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Title: Vanin-1 is a key activator for hepatic gluconeogenesis

Short title: Vanin-1 activates gluconeogenesis

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ABSTRACT

Vanin-1 (VNN1) is a liver-enriched oxidative stress sensor that has been implicated in the regulation of multiple metabolic pathways. Clinical investigations indicated that the levels of VNN1 were increased in the urine and blood of diabetic patients, but the physiological significance of this phenomenon remains unknown. In this study, we demonstrated that the hepatic expression of VNN1 was induced in fasted mice or mice with insulin resistance. Gain- and loss-of-function studies indicated that VNN1 increased the expression of gluconeogenic genes and hepatic glucose output, which led to hyperglycemia. These effects of VNN1 on gluconeogenesis were mediated by the regulation of the Akt signaling pathway. Mechanistically, vnnl transcription was activated by the synergistic interaction of PGC-1α and HNF-4α. A ChIP analysis indicated that PGC-1α was present near the HNF4α binding site on the proximal vnnl promoter and activated the chromatin structure. Taken together, our results suggest an important role for VNN1 in regulating hepatic gluconeogenesis. Therefore, VNN1 may serve as a potential therapeutic target for the treatment of metabolic diseases caused by over-activated gluconeogenesis.
Hepatic gluconeogenesis plays a crucial role in maintaining blood glucose homeostasis in mammals. Gluconeogenesis is activated by fasting, which means that the synthesis and secretion of glucagon and glucocorticoids are induced to enhance hepatic glucose production via the stimulation of \textit{pepck} and \textit{g6pase} expression (1,2). In contrast, insulin suppresses gluconeogenesis via the lipid kinase PI3K pathway and correspondingly activates glycogen synthesis when sufficient food is available (3). Thus, gluconeogenesis is finely regulated by positive and negative regulators to achieve a balance. However, gluconeogenesis is constitutively induced due to insulin resistance in certain pathophysiological conditions, such as type 2 diabetes, and leads to excess glucose secretion and aggravated hyperglycemia (4,5).

PGC-1α is one of the most important metabolic regulators and has been shown to stimulate various physiological processes by selectively activating nuclear receptors and transcriptional factors (6). Of note, PGC-1α robustly regulates gluconeogenesis. PGC-1α is elevated in the liver at a fasted or insulin-resistant state when gluconeogenesis is activated (7). In contrast, the liver-specific knockdown of PGC-1α significantly improves glucose intolerance during hyperglycemia (8). At the molecular level, PGC-1α facilitates the transcriptional activity of the glucocorticoid receptors (GR), FOXO1 and HNF4α on the promoter of gluconeogenic genes (9,10). These findings indicate that PGC-1α is a critical factor in the regulation of gluconeogenesis and glucose homeostasis.

Vanin-1 (VNN1) is a glycosylphosphatidyl inositol (GPI)-anchored pantetheinase that is highly expressed in the liver, gut, and kidney (11). Its pantetheinase activity
hydrolyzes pantetheine into pantotheic acid (vitamin B5) and cysteamine (12). Pantotheic acid acts as the structural component of Coenzyme A (13), which indicates that VNN1 may be involved in fatty acid metabolism. Conversely, cysteamine inhibits the gamma-glutamylcysteine synthetase (γ-GCS)-mediated regulation of the endogenous GSH pool and therefore affects the cellular redox status. VNN1-deficient mice exhibit significantly increased resistance to stress, and their intestinal inflammation is reduced (14-16). Importantly, the link between VNN1 and metabolic diseases has been recently revealed. For example, VNN1 protects islet beta cells from STZ-induced injury and regulates the development of type 1 diabetes (17). In the liver, the mRNA expression levels of VNN1, as well as its activity, are induced upon fasting (18,19). A clinical study showed that the concentrations of VNN1 in pooled human urine distinguished diabetic patients with macroalbuminuria from those with normal albuminuria (20). In addition, the combination of VNN1 and MMP9 has been suggested as a novel blood biomarker panel to discriminate pancreatic cancer-associated diabetes from type 2 diabetes (21). However, these findings are descriptive, and the detailed mechanism through which VNN1 regulates energy metabolism remains unknown.

Based on the findings mentioned above, we aimed to explore whether VNN1 regulates hepatic gluconeogenesis and if this process requires PGC-1α participation. To answer this question, we used the gain-of-function and loss-of-function strategy to manipulate the VNN1 expression levels both in vivo and in vitro based on adenoviral transduction and siRNA delivery. Our studies revealed an important role for VNN1 in
the regulation of gluconeogenesis and suggest that VNN1 inhibition may be a therapeutic target for the treatment of metabolic disorders associated with over-activated gluconeogenesis.
RESEARCH DESIGN AND METHODS

Male C57BL/6 mice and diabetic db/db mice on a C57BKS background were purchased from the Model Animal Research Center of Nanjing University, Nanjing, Jiangsu, China. All animal procedures in this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996 and the approved regulations set by the Laboratory Animal Care Committee at Nanjing Normal University (permit number 2090658, issued date April 20, 2008). For fasting-induced gluconeogenesis, the mice were subjected to 2 hours (h), 5 h, and 16 h fasting or 16 h fasting followed by 20 h re-feeding. To induce insulin resistance, 4-week-old mice were fed on a high-fat diet (HFD, fat content 60%, Research Diets, USA) for two months (22). To specifically manipulate the target gene expression in liver, we transduced adenoviruses targeting genes or siRNA oligonucleotides against the target genes into mice through tail-vein injection. For these experiments, adenoviruses were concentrated and purified at $1.5 \times 10^9$ plaque-forming units (pfu). Three days after the injection, all mice were killed and the livers were collected. Wortmannin (Sigma) was intraperitoneally injected into mice at a dose of 1.5 mg/kg body weight 12 h before killing when necessary (23).

**Pantetheinase activity assay.** The substrate, pantothenate-AMC, was chemically synthesized using β-alanine 7-amido-4-methylcoumarin trifluoroacetic acid (TFA) salt (H-β-Ala-AMC.TFA, 36 mg, 1eq) and R-(−)-pantolactone. We used
pantothenate-AMC as substrate at 37°C for 30 min to assay the pantetheinase activity; the hydrolysis catalyzed by VNN1 yields pantothenic acid and detectable free fluorescent AMC (excitation 340 nm, emission 460 nm) (24). The liver extracts were washed three times with PBS and lysed with potassium phosphate buffer (100 mM, pH 7.5) containing 0.1% Triton X-100 and 0.6% sulfosalicylic acid. The protein concentrations were determined with a BCA protein assay. The enzymatic assay was carried out using 10 µg of liver extract in phosphate buffer (100 mM potassium phosphate buffer, pH 7.5) containing 2 mM pantothenate-AMC, 0.01% BSA, 0.5 mM DTT, 5% DMSO, and 0.0025% Brij-35 in a total reaction mixture volume of 100 µl. The reactions were carried out at 37°C in the presence or absence of liver extract, and the fluorescence (Excitation 350 nm and emission 460 nm) was recorded every 2 min, with the change in fluorescence measured over a 30 min period. A standard curve was generated using purified recombinant VNN1 (Sino Biological, China) at the same buffer conditions described above. The VNN1 activity was normalized for total protein content. The pantetheinase activity was calculated by determining the slope at 30 min from fitting the data to the standard curve and normalizing it for total protein content.

Statistical analysis. The groups of data are presented as the mean ± standard deviation. The data were analyzed using a one-way ANOVA followed by Fisher’s LSD post-hoc test. The calculations were performed using the SPSS for Windows version 12.5S statistical package (SPSS, Chicago, USA). A value of \( P<0.05 \) was
considered statistically significant.
RESULTS

Gluconeogenic signals induce hepatic VNN1 expression. Fasting-re-feeding cycles are robust nutritional signals to regulate gluconeogenesis. As expected, the hepatic mRNA expression of *pgc-1α*, an important metabolic regulator, was induced by fasting and peaked at 5 h (Fig. 1A). Subsequently, its expression gradually declined. Two gluconeogenic genes (*pepck* and *g6pase*) were induced after 2 h of fasting, and this expression persisted during the entire starvation period. Interestingly, the hepatic expression of *vnn1* mRNA was also induced in mice subjected to fasting and decreased upon re-feeding at 20 h. The protein expression levels of VNN1 in the liver showed a similar pattern (Fig. 1B and Supplementary Fig. 1A). Conversely, diabetic *db/db* mice and high-fat diet (HFD) feeding-induced obese mice developed insulin resistance and showed over-activated hepatic gluconeogenesis. We found that the VNN1 mRNA and protein expression levels were increased in livers of these mice (Fig. 1C-E, Supplementary Fig. 1B and C). Furthermore, VNN1 is a pantetheianase, and the biological functions of VNN1 are thought to depend on its pantetheianase enzymatic activity. In our settings, the pantetheianase activity of VNN1 increased in the liver homogenates of fasted and *db/db* mice (Fig. 1F and G), which is consistent with the mRNA accumulation of VNN1.

*In vitro* studies indicated that 8-Br cAMP, an analogue of the natural signal molecule cAMP that activates gluconeogenesis, stimulated *vnn1* expression in a time-dependent manner in AML-12 mouse hepatocytes (Fig. 1H). 8-Br cAMP also increased the transcriptional activity of the *vnn1* promoter (Fig. 1I). In addition to
cAMP, fasting-induced hormones, such as glucocorticoids, can robustly activate gluconeogenesis. However, we found that 8-Br cAMP, but not glucocorticoids (DEX and hydrocortisone), induced VNN1 protein expression in mouse primary hepatocytes (Fig. 1J). These data suggested that cAMP is one of the major players to activate VNN1 expression, whereas glucocorticoids have a modest effect on VNN1 expression.

VNN3 is another member of the pantetheinase family. In contrast to the robust induction of VNN1 in gluconeogenic settings, the hepatic expression of vnn3 remained stable in fasted mice and was only moderately increased (2.1-fold) in db/db mice (Supplementary Fig. 2A and B). Functionally, the knockdown of VNN3 in the livers of db/db mice (Supplementary Fig. 2C) did not improve the glucose intolerance (Supplementary Fig. 2D) and insulin resistance (Supplementary Fig. 2E) of these mice. The hepatic expression levels of gluconeogenic genes also remained unchanged (Supplementary Fig. 2F and G). Taken together, these results suggested that VNN3 does not regulate gluconeogenesis.

**VNN1 activates hepatic gluconeogenesis.** As shown in Fig. 2A and B, the forced expression of exogenous VNN1 in AML-12 cells by adenovirus infection induced the mRNA and protein expression levels of gluconeogenic genes in a dose-dependent manner. In contrast, VNN1 overexpression did not alter the mRNA expression levels of baf60a (a subunit of SWI/SNF chromatin remodeling complex), cyp7a1 (a regulator involved in bile acid metabolism), and fas (fatty acid synthase). Functionally,
the overexpression of VNN1 in mouse primary hepatocytes increased the glucose production rate (Fig. 2C). *In vivo* studies indicated that the basal blood glucose levels during starvation were higher in mice that specifically over-expressed VNN1 in the liver (Supplementary Table 3). These mice also showed impaired glucose tolerance and insulin sensitivity (Fig. 2D and E). At the molecular level, the hepatic expression levels of gluconeogenic genes were increased in response to VNN1 overexpression (Fig. 2F and G), while *baf60a, cyp7a1*, and *fas* remained unchanged. These data suggest that the impact of VNN1 is specific on the regulation of gluconeogenesis and is not a general disturbance of the hepatic metabolism. Finally, the pantetheinase activity of VNN1 in the liver homogenates of these mice significantly increased (Fig. 2H). Taken together, our results strongly suggested that VNN1 activates gluconeogenesis.

**Knockdown of VNN1 ameliorates hyperglycemia.** We next used a loss-of-function strategy to confirm the above findings. The liver-specific knockdown of VNN1 led to improved glucose tolerance in normal fasted mice (Fig. 3A). Consistently, the expression of hepatic gluconeogenic genes was suppressed upon the depletion of VNN1 (Fig. 3B and C). In a pathophysiological setting, hepatic VNN1 knockdown reduced the food and water intake of *db/db* mice (Fig. 3D). This treatment also relieved glucose intolerance and insulin resistance in animals (Fig. 3E and F). Accordingly, the mRNA and protein expression levels of gluconeogenic genes were repressed (Fig. 3G and H). Finally, the pantetheinase activity of VNN1 in the liver
homogenates of these mice decreased (Fig. 3I). In agreement with these results, VNN1 knockdown in AML-12 cells repressed the 8-Br cAMP-induced increase of the transcriptional activity of gluconeogenic gene promoters (Supplementary Fig. 3A). VNN1 knockdown also inhibited gluconeogenic gene expression in AML-12 cells (Supplementary Fig. 3B and C) and decreased the glucose production rate in mouse primary hepatocytes (Supplementary Fig. 3D) upon 8-Br cAMP challenge.

**Akt signal pathway mediates VNN1 regulation of gluconeogenesis.** The insulin/Akt signaling axis plays an important role in the regulation of gluconeogenesis. Therefore, we investigated whether this signaling cascade is involved in mediating VNN1’s effects on glucose control. As shown in Fig. 4A and B, the overexpression of VNN1 reduced the expression levels of phosphorylated Akt both *in vivo* and *in vitro*. Conversely, the liver-specific knockdown of VNN1 in *db/db* mice resulted in a marked increase in phospho-Akt levels (Fig. 4C). Similarly, the transfection of VNN1 siRNA into AML-12 cells increased the basal level and showed synergistic effects with insulin on Akt phosphorylation (Fig. 4D). To further prove the causal relationship between the Akt signaling pathway and VNN1 regulation, we used wortmannin (a specific PI3K/Akt inhibitor) to treat *db/db* mice with liver-specific VNN1 knockdown. Our data demonstrated that wortmannin alone further increased the basal glucose level and worsened impaired glucose tolerance in *db/db* mice. Additionally, the beneficial effects of VNN1 knockdown on glucose intolerance and insulin resistance were attenuated when mice were subsequently treated with
wortmannin (Fig. 4E and F). These results strongly suggest that the Akt signaling pathway is required to regulate VNN1 in hepatic gluconeogenesis. Because VNN1 is a GPI-anchored enzyme, identifying the molecule that mediates the signal cascade from VNN1 to the insulin/Akt signaling pathway is of interest. Previous studies demonstrated that VNN1 prevents PPARγ nuclear translocation and antagonizes its transcriptional activity in epithelial cells (25). We also observed similar phenomena in our system. Confocal imaging and the Western blotting of nuclear extracts indicated that VNN1 knockdown led to an increase in PPARγ translocation from the cytoplasm to the nuclei in AML-12 cells (Supplementary Fig. 4A and B). Given that PPARγ activates the insulin-Akt signaling pathway (26) and inhibits gluconeogenesis (27), PPARγ may act as a mediator of signal transduction from VNN1 to the Akt pathway.

**PGC-1α and HNF4α synergistically activate VNN1 transcription.** PGC-1α is a key nuclear coactivator of gluconeogenesis and may serve as an upstream regulator for vnn1 transcription. Indeed, PGC-1α overexpression increased the VNN1 expression levels both *in vivo* (Fig. 5A) and *in vitro* (Supplementary Fig. 5A and B). In contrast, PGC-1α knockdown inhibited VNN1 expression in the liver (Fig. 5B) and in cultured cells (Supplementary Fig. 5C and D). Epigenetically, histone hyperacetylation is associated with transcriptional activation. Conversely, H3K9me2 is typically found in heterochromatin and silenced genes. Remarkably, we found that PGC-1α overexpression led to a robust increase in AcH3 levels with a corresponding decrease in H3K9me2 levels on the vnn1 promoter (Fig. 5C). The knockdown of PGC-1α
caused opposite results (Fig. 5D). These results indicate that PGC-1α stimulates vnnl transcription by altering the local chromatin environment from a repressive to an active state. We next attempted to identify the transcriptional factors that mediate the activation of vnnl transcription by PGC-1α. A bioinformatics analysis indicated that the vnnl promoter (-267 to +1) possessed two HNF4α binding sites (nrmotif.ucr.edu/NRBSScan/H4SBM.htm) (28). Reporter gene assays demonstrated that PGC-1α augmented the transcriptional activity of HNF4α on a vnnl promoter reporter (Fig. 5E). However, when the HNF4α binding sites were mutated, the synergistic effects of PGC-1α and HNF4α on the transcription of the vnnl promoter disappeared (Fig. 5E and Supplementary Fig. 6). To confirm that HNF4α is required by PGC-1α to activate VNN1, we knocked down HNF4α in AML-12 cells and found that HNF4α knockdown blocked the PGC-1α-induced epigenetic changes of the vnnl promoter towards activation (Fig. 5F). Consistently, the activation of vnnl transcription and translation by PGC-1α was repressed by HNF4α siRNA (Fig. 5G). Lastly, we explored whether VNN1 acts as a downstream effector for PGC-1α-controlled gluconeogenesis. We knocked down VNN1 in PGC-1α overexpressed AML-12 cells and found that VNN1 knockdown inhibited the positive effects of PGC-1α on gluconeogenic gene expression (Fig. 5H). Moreover, the glucose output of mouse primary hepatocytes was decreased by VNN1 knockdown (Fig. 5I).
**DISCUSSION**

VNN1 is assumed to primarily function as a pantetheinase, which hydrolyzes pantetheine into pantothenic acid (also called vitamin B5 or pantothenate) and cysteamine (12). Recent studies have revealed additional functions of VNN1. For example, VNN1-deficient mice are resistant to intestinal inflammation, oxidative stress, and experimental colitis (15,16). VNN1 also plays a critical role in malaria susceptibility, psoriasis, carcinogenesis, and cardiovascular disease (29-32). Importantly, the vnn1 gene is one of the major targets of PPARα in the mouse liver, which strongly implies that VNN1 is involved in the regulation of energy metabolism (18). In the current study, we characterized VNN1 as a novel factor that activates hepatic gluconeogenesis. This characterization was initially based on the induction of VNN1 at the transcriptional level during fasting. To unmask the impact of VNN1 during fasting glucose homeostasis *in vivo*, we adopted VNN1 gain- or loss-of-function manipulation methods in mouse models. Our data suggested that VNN1 activates gluconeogenesis and increases glucose output under the control of the PGC-1α/HNF4α complex, which is mediated by the Akt signaling pathway (Fig. 6).

In addition to its role in glucose homeostasis, VNN1 also affects lipid metabolism. Hepatic VNN1 expression is induced by the agonists of PPARα, a key transcriptional factor involved in fatty acid oxidation (FAO) (18,33). The oral administration of triglycerides or PPARα ligands, such as WY14643 and fenofibrate, leads to a robust increase of VNN1 expression in the mouse liver (18). In agreement with previous
findings, our study showed that hepatic VNN1 expression was induced in db/db and diet-induced obese mice, which exhibited severe hepatic steatosis. More importantly, the liver-specific knockdown of VNN1 improved the hepatic steatosis in these animal models (Chen et al., unpublished data). These findings suggested that VNN1 may be a culprit in the pathogenesis of lipid dysregulation and accumulation. Future studies are required to fully elucidate the role of VNN1 in lipid metabolism, including FAO, lipid synthesis, and the mobilization and/or conversion of triglycerides, fatty acids, and cholesterol.

The recognition of the physiological functions of VNN1 has been significantly extended in recent years. Due to its pantetheinase activity, VNN1 is actively involved in the progression of inflammatory reactions by generating cysteamine. Therefore, it plays important roles in the pathogenesis of diseases related to inflammation. For example, VNN1 protects pancreatic islets from streptozotocin-induced cell death and retards the development of type 1 diabetes (17). In the intestine, VNN1 promotes an inflammatory reaction and intestinal injury by decreasing the activity of γ-glutamylcysteine synthetase and reducing the stores of reduced glutathione (15,16). Finally, because cysteamine is a transglutaminase (TGase) inhibitor (34,35), VNN1 is implicated in the development of Huntington's disease, which is characterized by the accumulation of highly cross-linked insoluble proteins. These proteins may be associated with an up-regulation of TGase (Summarized in Fig. 6). Conversely, recent studies indicate that glucose homeostasis is modulated by the chronic inflammation associated with metabolic stress (36,37). Genetic models in mice have demonstrated
that the deletion of key inflammatory mediators improves glucose tolerance in obesity-induced insulin resistance (38). The detrimental effects of pro-inflammatory pathways on glucose homeostasis are partly achieved via the inhibitory serine phosphorylation of IRS1 by JNK. In turn, uncontrolled hyperglycemia could further contribute to chronic inflammation. For example, the activity of NF-κB is increased via the modification of O-GlcNAc under high-glucose conditions (39). In our study, we found that the pantetheinase activity of VNN1 was increased in liver homogenates from *db/db* mice, which have chronic inflammation. Therefore, exploring the role of VNN1 pantetheinase activity in the control of gluconeogenesis is of particular interest to future studies to integrate inflammation and glucose homeostasis via VNN1.

VNN1 knockout (KO) mice have been generated for more than 10 years and serve as a useful tool to elucidate the physiological functions of VNN1. However, C. Roisin-Bouffay and colleagues reported that the basal glucose levels and glucose tolerance do not change in VNN1 KO mice (17), which differs from our results. This inconsistency may be due to the microenvironment difference between whole-body KO and tissue-specific knockdown mice, which has been observed in many other studies. For example, *Bmal1* is a core component of the mammalian circadian clock machinery. Investigators reported that global *bmal1*-null mice exhibit impaired glucose tolerance. In contrast, liver-specific *bmal1* knockout mice show increased glucose tolerance (40). Because we mainly focused on the physiological function of VNN1 in the liver, we believe that our animal models are more straightforward and specific to address our question.
PGC-1α is intensively involved in the regulation of gluconeogenesis. In adults, starvation induces PGC-1α expression in the liver via glucagon and glucocorticoid signaling. Once activated, PGC-1α initiates hepatic gluconeogenesis by co-activating key transcription factors, such as HNF4α, GR, and FOXO1 (9,10). In accordance with these observations, PGC-1α KO mice and RNAi-mediated liver-specific PGC-1α knockdown mice display impaired gluconeogenic gene expression and hepatic glucose production (8,41). These mice develop hypoglycemia upon fasting. The strategy to target PGC-1α to regulate glucose homeostasis has been proposed; however, its specificity and safety cannot be guaranteed because PGC-1α is a versatile molecule. In contrast, the biological functions of VNN1 are relatively focused. In addition, it serves as a downstream effector of PGC-1α and is required for the action of PGC-1α in gluconeogenesis. Indeed, PGC-1α expression peaked after 5 h of fasting in our system. The expression of VNN1 increased later and peaked after 16 h of fasting. In addition, the fact that the expression levels of PGC-1α and VNN1 peak at different time-point suggests that these two factors may regulate gluconeogenesis at different stages of fasting. In the early stage of fasting when PGC-1α is robustly induced, gluconeogenesis is mainly orchestrated by PGC-1α. When fasting persists and PGC-1α expression levels decline, VNN1 will relay and perpetuate gluconeogenesis. Conversely because it is a membrane protein, VNN1 is more easily manipulated than PGC-1α, which localizes in the nuclei. Collectively, aiming at VNN1, the downstream target of PGC-1α, without disturbing PGC-1α itself, can lead to a more specific regulation of body glucose levels.
Inflammation, as well as oxidative stress, make important contributions to the pathogenesis of metabolic diseases (37). Obese patients suffer from chronic low-grade inflammation, as indicated by increased plasma levels of C-reactive protein, inflammatory cytokines, and multifunctional proteins, such as leptin and osteopontin (42). Inflammation is also prominent in diabetic elderly patients (43). Inflammatory mediators are known to affect liver function. For instance, TNF-α stimulates hepatic lipogenesis and promotes hyperlipidemia (44). Conversely, reactive oxygen species are pro-oxidant factors that are generated during diabetes development, and lipid peroxide-mediated tissue damage has been observed in both type 1 and type 2 diabetes (45). In detail, diabetes-associated free radical injury, i.e., the accumulation of lipid peroxidation products, depletion of GSH, decreases in GSH/GSSG ratio and down-regulation of key antioxidant enzymes, has been detected in various tissues, including the liver, with deleterious consequences on hepatic functions and cognitive impairments observed in different diabetes animal models. VNN1 plays a critical role in the regulation of inflammation and oxidative stress. VNN1-deficient mice display down-regulated intestinal inflammation and a tissue resistance to oxidative stress (15,16). This protection is a result of the enhanced gamma-glutamylcysteine synthetase activity in liver, which is due to the absence of cysteamine and leads to elevated stores of glutathione, the most potent cellular antioxidant (14). Based on these findings, VNN1 could serve as one of the nodes to interconnect inflammation, oxidative stress, and metabolic dysregulation. If so, the induction of PGC-1α, an upstream regulator of VNN1, would provide cell-autonomous protection to
antagonize the deleterious effects of VNN1 because PGC-1α is a master player to evoke antioxidant defense.

Akt is a crucial regulator of glucose transportation, glycolysis, glycogen synthesis, and gluconeogenesis. Impaired Akt activation is associated with abnormal gluconeogenesis and glucose intolerance (46). In contrast, the restoration of insulin-induced Akt phosphorylation improves insulin sensitivity and glucose tolerance in mice that are fed a high-fat diet-fed (47). In the present study, we found that VNN1 overexpression blocked insulin-induced Akt phosphorylation in AML-12 cells, while VNN1 knockdown restored Akt phosphorylation in db/db mice. More importantly, the Akt inhibitor Wortmannin attenuated VNN1 knockdown-induced inhibition in gluconeogenic gene expression. This finding provided correlative evidence that Akt inactivation may be involved in VNN1 signaling and its effects on glucose homeostasis and insulin sensitivity. The role of VNN1 in the regulation of other components involved in the insulin signaling pathway is interesting to study, such as insulin receptor substrate 1/2.

Histone undergoes extensive post-translational modifications in response to external signals, including acetylation, methylation, phosphorylation, and SUMOylation (48,49). These modifications allow the fine-tuning of the chromatin structure and gene expression in a context-dependent manner. To date, little is known about the epigenetic regulation of the vnnl promoter. Our findings showed that PGC-1α altered the local chromatin environment of the HNF4α binding site on the vnnl promoter from a repressive to an active state. Consistently, PGC-1α positively
regulates *vnn1* gene expression. Our findings extend the current recognition of the epigenetic regulation of gluconeogenesis via VNN1 and highlight the importance of PGC-1α in this process.

In summary, our results strongly implicate VNN1 as a central player that orchestrates gluconeogenic programs. The identification of the specific role of VNN1 in hepatic gluconeogenesis would expand our knowledge to understand the important metabolic coordination necessary for proper blood glucose control. Conversely, VNN1 may serve as a pharmaceutical target to treat metabolic diseases with over-activated gluconeogenesis. In fact, a recent report demonstrated the design, synthesis, and characterization of a novel pantetheine analogue, RR6, which acts as a selective, reversible, and competitive VNN1 inhibitor at nanomolar concentrations. The oral administration of RR6 in rats completely inhibits plasma VNN1 activity and alters the plasma lipid profile (50). More specific inhibitors of VNN1 will most likely be synthesized to attenuate obesity-related hyperglycemia.
AUTHOR CONTRIBUTION

S.C. and C.L. designed research; S.C., W.Z., C.T. and X.T. performed research; S.C.,

W.Z. and C.L. analyzed data; C.L. wrote the paper.
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Dr. Chang Liu is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Figure Legends

FIG. 1. Gluconeogenic signals induce hepatic VNN1 expression. A and B: RT-qPCR (A) and Western blot (B) analysis of VNN1 and gluconeogenic gene expression in the liver from mice subjected to fasting (2 h, 5 h, 16 h) or fasting 16 h followed by 20 h re-feeding. *P<0.05 and **P<0.01 vs. re-fed group, n=6. C and D: RT-qPCR (C) and Western blot (D) analysis in the liver from wild type or db/db mice. **P<0.01 vs. WT, n=5. E: mRNA and protein analysis in the liver from mice fed on normal diet (ND) or on high-fat diet (HFD) for 2 months. **P<0.01 vs. ND, n=6. F: Pantetheinase activity analysis in the liver homogenates from mice described in (A). **P<0.01 vs. re-fed group, n=6. G: Pantetheinase activity analysis in the liver from wild type or db/db mice. **P<0.01 vs. WT, n=5. H: Regulation of VNN1 expression by 8-Br cAMP in vitro. AML-12 mouse hepatocytes were treated with 1 mM 8-Br cAMP for an indicated time period and VNN1 expression was quantified by RT-qPCR (top) and Western blot (bottom) analysis. **P<0.01 vs. 0 h. I: Reporter gene analysis of transcriptional activity of mouse vnn1 promoter. mvnn1-luc was transfected into AML-12 cells. 36 h later, cells were treated with 8-Br cAMP (1 mM) for another 12 h. **P<0.01 vs. mvnn1-luc alone. J: Western blot analysis of VNN1 expression in the primary hepatocytes treated with dexamethasone (DEX, 1 µM), 8-Br cAMP (1 mM) or hydrocortisone (HCS, 1 µM) for 6 h. All the data are represented as the mean ± SD.

FIG. 2. VNN1 promotes hepatic gluconeogenesis. A and B: Regulation of gluconeogenic gene expression by VNN1 in vitro. RT-qPCR (A) and Western blot
analysis (B) of VNN1 and gluconeogenic gene expression in AML-12 cells transduced with adenoviruses encoding GFP (Ad-GFP, as control) or VNN1 (Ad-VNN1) for 48 h. **P<0.01 vs. Ad-GFP. C: Glucose output assay in mouse primary hepatocytes. Cells were infected by adenoviruses for 48 h and glucose production rate was assessed. 8-Br cAMP (1 mM, treatment time: 12 h) was used as a positive control. **P<0.01 vs. Ad-GFP. D: Glucose tolerance test (GTT). Mice were transduced with adenoviruses encoding GFP or VNN1 through tail-vein injection (0.1 absorbance units per mouse). Three days later, mice were fasted for 16 h and the GTT assay was performed. **P<0.01 vs. Ad-GFP, n=5. E: Insulin tolerance test (ITT). Mice were treated as (D). The fasting time was shortened to 6 h and the ITT assay was performed. *P<0.05 and **P<0.01 vs. Ad-GFP group, n=5. F and G: VNN1 and gluconeogenic gene expression in the liver. RT-qPCR (F) and Western blot (G) analysis in liver of mice treated as (D). *P<0.05 and **P<0.01 vs. Ad-GFP group, n=5. (H) Pantetheinase activity analysis in the liver homogenates from mice treated as (D). **P<0.01 vs. Ad-GFP group, n=5. All the data are represented as the mean ± SD.

FIG. 3. Liver-specific knockdown of VNN1 relieves hyperglycemia. A: GTT analysis. Normal mice were transduced with scrambled siRNA oligonucleotides (as control) or siRNA oligonucleotides against VNN1 through tail-vein injection (2.5 mg/kg). Three days later, mice were fasted for 16 h and the GTT assay was performed. scra, scrambled siRNA; siVNN1, VNN1 siRNA. B: RT-qPCR analysis of vnnl and gluconeogenic gene expression in the liver from mice treated as (A). C: Western blot
analysis. D: Analysis of food intake (g/mouse/d) and water drinking (ml/mouse/d) in
$db/db$ mice transduced with scrambled or VNN1 siRNA oligonucleotides for three
days. E: GTT analysis. F: ITT analysis. G: RT-qPCR analysis of $vnn1$ and
gluconeogenic gene expression in the liver from $db/db$ mice. H: Western blot analysis.
I: Pantetheinase activity analysis. For all the panels, $^*P<0.05$ and $^{**}P<0.01$ vs.
scrambled siRNA. Each group at least included 6 mice. All the data are represented as
the mean ± SD.

**FIG. 4.** Akt signaling pathway mediates VNN1’s regulation of gluconeogenesis. A:
Hepatic Akt phosphorylation levels in mice with liver-specific overexpression of
VNN1. Top, Western blot analysis of phospho-Akt levels. Bottom, quantitative results.
$^{**}P<0.01$ vs. Ad-GFP, n=5. B: Akt phosphorylation levels in AML-12 cells. Cells
were infected by adenoviruses encoding GFP or VNN1 for 48 h and then treated with
100 nM insulin for 0, 5, 10, 15 min. $^{**}P<0.01$ vs. 0 min; $^#P<0.05$ and $^{##}P<0.01$ vs.
Ad-GFP at each time point. C: Hepatic Akt phosphorylation levels in $db/db$ mice with
liver-specific knockdown of VNN1. $^*P<0.05$ vs. scrambled siRNA, n=5. D: Akt
phosphorylation levels in AML-12 cells. Cells were transfected with scrambled or
VNN1 siRNA oligonucleotides for 48 h and then treated with 100 nM insulin for 0, 5,
10, 15 min. $^{**}P<0.01$ vs. 0 min; $^#P<0.05$ and $^{##}P<0.01$ vs. scrambled siRNA at each
time point. E: GTT analysis. Scrambled or VNN1 siRNA oligonucleotides were
transduced into $db/db$ mice as described above and then Wortmannin (1.5 mg/kg) was
intraperitoneally injected. 12 h later, mice were fasted for 16 h and GTT analysis was
performed. F: RT-qPCR (top) and Western blot (bottom) analysis in the liver samples from db/db mice treated as above. For panel E and F, \(^nP<0.05\) and \(^{++}P<0.01\), \(P<0.05\) and \(^{**}P<0.01\) vs. db/db plus scrambled siRNA group; \(^{#}P<0.05\) and \(^{##}P<0.01\) vs. db/db plus VNN1 siRNA treatment. n=5. All the data are represented as the mean ± SD.

**FIG. 5.** PGC-1α and HNF4α activate vnn1 transcription. A: Gene expression levels in mice with liver-specific overexpression of PGC-1α. Mice were transduced with adenoviruses encoding GFP or PGC-1α through tail-vein injection (0.1 absorbance units per mouse). Three days later, the hepatic expression levels of VNN1 and gluconeogenic genes were assessed by RT-qPCR (top) and Western blot (bottom) analysis. \(*P<0.05\) and \(^{**}P<0.01\) vs. Ad-GFP, n=5. B: Gene expression levels in mice with liver-specific knockdown of PGC-1α. Mice were transduced with adenoviruses encoding scrambled siRNA (as control) or siRNA against PGC-1α through tail-vein injection. Three days later, mice were subjected to 16 h fasting followed by RT-qPCR (top) and Western blot (bottom) analysis. \(^{**}P<0.01\) vs. scrambled siRNA, n=5. C: ChIP assays with indicated antibodies using AML-12 cells infected with adenoviruses encoding GFP or PGC-1α. The enrichment was quantified by qPCR analysis. \(*P<0.05\) and \(^{**}P<0.01\) vs. Ad-GFP. D: ChIP assays in AML-12 cells. Cells were infected with adenoviruses encoding scrambled siRNA or siRNA against PGC-1α for 36 h and then treated with 8-Br cAMP for another 12 h. \(*P<0.05\) and \(^{**}P<0.01\) vs. 8-Br cAMP group. E: Reporter gene assays in AML-12 cells transfected with indicated plasmids. \(^{**}P<0.01\) vs. the basal levels; \(^{##}P<0.01\) vs. co-transfection of PGC-1α and HNF4α. F:
ChIP assays in AML-12 cells. The cells were transfected with scrambled or HNF4α siRNA oligonucleotides for 12 h and then infected by adenoviruses encoding GFP or PGC-1α for another 36 h. **P<0.01 vs. scrambled siRNA plus Ad-PGC-1α. G: RT-qPCR (top) and Western blot (bottom) analysis of PGC-1α, HNF4α and VNN1 expression in AML-12 cells treated as (F). **P<0.01 vs. the basal levels; ##P<0.01 vs. scrambled siRNA plus Ad-PGC-1α. H: RT-qPCR (top) and Western blot (bottom) analysis of gluconeogenic gene expression. AML-12 cells were transfected with scrambled or VNN1 siRNA oligonucleotides for 12 h and then infected by adenoviruses encoding GFP or PGC-1α for another 36 h. I: Glucose output assay. For panel H and I, *P<0.05 and **P<0.01 vs. the basal levels; #P<0.05 and ##P<0.01 vs. scrambled siRNA plus Ad-PGC-1α. All the data are represented as the mean ± SD.

**FIG. 6.** A model illustrating the physiological functions of VNN1 in various tissues, highlighting the role of VNN1 in the regulation of hepatic gluconeogenesis in a PGC-1α-dependent manner.
Gluconeogenic signals induce hepatic VNN1 expression.
VNN1 promotes hepatic gluconeogenesis.
Liver-specific knockdown of VNN1 relieves hyperglycemia.

219x284mm (300 x 300 DPI)
Akt signaling pathway mediates VNN1’s regulation of gluconeogenesis.
PGC-1α and HNF4α activate vnn1 transcription.

209x275mm (300 x 300 DPI)
A model illustrating the physiological functions of VNN1 in various tissues, highlighting the role of VNN1 in the regulation of hepatic gluconeogenesis in a PGC-1α-dependent manner.
SUPPLEMENTARY MATERIALS

Cell culture. Mouse AML-12 hepatocytes were cultured with DMEM/F-12 medium (Gibco) supplemented with insulin, transferrin, selenium (ITS; Gibco) and dexamethasone (DEX) (40 ng/ml; Sigma) in a humidified atmosphere containing 5% CO₂ at 37°C. Primary hepatocytes were prepared from C57BL/6 mice via the collagenase IV (Gibco) perfusion method as described previously (1).

Adenovirus, siRNA, and plasmid information. Adenoviruses that expressed the full-length mouse VNN1 complementary DNA coding sequence were constructed by Bioworld (Nanjing, China). For VNN1 and VNN3 knockdown, three sets of stealth siRNA for each gene were designed, validated, and synthesized by Invitrogen (Shanghai, China). To improve gene silencing efficiency, a siRNA cocktail comprising these three sets of siRNA oligonucleotides (an equal molar mixture) was used. The sequences of these siRNA oligonucleotides are listed in Supplementary Table 1. For the in vitro knockdown of HNF4α, commercial siRNA oligonucleotides against HNF4α were purchased from Santa Cruz Biotech (USA). Adenoviruses (Ad-GFP and Ad-PGC-1α, Ad-Scrambled, and Ad-PGC-1α RNAi) and plasmids encoding PGC-1α, HNF4α, mpepck-luc and mg6pase-luc promoters were kindly provided by Dr. Jiandie Lin (Life Sciences Institute, University of Michigan, Ann Arbor, USA).

mRNA and protein expression analysis. Total RNA was isolated using Trizol
reagents (Invitrogen), reverse transcribed, and analyzed by quantitative PCR (qPCR) using SYBR Green and the Mastercycler ep realplex2 system (Eppendorf, Hamburg, Germany). The primers for mouse 36B4 were included for normalization. A complete list of PCR primers is shown in Supplementary Table 2. For protein analysis, tissue samples were homogenized and the cells were lysed in RIPA buffer. The protein concentration was quantified with a DC protein assay reagent (Bio-Rad, Hercules, USA). Equal amounts of protein were loaded and separated by 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membranes were incubated overnight with appropriate primary antibodies. Bound antibodies were then visualized using alkaline phosphatase-conjugated secondary antibodies. A quantitative analysis was performed by using the NIH Image J 1.32j software. To detect the translocation of PPARγ protein to the nuclei, we extracted nuclear protein using a commercial kit (Shengxing, China) and then performed the Western blot analysis. Histone H3 was used as the loading control. Western blot results are shown as the representative blots from two or three animals randomly selected from each group. Anti-G6Pase, anti-PEPCK, and anti-HNF4α antibodies were purchased from Santa Cruz Biotech; anti-PGC-1α antibody was obtained from Calbiochem; anti-GAPDH, anti-phospho-Akt (Ser 473), anti-total Akt, and anti-PPARγ antibodies were purchased from Cell Signaling Technology; anti-VNN1 antibody was purchased from Proteintech.

**Transfection and reporter gene assays.** The *vnn1* promoter (-267 to +1) was
amplified from the mouse genomic DNA by using primers shown in Supplementary Table 2. Either individual or both of the two HNF4α binding sites were mutated using a commercial kit (Invitrogen). These sequences were validated by sequencing and cloned into a PGL3-basic vector using the KpnI and SacI restriction sites. All transient transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For the luciferase reporter assays, 200 ng of reporter plasmids were mixed with 400 ng of expression constructs for HNF-4α in the presence or absence of 1 µg PGC-1α expression construct. Equal amounts of DNA were used for all transfection combinations by adding the appropriate vector DNA. Relative luciferase activities were determined 48 h following transfection using the Dual Luciferase system (Promega). The data were representative of at least three independent experiments.

**Glucose and insulin tolerance tests.** For the glucose tolerance tests (GTT), the mice were fasted for 16 h and then intraperitoneally injected with 1 g/kg glucose. For the insulin tolerance tests (ITT), the WT mice were fasted for 6 h and intraperitoneally injected with 0.5 U/kg insulin. The dose of insulin used for ITT assays performed in *db/db* mice was increased to 2 U/kg. The blood glucose levels were measured before glucose/insulin injection and 0, 15, 30, 60, and 120 min after injection. The basal glucose levels are presented in Supplementary Table 3.

**Glucose output assay.** Mouse primary hepatocytes were isolated and seeded into
six-well plates (1×10^6 cells per well). For the glucose output assay, the cells were washed twice with PBS and incubated with glucose-free DMEM medium (pH 7.4, phenol red free, supplemented with 20 mM sodium lactate and 2 mM pyruvate). Six hours later, the medium was assayed for glucose production with a colorimetric kit (Sigma). The values were normalized to the protein content of the whole-cell lysates.

**ChIP assay.** ChIP assays were performed on AML-12 cells essentially as described by Upstate Biotechnology. Briefly, Chromatin lysates were prepared, pre-cleared with Protein-A/G agarose beads, and immunoprecipitated with antibodies against PGC-1α, HNF-4α, K9-dimethylated histone H3 (H3K9Me2) (Abcam), and acetylated histone H3 (AcH3) (Millipore), or normal mouse IgG (Santa Cruz Biotech) in the presence of BSA and salmon sperm DNA. The beads were extensively washed before reverse cross-linking. The DNA was purified using a PCR purification kit (Qiagen) and subsequently analyzed by PCR using primers flanking the proximal binding sites for HNF-4α on the mouse *vnn1* promoter, as detailed in Supplementary Table 2.

**Confocal fluorescence microscopy analysis.** AML-12 cells were transiently transfected with scrambled and VNN1 siRNA. Forty-eight hours later, they were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 minutes at room temperature. The samples were then treated with 0.5% Triton X-100 for 10 min and washed 3 times in PBS, followed by blocking in 1% BSA for 1 h. Subsequently, the samples were incubated with anti-PPARγ overnight, after repeated washing, the cells were
incubated with anti-Rabbit IgG (Alexa Fluor 488, Green) (Cell Signaling Technology).

DAPI (blue) staining for nuclear localization was performed simultaneously. The cells
were examined by confocal fluorescence microscopy (A1 Nikon).
Supplementary Figure Legends

Supplementary FIG. 1. A: Quantitative results for FIG. 1B. *P<0.05 and **P<0.01 vs. re-fed group. B: Quantitative results for FIG. 1D. *P<0.05 and **P<0.01 vs. WT mice. C: Quantitative results for the Western blot presented in FIG. 1E. **P<0.01 vs. ND group. All the data are represented as the mean ± SD.

Supplementary FIG. 2. VNN3 does not regulate gluconeogenesis. A: RT-qPCR analysis of vnn3 mRNA expression levels in the liver from mice subjected to 16 h fasting or 16 h fasting followed by 20 h re-feeding (n=6). B: RT-qPCR analysis of vnn3 mRNA expression in the liver from wild type (WT) or db/db mice. **P<0.01 vs. wild type (n=5). C: GTT analysis. db/db mice were transduced with scrambled or VNN3 siRNA oligonucleotides (2.5 mg/kg) through tail-vein injection. Three days later, mice were fasted for 16 h and the GTT assay was performed. scra, scrambled siRNA; siVNN3, VNN3 siRNA (n=5). D: ITT analysis. db/db mice were treated as (C). The fasted time was shortened to 6 h and the ITT assay was performed (n=5). E and F: RT-qPCR (E) and western blot (F) analysis of gluconeogenic gene expression in the liver from db/db mice treated as (C). *P<0.01 vs. scrambled siRNA group. All data are represented as the mean ± SD.

Supplementary FIG. 3. Knockdown of VNN1 inhibits 8-Br cAMP-induced gluconeogenic gene expression and glucose production in vitro. A: mpepck-luc or mg6pase-luc was co-transfected into AML-12 cells with scrambled siRNA or siRNA
oligonucleotides against VNN1 (siVNN1). 36 h later, cells were treated with 8-Br cAMP (1 mM) for another 12 h. Reporter gene assays were performed to measure the transcriptional activity of examined promoters. **P<0.01 vs. luciferase reporter alone, ### P<0.01 vs. scrambled siRNA plus 8-Br cAMP treatment. B: siRNA were transfected into AML-12 cells, followed by 8-Br cAMP treatment as described above. RT-qPCR analysis was performed to quantify pepck and g6pase mRNA expression. **P<0.01 vs. scrambled siRNA alone, ### P<0.01 vs. scrambled siRNA plus 8-Br cAMP treatment. C: Protein expression levels of VNN1 and gluconeogenic genes. D: Glucose output assay. **P<0.01 vs. scrambled siRNA alone, ### P<0.01 vs. scrambled siRNA plus 8-Br cAMP treatment. All data are represented as the mean ± SD.

**Supplementary FIG. 4.** VNN1 knockdown enhances PPARγ translocation to the nuclei. AML-12 Cells were transiently transfected with scrambled or VNN1 siRNA for 48 h. A: PPARγ translocation was assessed by a confocal fluorescence microscopy. The arrows indicate PPARγ nuclear localization (green dots). Magnification: 1000×. B: Western blot analysis of PPARγ expression levels in the nuclei. C: Quantitative results of (B). **P<0.01 vs. scrambled siRNA. All data are represented as the mean ± SD.

**Supplementary FIG. 5.** PGC-1α activates vnnl transcription in vitro. A: AML-12 cells were infected by adenoviruses encoding GFP (Ad-GFP) or PGC-1α (Ad-PGC-1α). vnnl mRNA expression levels were assessed by RT-qPCR analysis. **P<0.01 vs. Ad-GFP. B: Protein expression levels of VNN1 and gluconeogenic
genes. C: AML-12 cells were infected by adenoviruses encoding scrambled siRNA (Ad-scra) or siRNA against PGC-1α (Ad-siPGC-1α). 36 h later, cells were treated with 8-Br cAMP (1 mM) for another 12 h. vnn1 mRNA expression levels were assessed by RT-qPCR analysis. **P<0.01 vs. scrambled siRNA. D: Protein expression levels of VNN1 and gluconeogenic genes. All the data are represented as the mean ± SD.

Supplementary FIG. 6. HNF4α binding sites are essential for PGC-1α-induced vnn1 transcription. One of two HNF4α binding sites at the proximal region of mouse vnn1 promoter (-267 to +1) was mutated and cloned into a PGL3-basic vector. These vectors were co-transfected into AML-12 cells with plasmids encoding PGC-1α and HNF4α. **P<0.01 vs. wild-type vnn1-luc alone, ##P<0.01 vs. wild-type vnn1-luc plus PGC-1α and HNF4α. All the data are represented as the mean ± SD.
REFERENCE
VNN3 does not regulate gluconeogenesis.
Knockdown of VNN1 inhibits 8-Br cAMP-induced gluconeogenic gene expression and glucose production in vitro.
VNN1 knockdown enhances PPARγ translocation to the nuclei.

119x80mm (300 x 300 DPI)
PGC-1α activates vnn1 transcription in vitro.
HNF4α binding sites are essential for PGC-1α-induced vnn1 transcription.
Table S1. Sequences of siRNA oligonucleotides.

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<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
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Table S2. List of PCR primers.

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<td>5’-AGGGAAGACATACCAGGTTTC-3’</td>
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Table S3. Basal blood glucose levels after 16 h fasting

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<tr>
<th>Groups</th>
<th>Blood glucose (mmol/L)</th>
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<tbody>
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<td>4.1±0.49</td>
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<td>Ad-VNN1</td>
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<td>siVNN1</td>
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<td>db/db + siVNN1</td>
<td>6.5±0.3</td>
</tr>
</tbody>
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