Disruption of tumor suppressor gene* Hint1 leads to remodeling of the lipid metabolic phenotype of mouse liver

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Abstract A lipidomic and metabolomic investigation of serum and liver from mice was performed to gain insight into the tumor suppressor gene Hint1. A major reprogramming of lipid homeostasis was found in both serum and liver of Hint1-null (Hint1−/−) mice, with significant changes in the levels of many lipid molecules, as compared with gender-, age-, and strain-matched WT mice. In the Hint1−/− mice, serum total and esterified cholesterol were reduced 2.5-fold, and lysophosphatidylethanolamines (LPEs) were increased 7-fold in serum, with a corresponding fall in phosphatidylcholines (PCs). In the liver, MUFA s and PUFAs, including arachidonic acid (AA) and its metabolic precursors, were also elevated, as were mRNA encoding enzymes involved in AA de novo synthesis. There was also a significant 50% increase in hepatic macrophages in the Hint1−/− mice. Several hepatic ceramides and acylcarnitines were decreased in the livers of Hint1−/− mice. The changes in serum LPCs and PCs were neither related to hepatic phospholipase A2 activity nor to mRNAs encoding lysophosphatidylethanolamine acyltransferases 1-4. The lipidomic phenotype of the Hint1−/− mouse revealed decreased inflammatory eicosanoids with elevated proliferative media-DH disturbances of the tumor suppressor gene cholesterol (1). For hepatocellular carcinoma (HCC), for example, the activity around 20 TSGs was reported to be lost (1-4), both by mutation (1) and by hypermethylation of promoter CpG islands (1, 3-5). One such TSG is the histidine triad nucleotide binding protein 1 (HINT1) gene, an ancient gene of largely unknown physiological function that leads to a variety of tumors when disrupted in mice (6-8), with evidence suggesting that it is also a human TSG (3, 9). Research has focused on the consequences of TSG inactivation on cell signaling pathways in the liver. However, little, if anything, is known about the effects of inactivation of HINT1 and other TSGs on the hepatic metabolome and lipidome, which is surprising given the growing recognition of the role played by small metabolites (10, 11) and lipids (12, 13) in hepatocarcinogenesis.

A number of lipid signaling molecules deserve further consideration in relation to the modes of action of TSGs in carcinogenesis. Ceramide (CER) second messengers, which are derived from the catabolism of SMs or by de

Abbreviations: AA, arachidonic acid; CER, ceramide; COX2, cyclooxygenase 2; DG, diacylglycerol; ELVOL, fatty acid elongase; FADS, fatty acid desaturase; HCC, hepatocellular carcinoma; HINT1, histidine triad nucleotide binding protein 1; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase; MRM, multiple reaction monitoring; MSD, mass-selective detector; OPLS-DA, orthogonal projection to latent structures-discriminant analysis; PC, phosphatidylcholine; PCA, principal components analysis; PKC, protein kinase C; PLA2, phospholipase A2; QPCR, real-time quantitative PCR; PGE2, prostaglandin E2; PLA2, phospholipase A2; RT, retention time; TSG, tumor suppressor gene; UPLC, ultraperformance liquid chromatography; UPLC-QTOF-MS, ultraperformance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry.

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CERs can modulate apoptosis (15), cell proliferation, differentiation (16), and ER stress and senescence (17). Current opinion suggests that there are perhaps in excess of 200 CER species (17) and that CERs participate in the formation of plasma membrane rafts (18), which are involved in cell signaling and membrane trafficking (16). Regarding mediation of TSG activity, CERs were reported to regulate the TSG phosphatase PTEN in membrane rafts (19). Moreover, p53 downregulates sphingosine kinase 1, which may compete with ceramide synthase for the metabolism of sphingosine, thus leading to increased CER synthesis (20). Nothing is known regarding the effect of the TSG HINT1 on CER and sphingolipid metabolism.

Lysoosphosphatidic acid (LPA) species act as autocrine/paracrine signaling molecules via their plasma membrane G-protein-coupled LPA receptors (21–23), which trigger many cell signaling cascades, leading to cell proliferation, migration, and differentiation (22, 23). LPA is extracellular and intracellular molecules that are synthesized from their corresponding lysophosphatidylcholine (LPC) precursors by a secreted lysophospholipase D (24) known as autotaxin (23, 25). The relationship between LPA and TSGs is poorly understood, except for the interaction of p53 and LPA in the regulation of hypoxia-inducible factor (26). Similarly, little is known regarding the interactions between LPCs and TSGs, with the exception that 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine [LPC(16:0)] can trigger apoptosis in hepatocytes in a p53-dependent fashion (27). LPCs are also proinflammatory and may act through the induction of cyclooxygenase 2 (COX2) (28).

To shed further light on the functioning of HINT1 in relation to its activity as a TSG, a comprehensive metabolomic and lipidomic investigation of the HINT1-/- mouse and gender-, age-, and strain-matched WT controls was undertaken. These studies show that a major reprogramming of lipid metabolism occurred in the liver when the HINT1 gene was disrupted, an effect also detectable in serum. The net outcome was an increase in LPCs and mitogenic LPA, together with a shift away from proapoptotic CERS and inflammatory eicosanoids in the HINT1-/- mouse.

**MATERIALS AND METHODS**

**Animals**

All mice used in this study were of the 129SvJ strain. HINT1-null (HINT1-/-) mice were obtained from Weinstein and colleagues, who developed this mouse line (7). They were continuously bred at EPFL, Lausanne and maintained in our facility. Control WT (HINT1+/+) 129SvJ mice were also maintained in our facility and cohoused with the 129SvJ/HINT1-/- mice. Animals were housed with a 12-h light/dark cycle and provided with mouse chow and water ad libitum. Experimental animal procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by local regulatory authorities. For metabolomic and lipidomic investigations, serum and liver tissue were obtained from 9-week-old male HINT1+/+ (n = 6) and HINT1-/- (n = 6) mice between 8:00 AM and 12:00 PM under fed conditions and transported frozen on dry ice to Bethesda for analysis.

**Chemicals**

Palmitic (16:0), heptadecanoic (17:0) (internal standard), stearic (18:0), oleic (18:1), α-linoleic (18:2n-6), γ-linolenic (18:3), dihomo-γ-linolenic (20:3n-6), arachidonic (20:4n-6), eicosapentaenoic (20:5), and docosahexaenoic (22:6) acids were obtained from Sigma-Aldrich (Buchs, Switzerland). The following phospholipids and sphingomyelins were obtained from Avanti Polar Lipids (Alabaster, AL): lysophosphatidylcholines LPC(18:2), LPC(20:4), LPC(22:6), and LPC(17:0) (internal standard); phosphatidylcholines (PCs) PC(18:2/18:2), PC(16:0/20:4), PC(16:0/18:2), PC(18:0/18:2), PC(20:4/20:4), PC(22:6/22:6), and PC(17:0/17:0) (internal standard); lysophosphatidic acids LPA(16:0), LPA(18:0), LPA(18:1), and LPA(20:4); and SM(d18:1/16:0) and SM(d18:1/18:1). Inorganic reagents were of the highest possible grade, and solvents used were HPLC grade.

**Determination of serum cholesterol and triglyceride concentrations**

Serum cholesterol/cholesterol esters and triglycerides were quantitated using BioVision kits (K503-100, K622-100, respectively; LuBioScience GmbH, Luzern, Switzerland) according to the manufacturer’s instructions.

**Untargeted metabolomics and lipidomics and targeted quantitative methods**

For serum metabolomics, serum samples were diluted 20-fold with 66% aqueous acetonitrile containing 10 μM chloropropamide (Sigma-Aldrich) as internal standard, vortexed, microfuged at 18,000 g for 20 min, and transferred to ultraperformance liquid chromatography (UPLC) injection vials. Samples were analyzed by UPLC-electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) using an Acquity BEH C18 1.7 μm (2.1 × 50 mm) column (Waters Corp., Milford, MA) under the following conditions: solvent A, 0.1% aqueous formic acid; solvent B, 0.1% formic acid in acetonitrile; 95% solvent A for 0.5 min, to 40% solvent A at 4 min, to 1% solvent A at 8 min, flushed for 1 min, then equilibrated at initial conditions for 1.5 min using a Waters Acquity UPLC. Flow rate was 0.5 ml/min, and column temperature was maintained at 60°C. Mass spectrometry on UPLC eluates was conducted in positive (ESI+) and negative (ESI-) modes using a Waters Synapt HDMS QTOFMS, scanning 50–1,000 amu at a rate of 3.3 scans/s. Capillary voltage was 3 kV, source temperature was 120°C, sampling cone was 30V, and desolation gas (nitrogen) flow was 850 l/h at 400°C. Total run time was 10 min. Samples were randomized and included multiple pooled samples, blanks, and a standard mixture to monitor instrument stability.

For serum lipidomics, to serum (20 μl) was added cold chloroform/methanol (2:1 v/v; 100 μl) containing 5 μM each of LPC(17:0) and PC(17:0/17:0) as internal standards. The solution was vortexed for 30 s and stood for 5 min at room temperature, followed by additional vortexing. Samples were microfuged at 18,000 g for 10 min to separate the phases. Organic phase was removed and evaporated to dryness under nitrogen, and the dried samples were stored at −20°C until just before analysis, when evaporated extracts were dissolved in chloroform/methanol (11:1 v/v; 20 μl), propan-2-ol/water/acetonitrile (50:25:25 v/v; 200 μl) was added, and the solution was transferred to UPLC.
injection vials. UPLC-ESI-QTOFMS analysis was conducted using a Waters Acquity CSH 1.7 μm C18 column (2.1 x 100 mm) under the following conditions: solvent A, acetonitrile/water (60:40 v/v) containing 10 mM ammonium acetate and 0.1% formic acid; solvent B, propan-2-ol/acetonitrile (90:10 v/v) containing 10 mM ammonium acetate and 0.1% formic acid. Gradient was 60% A to 57% A at 2 min, to 50% A at 2.1 min (ballistic gradient), to 46% A at 12 min, to 30% A at 12.1 min (ballistic gradient), to 1% A at 18 min before returning to initial conditions at 18.5 min and equilibration for 2 min. Flow rate was 0.4 ml/min, and the column was maintained at 55°C. Conditions of the mass spectrometer were as above but with a run time of 18 min.

For liver lipidomics, the protocol was adapted in part from a published method (29). Accurately weighed liver samples (~50 mg) were homogenized in water-methanol (43:57 v/v) in a Precellys® 24 bead homogenizer (Bertin Corp., Rockville, MD). Chloroform [800 μl; containing 5 μM each of LPC(17:0) and PC(17:0/17:0) as internal standards] was added, and the mixture was shaken at 37°C for 30 min. Samples were microfuged at 18,000 g for 20 min to separate the phases. Organic phases were removed and evaporated to dryness under nitrogen, and the dried samples were stored at −20°C until just before analysis, when evaporated extracts were dissolved in chloroform/methanol (1:1 v/v; 20 μl), and propan-2-ol/water/acetonitrile (50:25:25 v/v; 200 μl) was added and transferred to UPLC injection vials. UPLC-ESI-QTOFMS was conducted as described above for serum lipidomics.

Quantitation of lipid species was performed using either multiple reactions monitoring (MRM) or parent ion scanning using a Waters Acquity UPLC coupled to a Waters Xevo TQ mass spectrometer (UPLC-ESI-TQMS). Samples were prepared as described above for serum metabolomics; however, only 5 μM LPA(17:0) was added as internal standard. A Waters Acquity BEH C18 column (2.1 x 100 mm) was used under the following conditions: solvent A, water containing 10 mM ammonium acetate and 0.1% formic acid; solvent B, acetonitrile/propan-2-ol (5:2 v/v) containing 10 mM ammonium acetate and 0.1% formic acid. Gradient was 70% A for 1 min, then linear gradient to 50% A at 3 min, to 1% A at 8 min, held until 15 min, returning to initial conditions over 1 min, and held for an additional 2 min for column equilibration. Flow rate was 0.4 ml/min, and the column was maintained at 50°C. The mass spectrometer was operated in MRM and parent ion scanning modes and, as appropriate, in ESI+ and ESI− modes. Capillary voltage was 2.2 kV, source temperature was 150°C, and desolvation gas (nitrogen) flow rate the phases. Organic phases were removed and evaporated to dryness under nitrogen, and the dried samples were stored at −20°C until just before analysis, when evaporated extracts were dissolved in chloroform/methanol (1:1 v/v; 20 μl), and propan-2-ol/water/acetonitrile (50:25:25 v/v; 200 μl) was added and transferred to UPLC injection vials. UPLC-ESI-QTOFMS was conducted as described above for serum lipidomics.

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lular giant cells was recorded. Immunohistochemistry was performed for detection of the murine F4/80 160 kDa cell surface glycoprotein that is expressed predominantly on murine macrophages. Paraffin-embedded, formalin-fixed sections were deparaffinized and hydrated with xyloc, ethanol, and then distilled water. Antigen retrieval was performed with proteinase K, followed by washing with distilled water and endogenous peroxidase quenching with 3% methanolic hydrogen peroxide, followed by washing with distilled water and blocking with 10% rabbit serum in PBS. Primary antibody incubation was conducted with F4/80 monoclonal antibody to mouse macrophages (clone BMS; BMA Biomedicals, Augst, Switzerland). Secondary antibody incubation used a polyclonal rabbit anti-rat immunoglobulin-biotinylated secondary antibody followed by incubation using the Vectastain Universal Elite ABC kit (Adipogen, Liestal, Switzerland). DAB was used as peroxidase substrate, and counterstaining was with hematoxylin.

**Real-time quantitative PCR**

Total RNA was extracted from liver tissues (30 mg) preserved with RNalater and using RNAeasy columns (QIAGEN AG, Hombrechtkon, Switzerland) with an on-column digestion step as described in the manufacturer’s protocol. Total RNA (2 μg) was reverse transcribed with Superscript III reverse transcriptase (Invitrogen AG, Basel, Switzerland). cDNA was used to a fast universal PCR master mix (#4382042; Applied Biosystems, Zug, Switzerland) and amplified using an Applied Biosystems 7500 Real-Time PCR system, with primers (Applied Biosystems) specific for mouse genes Lpcat1 (Mm00461015_m1), Lpcat2 (Mm00557141_m1), Lpcat3 (Mm00520147_m1), Lpcat4 (Mm01336593_m1), Fabp1 (Mm00444340_m1), Ste27a1 (Mm00449511_m1), Elvol5 (Mm00506717_m1), Fads1 (Mm00507605_m1), and Fads2 (Mm00517221_m1). The data were analyzed according to the 2^△△Ct method, and Ct values were normalized to 18S rRNA (Hs99999901_s1). All reactions were performed in duplicate.

**Multivariate data analysis**

For the UPLC-ESI-QTOFMS metabolomics/lipidomics experiments, mass spectral data were centroided and deconvoluted to generate a multivariate data matrix using MarkerLynx (Waters). Peak picking, alignment, deisotoping, and integration were performed automatically by the software and transformed into the multivariate data matrix containing aligned peak areas with matched m/z and Rt values. For the serum metabolomic data, the peak areas were normalized to the peak area of the internal standard chloropropamide (ESI+ = 277.041 and ESI− = 275.024 m/z; Rt = 5.3 min). Data were Pareto-scaled and analyzed by unsupervised principal components analysis (PCA), followed by orthogonal projection to latent structures-discriminant analysis (OPLS-DA) using SIMCA 13 (Umetrics AB, Malmö, Sweden) comparing data from Hint1+/− and Hint1−/− samples.

**Univariate statistics**

Statistical analysis of groups was made using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). In all cases, nonparametric Mann-Whitney U tests were used. All means are expressed ± standard deviation, including in the figures.

**RESULTS**

Untargeted metabolomic analysis of mouse sera was carried out to discover biomarkers associated with HINT1 expression. A clear separation of Hint1+/− and Hint1−/− sera was found in the PCA scores plots in ESI+ and ESI− modes (Fig. 1A). Further analysis of the data by OPLS-DA revealed loadings S-plots with positive and negative ions that were significantly elevated and depressed in the Hint1−/− mice relative to the Hint1+/− mice (Fig. 1A). Assignment of these ions is given in supplementary Table II. The overall metabolic pattern in Hint1−/− mice was of up-regulated LPCs.

Sixteen ions corresponding to 15 lipids were found to be lower in Hint1−/− mice in the serum metabolomics experiment, as well as 12 in ESI+ and four in ESI− mode. Assignment of these ions is given in supplementary Table III. The overall pattern in Hint1−/− mice was of downregulated phosphatidylcholine (PC) and SMs in the metabolomic (Fig. 1A) and lipidomic (Fig. 1B) investigations.

Only lipidomic analysis was performed on mouse liver. In these experiments, a clear separation of WT and Hint1−/− liver extracts was found in the PCA scores plots in ESI+ and ESI− modes (Fig. 1C). A total of 44 ions (37 in ESI+, 7 in ESI−) were annotated as 43 upregulated lipid constituents of the Hint1−/− liver (supplementary Table V). These comprised one LPC, three monoacylglycerols, four FAs (linoleic, arachidonic, docosapentaenoic, and docosahexaenoic acids), 11 PCs (red symbols in Fig. 1C), and 16 diacylglycerols (DGs) (orange symbols in Fig. 1C). A total of 38 ions (30 in ESI+, 8 in ESI−) were annotated as 28 downregulated lipid constituents of the Hint1−/− liver (supplementary Table V). These comprised one LPC, three PCs, four SMs (green symbols in Fig. 1C), 12 phosphatidylethanolamine species, and four ceramides (blue symbols in Fig. 1C). A summary of the lipidomic and metabolomic changes in the Hint1−/− mouse is given in Table 1.

First, the hypothesis was tested that elevated serum LPC arose from increased PLA2 activity in the liver. Because LPCs are released from membrane PCs by PLA2, the total PLA2 activity of Hint1+/− and Hint1−/− liver was determined in liver homogenates. There was no statistically significant difference in PLA2 activities in the two Hint1 genotypes. Therefore, formation of LPCs from PCs by PLA2 did not explain the large differences in serum LPCs and PCs (Fig. 2A) between the genotypes; nor did this explain the patterns of hepatic LPCs and PCs (Fig. 2B) observed.

Second, the hypothesis was tested that HINT1 might increase the synthesis of PC from LPC by lysophosphatidylcholine acyl transferase (LPCAT) enzymes by upregulating expression of one or more Lpeat genes. Quantitative PCR (QPPCR) determinations of Lpeat1, Lpeat2, Lpeat3, and Lpeat4 mRNAs in WT and Hint1−/− livers revealed no statistically significant differences in expression of any of these four Lpeat genes between Hint1 genotypes.

There occurred stark differences in hepatic fatty acid concentrations for PUFAs, for AA, and for its precursors in the de novo synthesis pathway, linoleic acid (18:2n-6) and dihomo-y-linolenic acid (20:3n-6) (Fig. 3). AA, 18:2n-6, and 20:3n-6 were highly statistically significantly elevated in the Hint1−/− livers compared with Hint1+/− livers. The 2-fold elevated concentration of AA was confirmed by an independent GCMS analysis, which yielded a significant 1.5-fold increase of AA in the Hint1−/− mice, with values
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[peak area ratio (20:4/17:0)/mg liver] of 0.23 ± 0.07 and 0.15 ± 0.01 (P = 0.02) for Hint1/−/− and Hint1+/+ livers, respectively. The intermediate precursor γ-linolenic acid (18:3n-6) was not detected in livers of either genotype (Fig. 3B). Because of these metabolic findings, QPCR was conducted to evaluate mRNA expression for the three enzymes involved in the conversion of linoleic acid (18:2) to arachidonic acid (20:4). The findings for Fads1, Elovl5, and Fads2 are given in Fig. 3B. The expression of mRNA for these three genes was 1.5- to 2-fold higher in the null mice, consistent with the observed increased hepatic AA levels. The increased hepatic levels of 18:2 and 20:4 were consistent with the observations that LPC(18:2), LPC(20:4), and PC(20:4) were also significantly higher in livers of Hint1/−/− mice. It therefore appeared that one phenotype of the Hint1/−/− mouse was a small (1.5- to 2-fold) accumulation of AA, together with AA metabolic precursors and their phospholipid derivatives in the liver (Fig. 2B).

Third, because of the significantly elevated hepatic levels of AA and its phospholipid derivatives, the eicosanoid profile of Hint1/−/− and Hint1+/+ livers was determined using LC-ESI-TQMS. Table 2 gives the concentrations (pg/mg liver) of 37 eicosanoids or groups of unresolved eicosanoids.
that represent 44 distinct prostaglandin and leukotriene metabolites. Contrary to expectations, prostaglandins and their metabolites were significantly lower in the null mice. Of particular note was the 4-fold decrease in hepatic 20-hydroxy-PGE2 from 14 ± 4 to 114 ± 63 pg/mg liver (P = 0.01). The only statistically significant increase observed for the null livers was the level of 20-hydroxy-PGE2 from 14 ± 4 to 14 ± 2 pg/mg liver (P = 0.026). This metabolite arises by the ω-hydroxylation of prostaglandin E2 (PGE2) by murine CYP4A14 (32). The downregulated eicosanoids in null livers all arose from COX1/COX2 metabolism of AA (supplementary Fig. II). Eicosanoid metabolites arising from cytochrome P450 or lipoxygenase metabolism of AA were unaltered in the null livers (Table 2), showing a degree of selectivity for the COX1/COX2 pathway.

Fourth, increased FFA uptake by the liver might explain the differences observed in hepatic fatty acids in Hint1−/− mice (33). Increased FFA hepatic uptake has been proposed as a mechanism of liver inflammation (34). However, mRNA quantitation by QPCR could not detect a difference in expression of Sre27a1 or Fapb1, two genes involved in the uptake of long-chain FFA.

Fifth, representatives of two other groups of phospholipid were determined in serum of the two mouse genotypes, SMs and LPAs. There were no statistically significant differences in the serum µM concentrations of SM(d18:1/16:0) and SM(d18:1/18:1) as determined by UPLC-ESI-TQMS (Fig. 2A). Six ions corresponding to four hepatic CERS had already been observed in the lipidomic experiment to be downregulated in Hint1−/− livers (supplementary Table V). Because SMs are metabolized to proapoptotic CERS, the hepatic concentrations of these four ceramides was determined from the UPLC-ESI-QTOFMS data and expressed in arbitrary units/mg wet liver. All four CERS were statistically significantly decreased in the Hint1−/− livers, by 35% to 60% (Fig. 2C). Regarding LPAs, up to 10-fold elevated serum concentrations of LPA(16:0), LPA(18:0), LPA(18:1), and LPA(20:4) were found in Hint1−/− mice as compared with their Hint1+/+ counterparts (Fig. 2A).

Sixth, because of the lipid changes observed in the livers of the null mice and the observation that C4- and C18-carnitines were depressed in the null livers (supplementary Table V), hepatic concentrations of C2-, C4-, and C18-carnitines were determined from the UPLC-ESI-QTOFMS dataset using extracted ion chromatograms for each liver sample. The results showed a highly statistically significant decrease in all three acyl carnitines, but not in C16-carnitine, in the null livers (supplementary Fig. III). This pattern is suggestive of increased fatty acid oxidation in the null mouse, but this is not supported by the hepatic fatty acid data in Fig. 3A.

Finally, because of the increase in AA, its metabolic precursors, and phospholipid metabolites in the Hint1−/− livers, we decided to examine formalin-fixed sections of WT and Hint1−/− livers for evidence of inflammation. Histological examination of liver sections from six Hint1−/− mice showed one liver with a single infiltrate with a lobular focus of 10 or more lymphocytes. The other five mice were negative in this regard. For six Hint1−/− mice, three livers were negative, but three showed 2–3 foci of lymphocytic infiltration. One of these livers displayed hepatocellular giant cells (Fig. 4). However, these differences were not statistically significant. In contrast, immunohistochemistry for the mouse macrophage surface glycoprotein F4/80 demonstrated a larger number of macrophages resident in the Hint1−/− liver than the Hint1+/+ liver (Fig. 4A,B). The macrophage cell counts were highly statistically significantly.

### Table 1. Summary of lipidomic changes in serum and liver for Hint1−/− mice

| Serum | | Liver | | |
|-------|-------|-------|-------|-------|
| ↑ | ↓ | ↑ | ↓ |
| LPC (n = 9) | LPC (n = 2) | LPC (n = 1) | | |
| LPE (n = 5) | LPE (n = 2) | | | |
| LPA (n = 4) | PC (n = 12) | PC (n = 10) | PC (n = 3) | |
| PE (n = 5) | PE (n = 2) | PE (n = 12) | PE-P (n = 1) | |
| PE-P (n = 4) | PE-P (n = 1) | PE-P (n = 1) | | |
| MG (n = 5) | MG (n = 3) | | | |
| DG (n = 1) | DG (n = 1) | DG (n = 17) | | |
| SM (n = 7) | SM (n = 3) | | | |
| CER (n = 1) | CER (n = 2) | CER (n = 1) | CER (n = 4) | |
| SM (n = 3) | | | | |
| 20:4d | 18:2d, 20:4d, 22:5d, 22:6d | retinylacetate | butyrylcarnitine, stearoylcarnitine, retinoldehyde, retinoic acid | |

LPE, lysophosphatidylethanolamine; MG, monoacylglycerol; PE, phosphatidylethanolamine; PE-P, phosphatidylethanolamine plasmalogon.  
↑ and ↓ represent increases and decreases, respectively, in molecules determined by the multivariate data analysis of UPLC-ESI-TQMS data. The exact fold changes were not determined, except in cases where quantitation by UPLC-ESI-TQMS or extracted ion chromatograms from the UPLC-ESI-QTOFMS data were performed. Such data appear in Figs. 2, 3, and supplementary Fig. III.  
†n is the number of molecules of a particular class that were found to be either increased or decreased in the Hint1−/− mice.  
‡Glycosylceramide (see supplementary Table IV).  
§Fatty acid nomenclature: 18:2, linoleic acid; 20:4, arachidonic acid; 22:5, docosapentaenoic acid; 22:6, docosahexaenoic acid.
Murine Hint1 disruption and remodeling of lipid metabolism

Precursors of de novo AA synthesis, in particular linoleic acid (18:2), were elevated. There was a decline in hepatic CERs d18:1/22:1, d16:1/23:0, d18:1/22:0, and d18:1/24:0; the C2-, C4-, and C18-acyl carnitines; and various eicosanoids derived from the COX1/COX2 pathway through PGH2. Furthermore, the Hint1−/− mouse phenotype involved expansion of the different (Fig. 4D). Therefore, a previously undisclosed phenotype of the Hint1−/− mouse liver is an enhanced population of hepatic macrophages.

DISCUSSION

The current study revealed that the Hint1−/− mouse is characterized by a phenotype involving elevated serum LPC and LPA, with increased hepatic concentrations of AA, LPC(20:4), and PC(20:4/20:4). Precursors of de novo AA synthesis, in particular linoleic acid (18:2), were elevated. There was a decline in hepatic CERs d18:1/22:1, d16:1/23:0, d18:1/22:0, and d18:1/24:0; the C2-, C4-, and C18-acyl carnitines; and various eicosanoids derived from the COX1/COX2 pathway through PGH2. Furthermore, the Hint1−/− mouse phenotype involved expansion of the hepatic phospholipids (nmol/mg wet liver) between KO mice and WT mice. C: Changes in ceramide concentrations (units/mg wet liver) between KO mice and WT mice. Analyses were performed in duplicate for WT (n = 6) and KO (n = 6) mice. * P < 0.05; ** P < 0.001; *** P < 0.0001.

Fig. 2. Quantitation of lipids in serum of Hint1−/− (KO) and WT mice. A: Rise in serum LPC concentrations (μM), fall in serum PC concentrations and rise in serum LPA concentrations in KO mice compared with WT mice. B: Changes in hepatic phospholipids (nmol/mg wet liver) between KO mice and WT mice. C: Changes in ceramide concentrations (units/mg wet liver) between KO mice and WT mice. Analyses were performed in duplicate for WT (n = 6) and KO (n = 6) mice. * P < 0.05; ** P < 0.001; *** P < 0.0001.
hepatic macrophage population, presumably mostly Kupffer cells. The possible significance of these findings regarding the loss of tumor suppressor function is discussed below.

Disruption of Hint1 increased mediators proliferative signaling in mouse liver while decreasing inflammatory eicosanoids. The prominent elevation of serum LPA concentrations into the μM range can be expected to have a significant mitogenic effect. LPAs stimulate cell surface G-protein-coupled receptors that activate multiple cell signal transduction pathways, including those involving RAS and RHO family GTPases, which regulate cell proliferation (35). LPAs are produced extracellularly from LPC by autotaxin and intracellularly from PA by PLA2 (35, 36). However, total PLA2 activity did not differ between Hint1−/− and Hint1+/+ livers. Studies in hepatoma cell lines Hep3B and Huh7 have indicated that the proinflammatory cytokine TNFα promoted autotaxin expression and secretion, leading to an increase in LPA (37). Thus, inflammatory signaling within the liver, but not involving eicosanoids, might be expected to lead to LPA-directed proliferation.

Other investigations have established that the γ isotype of protein kinase C (PKCγ) was elevated in the brains of Hint1−/− relative to WT mice, with a concomitant 2-fold increase in PKC activity in Hint1−/− brain lysates (38). In addition, cytosolic PLA2, the principal form of PLA2 responsible for AA release from membrane phospholipids (39), is activated by phosphorylation by either p42 MAPK or PKC (40). Therefore, increased PKC activity in Hint1−/− liver due to the absence of a HINT1-PKC inhibitory interaction, as reported for Hint1−/− brain (38), would be expected to lead to increased cytosolic PLA2 phosphorylation, metabolic activity, and AA release. This may in part explain the increase in the AA pool observed in Hint1−/− mice. The enriched hepatic AA pool was expected to drive prostaglandin E2 (PGE2) synthesis via COX2. However, contrary to expectation, it was found that inflammatory eicosanoids were depressed in the null mouse livers. Decreased COX1/COX2 activity might lead to a backlog of AA and possibly explain the observed slight increase in hepatic AA.

Another observation of relevance was the downregulation of all PCs studied and a concomitant upregulation of all LPCs that were quantitated because several studies have reported that LPCs activate PKC (28). Studies in hepatoma cell lines Hep3B and Huh7 have indicated that the proinflammatory cytokine TNFα promoted autotaxin expression and secretion, leading to an increase in LPA (37). Thus, inflammatory signaling within the liver, but not involving eicosanoids, might be expected to lead to LPA-directed proliferation.

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Another observation of relevance was the downregulation of all PCs studied and a concomitant upregulation of all LPCs that were quantitated because several studies have reported that LPCs activate PKC (28). These observations did not appear to be due upregulated PLA2. Nor were differences detected in the expression of LPCAT genes, Lpcat1, Lpcat2, Lpcat3, and Lpcat4. Thus, an attenuated conversion of LPC to PC by LPCAT enzymes did not explain the pattern of LPCs and PCs observed. The origins of elevated serum LPCs in the Hint1 KO mouse remain unknown.

Disruption of the Hint1 gene may not only remove the inhibition of PKC by HINT1 but also may cause PKC to become...
The aforementioned findings are consistent with a phenotype in the \( \text{Hint}^{+/-} \) liver of increased proliferative signaling with reduced proapoptotic signaling. Although inflammation was shown to be required for diethylnitrosamine-induced liver cancer (45) and is thought to play a role in human HCC (46), no increase in prostaglandin inflammatory mediators was found in this study; in fact, a decrease in these molecules was observed in the livers of the null mice. It is not known which components of the lipidomic phenotype of the \( \text{Hint}^{+/-} \) mouse are key to cancer susceptibility in these animals. Future investigations will consolidate these findings and lead to a fuller understanding of the role played by hepatic LPC, LPA, AA, CER, DG, and macrophages in hepatocarcinogenesis in the \( \text{Hint}^{+/-} \) mouse. Moreover, the role played by \text{HINT1} in the cellular physiology of the liver remains to be elucidated, but lipidomics has provided relevant new insights. As noted by Weinstein and colleagues (6), there is no other TSG that has a prokaryotic ortholog. \text{HINT1}, on the other hand, is highly conserved, and in the smallest genome of any cellular organism, \textit{Mycoplasma genitalium} (580 kb), there is a homolog that shares >30% identity with the full human \text{HINT1} sequence (6). From the current investigations, it is clear that the \text{HINT1} protein has a

### Table 2. Occurrence of eicosanoids in \( \text{Hint}^{+/-} \) and \( \text{Hint}^{+/-} \) liver

| Eicosanoid                        | \( \text{Hint}^{+/-} \) (n = 6) | \( \text{Hint}^{+/-} \) (n = 6) | \( P \) (Mann-Whitney) |
|----------------------------------|--------------------------------|--------------------------------|------------------------|
| Tetranor-PGEM/Tetranor-PGDM      | 11 ± 7                         | 16 ± 6                         | —                      |
| Tetranor-PGFM                    | 58 ± 17                        | 70 ± 22                        | —                      |
| 20-Hydroxy-PGE2                  | 14 ± 4                         | 114 ± 63                       | 0.026                  |
| 20-Hydroxy-PGF2a                 | 20 ± 9                         | 38 ± 16                        | —                      |
| 6-Keto-PGE1a                     | 92 ± 104                       | 1 ± 0                          | 0.01                   |
| 8-IsopGF2a                       | 16 ± 4                         | 10 ± 3                         | 0.009                  |
| TxB2                             | 97 ± 26                        | 53 ± 30                        | 0.026                  |
| PGF2a                            | 409 ± 142                      | 101 ± 58                       | 0.002                  |
| PGF1a                            | 35 ± 8                         | 14 ± 6                         | 0.002                  |
| 13-Keto-PGF2a/8-IsopGE2/PGE2      | 37 ± 12                        | 9 ± 3                          | 0.002                  |
| 8-IsopGE2/PGE2                   | 54 ± 23                        | 17 ± 5                         | 0.015                  |
| PGI1                             | 21 ± 5                         | 1 ± 4                          | 0.002                  |
| PGD2                             | 74 ± 17                        | 37 ± 15                        | 0.009                  |
| 15-Keto-PGE2/8-Isop-15-keto-PGE2  | 5 ± 1                          | 4 ± 1                          | —                      |
| 13,14-Dihydro-15-keto-PGF2a       | 15 ± 5                         | 19 ± 10                        | —                      |
| 5,6S/LXA4                        | 6 ± 1                          | 6 ± 1                          | —                      |
| 13,14-Dihydro-15-keto-PGE2       | 15 ± 5                         | 19 ± 10                        | —                      |
| 8-IsopGA2/PGA2/PGJ2              | 7 ± 1                          | 9 ± 12                         | —                      |
| 8X15SDBHETE                      | 58 ± 55                        | 84 ± 30                        | —                      |
| 5X15SDHETE                       | 14 ± 5                         | 34 ± 24                        | —                      |
| 10S,17S-DHDoHe                    | 22 ± 13                        | 28 ± 7                         | —                      |
| LTB4                             | 21 ± 2                         | 22 ± 3                         | —                      |
| Tetranor-12S4HETE (x)-14,15-DHET  | 165 ± 60                       | 237 ± 106                      | —                      |
| 12S-HHT                          | 603 ± 179                      | 365 ± 224                      | —                      |
| (x)-11,12-DHET                   | 60 ± 18                        | 94 ± 42                        | —                      |
| (x)-8,9-DHET                     | 40 ± 10                        | 50 ± 14                        | —                      |
| 5S6S-DiHETE                      | n.d.                           | n.d.                           | —                      |
| 19S-HETE                         | 72 ± 15                        | 53 ± 14                        | —                      |
| 5,6-DHET                         | 25 ± 3                         | 28 ± 5                         | —                      |
| (x)-18-HETE                      | 53 ± 12                        | 50 ± 13                        | —                      |
| 12S-HEPE                         | 367 ± 117                      | 381 ± 226                      | —                      |
| 17S-HETE                         | 28 ± 3                         | 27 ± 4                         | —                      |
| 16S-HEPE                         | 54 ± 14                        | 45 ± 11                        | —                      |
| 5SHEPE                           | 17 ± 2                         | 19 ± 1                         | —                      |
| (x)-13-HODE                      | 2,392 ± 1210                   | 2,080 ± 738                    | —                      |
| (x)-9-HODE                       | 2,710 ± 578                    | 2,300 ± 902                    | —                      |

n.d., not determined

activated. It is reported here that 16 discrete DGs were upregulated in the livers of the null mice. DGs are physiological activators of PKC and have long been considered as important second messengers in carcinogenesis (41).

CERs play an important role in apoptosis signaling. CERs also play a role in fatty acid synthesis by upregulating SREBP1c expression. In a recent report, CER(d18:1/22:0) and CER(d18:1/24:0) were two of the three most abundant CERs in human plasma (42). All of these CERs were downregulated in serum and liver tissue of \( \text{Hint}^{+/-} \) mice. The principal ceramide synthase in mouse liver is CerS2, which can synthesize these two CERs (43). There are many transcriptional stimuli of ceramide synthase genes, including stress, DNA damage, and hypoxia (43). Use of the ceramide synthase inhibitor fumonisin B1 in a variety of cultured cells led to decreased CER concentrations and the inhibition of apoptosis in all cases (43). Moreover, \( \text{Cers}^{2}\)-deficient mice developed hepatic adenomas, many of which progressed to HCC (44), showing that impaired specific CER synthesis can lead to HCC. Thus, the downregulation of CER serum and liver concentrations, including for CER(d18:1/22:0) and CER(d18:1/24:0), in \( \text{Hint}^{+/-} \) mice may partly explain the action of \text{Hint1} as a TSG.
major impact on lipid homeostasis in liver and serum and, in all probability, in many other tissues. The lipid reprogram-
ing that is associated with Hint1 inactivation provides clues to its activity as a tumor suppressor gene.\textsuperscript{14}

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