one independent repeat. An Olympus IX81 confocal microscope or Zeiss LSM-880 confocal microscope was used to image samples.

Mice serum samples analysis. The bone turnover marker pro-collagen type I N-terminal peptide (PINP) were measured in the serum using ELISA kits (Elabscience Biotechnology, Wuhan, China) according to the manufacturer’s instructions. Serum ADMA and bone tissue ADMA concentrations were determined by LC-MS assay.

RNA isolation, reverse transcription, and real-time PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) or RNeasy Mini Kit (Qiagen), and reverse transcription was performed with the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). Real-time PCR analysis was performed using the PRISM 7500 real-time PCR system (Applied Biosystems). All experiments were conducted in triplicate samples, and the data were expressed as the fold change compared to the control group.
Transcription Kit from Applied Biosystems according to the manufacturer’s instructions. We performed quantitative analysis of gene expression using SYBR® Green PCR Master Mix (Applied Biosystems) with the LightCycler 480 real-time PCR system (Roche Life Science, China). Gapdh expression was used as an internal control. The sequence of the primers used for PCR is listed in Supplementary table 3.

Western blot assay. Western blot assay was performed according to previously described standard protocol. Primary antibodies were specific for DDH1 (1:1000; SAB, 57268), phospho-Ser-179 YAP (1:1000; Cell Signaling Technology, #4911), YAP (1:1000; Cell Signaling Technology, #14074), TAZ (1:1000; Cell Signaling Technology, #83669), ALP (1:500; Santa Cruz, sc-271431), SMAD4 (1:1000; Cell Signaling Technology, #46353), GAPDH (1:5000; Protextech, #66004-1-lg), alpha-tubulin (1:5000; Protextech, #66031-1-lg), and Histone H3 (1:5000; Protextech, #171682). Secondary anti-mouse/rabbit HRP-conjugated antibodies were subsequently applied.

Co-immunoprecipitation assay. As performed in our previous study, briefly, cell extracts were first precleared with 25 μL of protein A/G-agarose (50% v/v). The supernatants were immunoprecipitated with 2 μg of anti-TAZ antibodies for overnight at 4 °C, followed by incubation with protein A/G-agarose for 4 h at 4 °C. Protein A/G-agarose-antigen-antibody complexes were collected by centrifugation at 300 g for 60 s at 4 °C. The pellets were washed five times with 1 mL IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1 mM PMFS), for 5 min each at 4 °C. Bound proteins were resolved by SDS-PAGE, followed by western blotting with the anti-TAZ, anti-SMAD4 or anti-GAPDH antibodies. The experiments were replicated at least three times.

Chromatin immunoprecipitation assay. Transcription factor binding sites within ~2000bps before the murine Ddah1 coding start site was searched using TFSEARCH software to identify putative Ddah1 binding sites. ChIP assays were performed to test for binding of Smad4 to each of the seven Ddah1 binding sites, following our published procedure. Briefly, the sheared chromatin from control and tensile-treatment pre-obs that had been fixed with 1% formaldehyde was immunoprecipitated with antibodies to SMAD4, or rabbit IgG as a negative control. The precipitated DNA was used as a template for PCR using primers specifically designed to amplify a segment of 100–200 bps containing the putative Ddah1 binding sites. The sequences of the primers are listed in Supplementary table 4.

Luciferase reporter gene assay. Luciferase reporter gene assay was performed as the instruction indicated (Beiyue Technology #RG028, Shanghai, China). Briefly, pre-obs were plated in 24-well plates in triplicate, and the cells were transfected with different concentrations of pcDNA3 basic or PC-3Ddah1-Luc. After 24 h, the transfected cells were lysed with the lysis buffer (Promega, Madison, WI, USA). Renilla luciferase expression was determined, and luciferase activity was measured using a luciferase assay system (Promega, USA).

Metabolomics sample preparation, quality control, data extraction and analysis

Sample preparation. Samples were prepared using the automated MicroLab STAR system from Hamilton Company. The sample extracts were stored overnight under nitrogen before preparation for further experiments. QA/QC. Several types of control were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample served as a technical replicate throughout the data set, extracted water samples served as process blanks, and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds was spiked into every analyzed sample to allow instrumental performance monitoring and aided chromatographic alignment. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections.

Ultra-high performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS). All methods utilized a Waters ACQUITY ultra-performance liquid chromatography and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent containing a series of standards at fixed concentrations to ensure injection and chromatographic consistency. The extract was gradient eluted from a C18 column (Waters UPLC BEH C18 2.1 × 100 mm, 2.5 μm) using water and methanol containing 0.05% perfluorooctanoic acid (PFA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, it was optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% PFFA and 0.01% FA and was operated at an overall higher flow for data analysis. Log aliquot was analyzed using negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, with 6.5 mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1 × 100 mm, 2.5 μm) using a gradient consisting of water and acetonitrile with 10 mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slightly between methods but covered 70–1000 m/z.

Data preprocessing and statistical analysis. The acquired LC-MS raw data were analyzed by the progenss QI software (Waters Corporation, Milford, USA) using the following parameters. The precursor tolerance was set to 5 ppm, fragment tolerance was set at 10 ppm, and retention time (RT) tolerance was set at 0.02 min. Internal standard detection parameters were deselected for peak RT alignment, isotope peaks were excluded for analysis, and noise elimination level was set at 10.00, the minimum intensity was set to 15% of base peak intensity. The Excel file was obtained with 3D data sets including m/z, peak RT and peak intensities, and RT-m/z pairs were used as the identifier for each ion. The resulting matrix was further reduced by removing any peaks with a missing value (ion intensity = 0) in more than 50% of samples. The internal standard was used for data QC (reproducibility). Metabolites were identified by the progess QI (WatersCorporation, Milford, USA) Data Processing Software, based on public databases and self-built databases. The positive and negative data were combined to get a combined data which was imported into R packages. Principle component analysis (PCA) and (orthogonal) partial least-squares-discriminant analysis (OPLS-DA) were carried out to visualize the metabolic alterations among experimental groups, after mean-centering (Cteto) and Pareto variance (Par) scaling, respectively. The Hotelling’s T2 region, shown as an ellipse in score plots of the models, defines the 95% confidence interval of the modeled variation. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the OPLS-DA model, and those variables with VIP > 1 are considered relevant for group discrimination. In this study, the default 7-round cross-validation was applied with 1/seventh of the samples being excluded from the mathematical model in each round, in order to guard against overfitting. The differential metabolites were selected on the basis of the combination of a statistically significant threshold of variable influence on projection (VIP) values obtained from the OPLS-DA model and p values from a two-tailed Student’s t test on the normalized peak areas, where metabolites with VIP values >1.0 and p values <0.05 were considered as differential metabolites.

All reagents were analytical or HPLC grade, which were from CNW Technologies GmbH (Düsseldorf, Germany). L-2-chlorophenylalanine was from Shanghai Hengchuang Bio-tecnoogy Co., Ltd. (Shanghai, China).

Collection and determination of human serum and hemocytes sample. Venous blood samples were taken from the cubital vein of patients. The sampling was performed between 7:00 a.m. and 9:00 a.m. 7 days after the intervention. Blood samples were centrifuged for dividing serum aliquots and hemocytes. Both of them were stored at −80 °C. The information of patients was provided in Supplementary table 2. In brief, all patients and controls were of Han Chinese ancestry, and the three different groups were determined by T values (T ≥ −1.0 to control group, −1.0 ≤ T < −3.5) in osteoporosis group, and T ≤ −3.5 to osteoporosis group).

Serum samples were determined on the LC-MS assay to investigate the concentrations of ADMA, ADMA standard was used as a control, and we determined used SDMA standard to distinguish the differences between ADMA and SDMA. In our analysis, the linear associated results of ADMA and SDMA are good in the range of 0.1–1000 ng/ml. The QC results of serum samples and standard samples were verified.

DNA samples were extracted from hemocytes, and the characteristics of individuals were provided in Supplementary table 1. Then, the polymorphism genotyping analysis was performed by the SNaPshot assay method. The polymorphism of Ddah1 (Probe sequences were listed in Supplementary table 5) by LightCycler 480 real-time PCR system (Roche Life Science, China), and SNaPshot assay (Supported by GENESKY, Shanghai, China).

Statistical and reproducibility. Each experiment was performed at least three times, data are presented as mean ± standard deviation (S.D.). The variance was similar between groups for most parameters assessed. The normality of data was determined using the Kolmogorov-Smirnov test. In cases where data were normally distributed, the one-way analysis of variance (ANOVA) was used to determine the significance of the polymorphism of Ddah1 (Supported by GENESKY, Shanghai, China).

Graphs and statistics were prepared using GraphPad Prism 8.0 software.
Supplementary Information

Source data are provided with this paper as supplementary information. Source data are provided with this paper.

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Competing interests
The authors declare no competing interests.

Additional information
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