Mitogen-Activated Protein Kinase Phosphatase 3 (MKP-3)–Deficient Mice Are Resistant to Diet-Induced Obesity

Mitogen-activated protein kinase phosphatase 3 (MKP-3) is a negative regulator of extracellular signal–related kinase signaling. Our laboratory recently demonstrated that MKP-3 plays an important role in obesity-related hyperglycemia by promoting hepatic glucose output. This study shows that MKP-3 deficiency attenuates body weight gain induced by a high-fat diet (HFD) and protects mice from developing obesity-related hepatosteatosis. Triglyceride (TG) contents are dramatically decreased in the liver of MKP-3−/− mice fed an HFD compared with wild-type (WT) controls. The absence of MKP-3 also reduces adiposity, possibly by repressing adipocyte differentiation. In addition, MKP-3−/− mice display increased energy expenditure, enhanced peripheral glucose disposal, and improved systemic insulin sensitivity. We performed global phosphoproteomic studies to search for downstream mediators of MKP-3 action in liver lipid metabolism. Our results revealed that MKP-3 deficiency increases the phosphorylation of histone deacetylase (HDAC) 1 on serine 393 by 3.3-fold and HDAC2 on serine 394 by 2.33-fold. Activities of HDAC1 and 2 are increased in the livers of MKP-3−/− mice fed an HFD. Reduction of HDAC1/2 activities is sufficient to restore TG content of MKP-3−/− primary hepatocytes to a level similar to that in WT cells.

Obesity has become a major public health issue and is mainly caused by a sedentary lifestyle and energy-dense diet. Massive expansion of white adipose tissue (WAT) is the hallmark of obesity. Excessive energy is normally stored in WAT as triglycerides (TGs). Lipids are deposited into nonadipose tissues such as liver and muscle when the limit of WAT storage capacity is reached. Lipotoxicity is a major causal factor for the development of insulin resistance; it promotes the production of inflammatory mediators in WAT and the liver (1,2). The effect of insulin on glycemic control is implemented through coordinated actions in multiple tissues. In the liver, insulin represses glucose output (3); in muscle and fat, insulin stimulates glucose uptake (4,5). In the state of insulin resistance, glucose output from the liver increases, and glucose disposal in muscle and fat decreases. As a result, circulating insulin levels must increase to maintain a blood glucose concentration within the normal range. Whether an individual with insulin resistance ultimately develops diabetes depends on the capacity of pancreatic β-cells to sustain increased insulin secretion (6).

The liver is an important organ for maintaining energy homeostasis. It keeps blood glucose concentrations within a normal range by promoting glycogenesis (sequestering glucose by glycogen synthesis) after a meal and activating glycogenolysis (breakdown of glycogen) and gluconeogenesis from noncarbohydrate precursors during starvation (7). The liver is also central for lipid metabolism. It is highly active in oxidizing TGs to produce energy and...
exports through circulation large quantities of acetoacetate to be metabolized by other tissues. It is also the main site for converting excessive carbohydrates and proteins into free fatty acids and TGs, which are subsequently transported to adipose tissue for storage. In the setting of overnutrition, liver and adipose tissue have been considered the primary organs for systemic metabolic disorders, whereas skeletal muscle and pancreatic islets are thought of as responder systems (8).

Mitogen-activated protein (MAP) kinase phosphatases (MKPs) are negative regulators of the MAP kinase pathway in the regulation of mitogenesis. Our study is the first to reveal the involvement of MKP in insulin-regulated metabolic pathways. We previously reported that MKP-3 is highly upregulated in the liver of obese and diabetic mice and promotes glucose output in both cultured liver cells of rats and in the livers of mice (9,10). In this study, we characterized the metabolic phenotype of MKP-3−/− mice in response to overnutrition. Global MKP-3 deficiency not only decreases blood glucose concentrations and significantly improves systemic insulin sensitivity but also reduces TG contents in both the liver and WAT, indicating that MKP-3 is involved in both glucose and lipid metabolism. Through phosphoproteomic study, we identified that MKP-3 regulates lipogenesis in the liver through modulation of histone deacetylase (HDAC) 1/2 phosphorylation.

RESEARCH DESIGN AND METHODS

Reagents and Cells

Hepa1–6 mouse hepatoma cells were provided by Dr. Gökhan Hotamisligil (Harvard School of Public Health). 3T3-L1 cells were purchased from the American Type Culture Collection. 3T3-L1 coxsackie and adenovirus receptor (3T3-L1 CAR) cells were provided by Dr. David Orlicky (University of Colorado Health Sciences Center) (11). Preadipocytes were differentiated using a previously described method (12). Mouse insulin ELISA kits were purchased from ALPCO (Salem, NH). MKP-3 antibody (Ab), HDAC1 and 2 Abs, and hemagglutinin Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Flag Ab was purchased from Sigma-Aldrich (St. Louis, MO). Tubulin Ab was purchased from Abcam. Insulin, dexamethasone, isobutyl methyl xanthine, ATP, horseradish peroxidase, glycerol phosphate oxidase, glycerol kinase, and lipase were purchased from Sigma-Aldrich. Acyl CoA oxidase was purchased from Wako Diagnostics (Richmond, VA). Amplex red was purchased from Invitrogen (Carlsbad, CA). Humulin R was purchased from Eli Lilly (Indianapolis, IN). Glucose test strips and AlphaTRAK meters were purchased from Abbott Animal Health (Abbott Park, IL). Fluorogenic HDAC Assay Kits were purchased from BPS Bioscience (San Diego, CA). HDAC1 and 2 constructs were provided by Dr. Matthew S. Walters (Weill Cornell Medical College). Luciferase kits were purchased from Promega (Madison, WI). FK228 and SAHA were purchased from Selleckchem.com (Houston, TX).

Isolation of Primary Cells

Adipocytes and stromal vascular cells were isolated from gonadal fat pads of mice using a method described previously (12). For in vitro adipogenesis, stromal vascular cells were seeded on 24-well plates at a density of 4 × 10^5/well and induced by DMEM containing 10% fetal calf serum, 0.5 mmol/L isobutyl methyl xanthine, 1 μmol/L dexamethasone, and 1 μg/mL insulin. Primary hepatocytes were isolated by infusing mouse liver with collagenase buffer, and cells were filtered through a 150-μm tissue sieve. Cells were seeded in collagen-coated 6-well plates at a density of 1 × 10^6/well.

Hyperinsulinemic-Euglycemic Clamp Study

Catheters were implanted into the left jugular vein of mice 5–10 days before the clamp study. After an overnight fast, a hyperinsulinemic-euglycemic clamp study was performed using a method described previously (10).

RNA Extraction and Real-Time PCR Analysis

RNA samples were extracted from tissues or isolated cells using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Real-time PCR analysis was performed using a method described previously (10).

Immunoprecipitation and Immunoblot Blot Analysis

Tissues and cells were homogenized and lysed with ice-cold lysis buffer supplemented with protease inhibitors. MKP-3 was immunoprecipitated using a method described previously (10). Protein lysates were separated on 4–12% Bis-Tris-PAGE gel and transferred onto polyvinylidene difluoride membranes. Following blocking, membranes were incubated with the corresponding primary antibodies. After thorough washes, membranes were exposed to corresponding horseradish peroxidase–linked secondary antibodies, and signals were detected with a chemiluminescence Western blotting detection solution (PerkinElmer, Waltham, MA) using the α-Innotech fluorochrom image system.

Mice Maintenance and Analysis

MKP-3–deficient male mice were purchased from The Jackson Laboratory (Bar Harbor, ME); they were maintained on a mixed B6/129 background. We backcrossed them with C57BL/6 female mice purchased from The Jackson Laboratory for nine generations. Mice were kept on a 12-h light/dark cycle and given ad libitum access to food and water. Mice were fed on either a normal chow diet or a high-fat diet (HFD; 60% kcal from fat; D12492, Research Diets) at 4 weeks of age. The HFD was maintained for up to 28 weeks. Body weights were measured biweekly. At the end of study, mice were killed by asphyxiation with carbon dioxide (CO2) after an overnight fast or under fed conditions. Plasma and tissues were collected for further analysis. All animal procedures used in our studies were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital.

Histological Analysis

Livers were fixed in 10% neutral-buffered formalin for 1 day then transferred to 70% ethanol and embedded in
paraffin for sectioning. Hematoxylin-eosin and Oil red O staining were performed at the Rhode Island Hospital’s core laboratory.

**Measurement of TG Contents**

The TG contents in tissue were determined using homogenates of mesenteric WAT and liver samples. Frozen tissues were weighed, pulverized in liquid nitrogen, homogenized in ethanol, vortexed, and centrifuged; the supernatant was collected for measurement. To extract plasma TGs, plasma was vortexed in ethanol and centrifuged, and supernatant was gathered for measurement. To measure TG contents, TG standards or samples were mixed with reaction buffer and incubated for 30 min at 37°C.

**Indirect Calorimetry**

Oxygen consumption (VO_{2}), CO_{2} production (VCO_{2}), and food intake were measured individually for 24 h using the comprehensive laboratory animal monitoring system (Columbus Instruments, Columbus, OH) after 1 day of acclimation. During the experiment, mice had free access to food and water. Energy expenditure was calculated using the following formula: VO_{2} × (3.815 + 1.232 × Respiratory Quotient), and normalized to (body mass)^{0.75}.

**Insulin Tolerance Test**

For the insulin tolerance test (ITT), mice were fasted for 6 h, and male mice fed an HFD were injected with insulin at a dose of 1 U/kg. Blood glucose concentrations were measured at 0, 15, 30, 45, 60, and 90 min after injection.

**Phosphoproteomic Study**

Hepa1–6 cells were cultured in stable isotope labeling in cell culture (SILAC) medium for eight doublings and then seeded in SILAC medium on 6-well plates. Half of the cells were labeled with light arginine and lysine and infected with adenovirus expressing short hairpin interfering RNA against MKP-3. For each sample, 2.5 mg of protein lysates labeled with light arginine and lysine were mixed with 2.5 mg of protein lysates labeled with heavy arginine and lysine. Samples were reduced with 45 mmol/L dithiothreitol at 60°C for 20 min, alkylated with 100 mmol/L iodoacetamide for 15 min in the dark, and digested overnight with TPCK-treated trypsin. Peptides then were desalted using Sep-Pak C18 columns and enriched for phosphopeptides using a TitanSphere Phos-TiO kit before loading to an Orbitrap Velos electron transfer dissociation mass spectrometer. Results from five biological replicates were used for data analysis. Q values for multiple hypothesis tests also were calculated based on the determined P values using the R package QVALUE, as previously described (13).

**HDAC Activity Assay**

HDAC1 or 2 was immunoprecipitated, and activity was assayed using the fluorogenic HDAC assay kit from BPS Bioscience, according to the manufacturer’s instructions. Immunoprecipitation beads were washed four times with lysis buffer and twice with HDAC assay buffer then incubated with 50 µL HDAC substrate at 37°C for 30 min. After centrifugation, 30 µL supernatant was mixed with 30 µL developer for 15 min of incubation at room temperature. Fluorescence was read at excitation 360 nmol/L and detection 450 nmol/L.

**Statistical Analysis**

Results are presented as mean ± standard errors. Statistical significance was determined at P < 0.05. Student t test was used to compare differences between two groups. Two-way ANOVA and Bonferroni posttests were used to analyze multiple experimental groups.

**RESULTS**

**MKP-3 Deficiency Protects Mice From Developing Diet-Induced Obesity**

MKP-3−/− mice are significantly lighter than wild-type (WT) littermates (controls) at 4 weeks of age (Fig. 1A). When fed a chow diet, MKP-3−/− mice became indistinguishable from WT mice between 6 and 20 weeks of age (Fig. 1A). After 23 weeks of age, male MKP-3−/− mice became significantly leaner than WT mice (Supplementary Fig. 1A). When challenged with an HFD, body weight gain is attenuated by MKP-3 deficiency in both sexes (Fig. 1B), and mice are protected from developing diet-induced obesity (DIO). Concentrations of fasting blood glucose and fasting plasma insulin are significantly lower in MKP-3−/− mice fed an HFD compared with WT mice (Fig. 1C and D). There is no difference in fasting blood glucose and fasting plasma insulin concentrations between WT and MKP-3−/− mice fed a chow diet (Supplementary Fig. 1B). The ITTs indicate improved systemic insulin sensitivity in MKP-3−/− mice fed an HFD (Fig. 1E).

The weights of liver and total adipose tissue are significantly reduced in MKP-3−/− mice fed an HFD compared with WT controls (Fig. 2A). Weights of adipose tissue are significantly decreased in all fat depots of female MKP-3−/− mice fed an HFD and in all fat depots (except gonadal) of male MKP-3−/− mice fed an HFD (Supplementary Fig. 2A). MKP-3 deficiency did not change circulating lipid profiles (Fig. 2B). Liver weights also are significantly reduced in MKP-3−/− mice fed a chow diet, but adipose tissue weights are only significantly decreased in male MKP-3−/− mice fed a chow diet; there is no significant change in female MKP-3−/− mice receiving chow (Supplementary Fig. 2B and C). TG contents in the livers of MKP-3−/− mice fed an HFD are drastically reduced to levels comparable to those in the livers of lean WT mice (Fig. 2C). TG contents in the livers of MKP-3−/− mice fed chow also are significantly reduced (Fig. 2C). Histology study reveals that MKP-3 deficiency protects mice from developing HFD-induced hepatosteatosis (Fig. 2D). Gene expression analysis demonstrates significant reduction of lipogenic gene expression, including fatty acid synthase (FAS), stearoyl CoA desaturase 1 (SCD1), acetyl CoA carboxylase 2 (ACC2), and peroxisome proliferator-activated receptor γ (PPARγ) in MKP-3−/− mice fed an HFD.
compared with WT mice fed an HFD (Fig. 2E). To determine whether MKP-3 is required for transcription of lipogenic genes, FAS promoter-driven luciferase reporter construct was transfected into cultured mouse hepatoma Hepa1–6 cells expressing interfering RNA for green fluorescent protein (shGFP) or MKP-3 (shMKP-3). Transcription of FAS promoter is significantly repressed when expression of MKP-3 is reduced (Fig. 2F).

**Absence of MKP-3 Increases Energy Expenditure and Improves Glucose Homeostasis**

To understand the effect of MKP-3 deficiency on whole-body energy homeostasis, metabolic cages were used. Oxygen consumption, CO₂ release, and energy expenditure are significantly increased during a 24 h period in both male and female MKP-3⁻/⁻ mice fed an HFD (Fig. 3A). Further analysis indicates that these parameters significantly increase during both dark and light cycles (data not shown). Food intake is not changed in male MKP-3⁻/⁻ mice fed an HFD but is significantly increased in female MKP-3⁻/⁻ mice fed an HFD (Fig. 3A). The respiratory exchange ratio remains unchanged, indicating that the balance between lipid and carbohydrate oxidation does not shift. Oxygen consumption, CO₂ release, and energy expenditure also are significantly increased during the dark period in MKP-3⁻/⁻ male mice fed a chow diet but not changed in MKP-3⁻/⁻ female mice fed a chow diet (data not shown). Food intake does not change in either sex when fed a chow diet (data not shown).

Hyperinsulinemic-euglycemic clamp study demonstrates significantly increased glucose infusion rate in MKP-3⁻/⁻ female mice fed an HFD (Fig. 3B), supporting our previous conclusion, based on the results of ITTs, that systemic insulin sensitivity is improved in the absence of MKP-3. Hepatic glucose production can be more effectively suppressed by insulin in MKP-3⁻/⁻ mice (Fig. 3B). Glucose disposal rate also is significantly improved in MKP-3⁻/⁻ mice; this is most likely attributable to increased muscle glucose uptake and glycolysis (Fig. 3B). Glucose uptake in WAT increased 54.8% but did not reach statistical significance (Fig. 3B). Plasma insulin levels in MKP-3⁻/⁻ female mice fed an HFD are significantly lower than those of WT mice during the clamp period (Fig. 3C).

**MKP-3 Plays a Role in Adipocyte Differentiation**

MKP-3 mRNA level has been previously reported to increase in WAT of ob/ob and db/db mice (9). In this study, levels of MKP-3 mRNA and protein expression are shown to significantly increase in WAT of mice with DIO (Fig. 4A and B). Interestingly, the level of MKP-3 protein expression significantly increases at the early stage of adipogenesis (2 days after induction; Fig. 4C), suggesting that MKP-3 may play a role in adipocyte differentiation. Adipogenesis of 3T3-L1 coxsackie and adenovirus receptor adipocytes (a subline of 3T3-L1 that stably expresses the truncated adenovirus receptor) is impaired when MKP-3 expression was reduced in preadipocytes by adenovirus-mediated expression of interfering RNA against MKP-3 (shMKP-3), as shown by decreased TG accumulation (Fig. 4D). Analysis of gene expression reveals significant reduction of peroxisome proliferator-activated receptor γ, FAS, and SCD1 (Fig. 4E). The stromal vascular cells isolated from gonadal adipose tissue of MKP-3⁻/⁻ mice also display impaired adipogenesis, which
was reflected by significantly decreased expression of adipocyte genes such as CCAAT/enhancer binding protein α (C/EBPα), FAS, and SCD1 (Fig. 4F). That baseline extracellular signal–related kinase (ERK) activity mildly increases in the heart of MKP-3 \(^{-/-}\) mice and promotes myocyte proliferation has been previously reported (14). ERK phosphorylation significantly increased in gonadal fat, but was unchanged in mesenteric fat, of MKP-3 \(^{-/-}\) male mice fed an HFD (Fig. 5A and data not shown).

Because MKP-3 deficiency did not affect the weight of gonadal fat but did decrease mesenteric fat, the reduction of adiposity is unlikely due to ERK activation. Phosphorylation status of ERK2 but not ERK1 is significantly increased in the livers of MKP-3 \(^{-/-}\) mice fed an HFD (Fig. 5B).

**HDAC1 and 2 Are Downstream Targets of MKP-3 Signaling in Hepatic Lipid Metabolism**

To identify potential downstream mediator(s) of MKP-3 action in lipid metabolism, global phosphoproteomics was studied using the quantitative SILAC method. Cultured mouse hepatoma Hepa1–6 cells expressing shMKP-3 via adenovirus-mediated gene transfer were labeled with heavy arginine/lysine, and control cells expressing shGFP were labeled with light arginine/lysine. Changes in serine/threonine phosphorylation caused by reduced MKP-3...
expression were expressed as ratios of shMKP-3 to shGFP. Under the condition of reduced expression of a phosphatase, we assume that phosphorylation levels of downstream mediators are increased if other phosphatases are not sufficient to compensate. Table 1 shows the average results of five biological replicates, which contain a total of 10 proteins with an increase in serine/threonine phosphorylation of 50% or more. Among these candidates, HDAC1 stood out as an interesting target, with significantly increased phosphorylation on serine 393 (S393) by 3.3-fold. Interestingly, our label-free global phosphoproteomic study using livers from WT and MKP-3\(^{-/-}\) mice fed an HFD demonstrates significantly increased phosphorylation on serine 394 (S394) of HDAC2 by 2.33-fold due to the absence of MKP-3 (Supplementary Table 1).

HDAC1 and 2 proteins are 86% identical and 93% positive. S394 of HDAC2 is the equivalent of S393 of HDAC1. These two proteins are functionally redundant in many tissues (15–18). To understand the meaning of S393/S394 phosphorylation on HDAC1/2 activity, alanine and glutamic acid mutants were used in an activity assay. As shown in Fig. 6A and B, HDAC1 S393A/HDAC2 S394A mutants are significantly less active compared with WT proteins. Glutamic acid mutants partially mimic phosphorylation and are more active than alanine mutants but less active than WT proteins. The endogenous HDAC1/2 activities also are significantly increased in the livers of MKP-3\(^{-/-}\) mice fed an HFD compared with WT controls, and this activity can be completely blocked by a pan HDAC inhibitor trichostatin A (Fig. 6C). Furthermore, endogenous HDAC1/2 activities are significantly decreased in the liver of WT mice fed an HFD compared with lean controls (Fig. 6D), consistent with our previous report that MKP-3 expression is markedly increased in the liver of mice with DIO (10). These results indicate that MKP-3 may repress HDAC1/2 activity by dephosphorylating HDAC1/2 on S393/S394. MKP-3 is predominantly localized in the cytoplasm but can shuttle between the nucleus and the cytosol (19). HDAC1/2 are nuclear enzymes but can be exported to the cytoplasm under pathological conditions (20,21). To test whether HDAC1 and 2 interact with MKP-3, HDAC1 and 2 were coexpressed with MKP-3 in Hepa1-6 cells. Both HDAC2 and MKP-3 could be immunoprecipitated with HDAC1 (Fig. 6E). Overexpression of the HDAC1 WT and S393E mutant, but not the S393A mutant, significantly suppresses expression of SCD1 gene in Hepa1–6 cells (Fig. 6F).
To investigate whether increased HDAC1 and 2 activity in the liver of MKP-3+/− mice is responsible for decreased TG accumulation, we used both interfering RNA and a selective HDAC1/2 inhibitor to repress HDAC1/2 activity in liver cells. HDAC1 knockdown alone does not significantly increase the expression of lipogenic genes in Hepa1–6 cells. Reduction of HDAC2 expression alone significantly induces expression of both SCD1 and ACC2 genes. Reduction of both HDAC1 and HDAC2 significantly increases expression of FAS, SCD1, and ACC2 genes (Fig. 7A). These results indicate that HDAC1 and 2 have a redundant function in repressing lipogenic gene expression. A potent and selective HDAC1/2 inhibitor, FK228, also was used to confirm the results obtained from interfering RNA against HDAC1 and 2. FK228 inhibited class I HDAC activity by more than 50% at a concentration of 5 nmol/L, whereas the pan HDAC inhibitor SAHA did not have any effect at the same concentration (Fig. 7B). FK228, not SAHA, significantly increases expression of FAS, SCD1, and ACC2 genes at 5 nmol/L (Fig. 7B). Primary hepatocytes isolated from MKP-3−/− mice have a significantly reduced TG concentration (by 33%) compared with WT hepatocytes (Fig. 7C). Treatment with FK228 significantly increased TG concentration by 45% in WT hepatocytes but by 96% in MKP-3−/− hepatocytes, which completely abolishes the effect of MKP-3 deficiency on reducing TG accumulation (Fig. 7C). These results indicate that HDAC1 and 2 are likely the downstream mediators of MKP-3 action in lipid homeostasis.

DISCUSSION

MKP-3 has a well-established role in the attenuation of MAP kinase signaling by selectively dephosphorylating ERK1/2 (22–24). Expression of hepatic MKP-3 drastically increases in response to overnutrition (10). One potential mechanism could be due to overnutrition-induced ERK activation (25). Our previous studies show that MKP-3 is an important regulator of hepatic gluconeogenesis by acutely overexpressing and knocking down MKP-3 in liver cells and in the liver of lean mice and mice with DIO via adenovirus-mediated gene transfer (9,10,26). In this study, the role of MKP-3 in whole-body energy homeostasis was investigated using global MKP-3−/− mice. As expected, MKP-3 deficiency significantly lowered fasting blood glucose concentrations and improved systemic insulin sensitivity in mice with DIO. In addition to the beneficial effect of phenotype on glycemic control, MKP-3 global deficiency protected mice from developing DIO, which could attribute to reduced adiposity, decreased hepatic lipid accumulation, and increased energy expenditure.
Experiments with cultured adipocytes and isolated pre-adipocytes demonstrate that MKP-3 deficiency impairs in vitro adipocyte differentiation.

The major focus of this study is to investigate the underlying molecular mechanism of the protective effect of MKP-3 deficiency on HFD-induced hepatosteatosis. The liver TG contents in MKP-3$^{-/-}$ mice fed an HFD are similar to those in lean WT mice. These results indicate that MKP-3 plays a role in lipid homeostasis, which is a previously unrecognized and novel function. Considering the fact that the respiratory exchange ratio is not changed in MKP-3$^{-/-}$ mice, it is more likely that MKP-3 deficiency decreases lipogenesis. ERK2 activity is significantly increased in the liver of MKP-3$^{-/-}$ mice, but ERK2 is not likely to be responsible for the liver phenotype because, as we have previously shown, overexpression of the constitutively active mitogen-activated protein kinase actually significantly increases liver weight (25).

**Table 1**—Fold increase of serine/threonine phosphorylation in mouse hepatoma cells with reduced MKP-3 expression vs. control cells

| Symbol            | Name                                      | Phosphorylation sites | KD-to-control ratio | Q value for SILAC |
|-------------------|-------------------------------------------|-----------------------|---------------------|-------------------|
| Transcription     |                                           |                       |                     |                   |
| HDAC1             | Histone deacetylase 1                     | S393                  | 3.3                 | 0.02              |
| THRAP3            | Thyroid hormone receptor–associated protein 3 | S395                  | 1.5                 | 0.04              |
| Translation repression |                                |                       |                     |                   |
| EEF1D             | Eef1d protein                             | S162                  | 2.7                 | 0.02              |
| Ribosomal biogenesis |                                      |                       |                     |                   |
| NOP58             | Nucleolar protein 58                      | S509                  | 1.6                 | 0.03              |
| Protein folding   |                                           |                       |                     |                   |
| Ero1l             | Ero1l protein                             | S106                  | 2.6                 | 0.047             |
| Cytoskeleton      |                                           |                       |                     |                   |
| PTK9              | Twinfilin-1                               | S142                  | 179                 | 0.02              |
| Membrane trafficking |                                |                       |                     |                   |
| SNX2              | Sorting nexin-2                           | S97T101T104           | 9.4                 | 0.03              |
| RNA processing    |                                           |                       |                     |                   |
| PCBP1             | Poly(rC)-binding protein 1                | S173                  | 2.5                 | 0.047             |
| Function unknown  |                                           |                       |                     |                   |
| RSRC2             | Rsr2 arginine/serine-rich coiled-coil protein 2 isoform 1 | S32 | 2.0 | 0.02 |
|                   | AHNNAK nucleoprotein isoform 1            | S136                  | 1.5                 | 0.03              |

KD, knockdown; SILAC, stable isotope labeling with amino acids in cell culture.
search for the downstream targets of MKP-3, we performed two phosphoproteomic studies to identify proteins with significantly increased phosphorylation in the absence of MKP-3: 1) we compared stable isotope-labeled Hepa1–6 cells expressing shMKP-3 with cells expressing shGFP; 2) we compared label-free liver lysates from MKP-3−/− mice fed an HFD with liver lysates from WT mice fed an HFD. The first experiment has less variation and represents acute reduction of MKP-3 expression. The second experiment is more physiologically relevant and reflects the status of chronic MKP-3 deficiency. Interestingly, one overlap is significantly increased phosphorylation of HDAC1 on S393 in experiment 1 and significantly increased phosphorylation of HDAC2 on S394 in experiment 2. We also demonstrate that activities of both HDAC1 and 2 are significantly decreased by an HFD. The expression of casein kinase 2α, which was reported to phosphorylate HDAC2 on S394 (27), is also significantly decreased by HFD (data not shown). This indicates that both MKP-3 and casein kinase 2α may be responsible for decreased HDAC1/2 activity in response to overnutrition. Consistent with this hypothesis, HDAC1/2 activity is significantly increased by MKP-3 deficiency in the liver of mice with DIO, which is accompanied by decreased expression of lipogenic genes. These results indicate that HDAC1 and 2 may repress lipogenesis by inhibiting expression of lipogenic genes and that MKP-3 is required to keep HDAC1 and 2 in an unphosphorylated status on residues S393 and S394, respectively.

**HDACs function as transcription repressors by deacetylating lysine residues on histone proteins, which results in chromatin condensation (28). HDACs are divided into three classes based on homology and function: class I (HDAC1–3, 8, 10), class II (4–7,9,11), and class III (sirtuins 1–7) (29). Among class I HDACs, HDAC1 and 2 are highly homologous and functionally redundant. Activities of HDAC1 and 2 can be modified by phosphorylation. It has been reported that phosphorylation on residues S421 and S423 promotes HDAC1 enzymatic activity (30). Previous publications also showed that casein kinase-2α promotes hypertrophic response in the heart by phosphorylating HDAC2 on S394. Our study reveals that phosphorylation on residue S393 of HDAC1 and S394 of HDAC2 are essential for their activity. The role of HDAC1 and 2 in liver lipid metabolism has not been investigated previously. Liver-specific deletion of HDAC3, also a class I HDAC, has been reported to cause hepatic lipid accumulation (31).**
Our results reveal for the first time that inactivation of HADC1/2 by either interfering RNA or the selective HDAC1/2 inhibitor FK228 is sufficient to promote lipogenic gene expression in liver cells. Most important, inactivation of HDAC1/2 in primary hepatocytes isolated from MKP-3\(^{-/-}\) mice abolished the protective effect of MKP-3 deficiency on reducing TG content. These results support our hypothesis that MKP-3 is involved in lipid metabolism by regulating HDAC1/2 activity through modifying phosphorylation status on residues S393/S394. In an obese state, elevated MKP-3 expression in the liver likely contributes to hepatosteatosis by repressing the activity of HDAC1/2.

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**Figure 7**—Reduction of HDAC1/2 activity increases lipogenesis in liver cells. A: Effects of HDAC1/2 knockdown on lipogenic gene expression in Hepa1–6 cells. B: Effects of selective HDAC1/2 inhibitor FK228 on lipogenic gene expression in Hepa1–6 cells. C: Effects of selective HDAC1/2 inhibitor FK228 on lipogenesis in primary hepatocytes isolated from WT or MKP-3\(^{-/-}\) mice. For cell-based experiments, results shown here represent 3 independent experiments. *P < 0.05, as indicated; **P < 0.001, as indicated.
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