



# Lactic acid bacteria–derived $\gamma$ -linolenic acid metabolites are PPAR $\delta$ ligands that reduce lipid accumulation in human intestinal organoids

Received for publication, July 9, 2022, and in revised form, September 13, 2022. Published, Papers in Press, September 24, 2022, <https://doi.org/10.1016/j.jbc.2022.102534>

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Edited by Qi-Qun Tang

Gut microbiota regulate physiological functions in various hosts, such as energy metabolism and immunity. Lactic acid bacteria, including *Lactobacillus plantarum*, have a specific polyunsaturated fatty acid saturation metabolism that generates multiple fatty acid species, such as hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and *trans*-fatty acids. How these bacterial metabolites impact host physiology is not fully understood. Here, we investigated the ligand activity of lactic acid bacteria–produced fatty acids in relation to nuclear hormone receptors expressed in the small intestine. Our reporter assays revealed two bacterial metabolites of  $\gamma$ -linolenic acid (GLA), 13-hydroxy-*cis*-6,*cis*-9-octadecadienoic acid ( $\gamma$ HYD), and 13-oxo-*cis*-6,*cis*-9-octadecadienoic acid ( $\gamma$ KetoD) activated peroxisome proliferator-activated receptor delta (PPAR $\delta$ ) more potently than GLA. We demonstrate that both  $\gamma$ HYD and  $\gamma$ KetoD bound directly to the ligand-binding domain of human PPAR $\delta$ . A docking simulation indicated that four polar residues (T289, H323, H449, and Y473) of PPAR $\delta$  donate hydrogen bonds to these fatty acids. Interestingly, T289 does not donate a hydrogen bond to GLA, suggesting that bacterial modification of GLA introducing hydroxy and oxo group determines ligand selectivity. In human intestinal organoids, we determined  $\gamma$ HYD and  $\gamma$ KetoD increased the expression of PPAR $\delta$  target genes, enhanced fatty acid  $\beta$ -oxidation, and reduced intracellular triglyceride accumulation. These findings suggest that  $\gamma$ HYD and  $\gamma$ KetoD, which gut lactic acid bacteria could generate, are naturally occurring PPAR $\delta$  ligands in the intestinal tract and may improve lipid metabolism in the human intestine.

Gut microbiota plays diverse critical roles in host physiological processes including energy metabolism. Dysbiosis, that is, an imbalance in microbiota, is associated with

metabolic disorders such as obesity (1, 2). Human gut microbiota mainly comprises of four phyla: *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* (3, 4). Although a relationship between the gut microbiota and metabolic diseases is not fully understood, alteration of the proportion of *Bacteroidetes* and *Firmicutes* may associate with the development of obesity (5). Previous studies provided evidence that gut microbiota alters host energy harvest. For example, fecal transplantation from *ob/ob* mice to germ-free mice leads to a higher increase in body fat in the WT mice (6). Gut microbiota–derived metabolites mediate host energy metabolism. For instance, changes in the amount of short-chain fatty acids (SCFAs) produced from dietary fibers have been detected in the intestinal lumen of obese patients (7, 8). SCFAs reportedly activate GPR41 and GPR43 as mediators and energy resources for host tissues (9). Activated GPR41 and GPR43 by SCFAs may function as energy sensors in gut microbiota communication connecting with the sympathetic nervous system and white adipose tissue (10, 11). In addition, long-chain fatty acids (LCFAs) may also function as nutrients and signaling molecules that target some G protein–coupled receptors (GPCRs) and are involved in systemic energy metabolism (12). However, whether LCFA derivatives converted by gut microbiota regulate host energy metabolism is unclear. In a previous study, we reported that lactic acid bacteria can enzymatically convert and produce certain fatty acids from diet-derived polyunsaturated fatty acids (PUFAs), including  $\gamma$ -linolenic acid (GLA) (13). *Lactobacillus acidophilus* and *Pediococcus* sp. exhibit saturation metabolism on  $\Delta 9$  and  $\Delta 12$  double bonds in PUFAs, producing hydroxy- and oxo-fatty acids (Fig. S1) (14). These metabolites occur at significantly higher levels in specific pathogen-free mice relative to the levels in germ-free mice (13). Although these compounds are presumed to function in the human intestine at physiological concentrations, such biological activities are yet to be studied in detail.

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## GLA metabolites are PPAR $\delta$ ligands

Nuclear hormone receptors (NRs) are transcription factors activated by lipophilic ligands, including steroid hormones, fat-soluble vitamins, and dietary lipids. The NR superfamily comprises 48 members in humans (15). An N-terminal domain characterizes these receptors with activation function 1 which can activate transcription constitutively in the absence of ligand, a central DNA-binding domain, and a C-terminal ligand-binding domain (LBD) with activation function 2 (16). The transcriptional activity of NRs is regulated by the recruitment of coactivators such as steroid receptor coactivator-1 (SRC-1) and corepressors such as nuclear receptor corepressor (NCoR) (17). Ligand binding induces a conformational change of the LBD of NR and releases bound corepressors. Subsequently, coactivators are associated with and activate NRs, leading to transcriptional activation. Although the classical NRs such as steroid hormone receptors are translocated from the cytoplasm to the nucleus in a ligand-dependent manner, other NRs including peroxisome proliferator-activated receptors (PPARs) are constitutively localized in the nucleus and associated with their response elements (18). NRs govern the expression of genes involved in diverse physiological functions including reproduction, immune response, and energy metabolism. In several studies, NRs reportedly play an important role in energy metabolism. For example, NRs that regulate energy metabolism are highly expressed in metabolic tissues, including the liver, adipose tissue, skeletal muscle, and intestines (19). In the intestines, higher levels of dietary lipophilic molecules such as fatty acids, bile acids, and fat-soluble vitamins, which can serve as physiological ligands for NRs, are observed relative to the levels found in other tissues (18, 20).

PPARs play a major regulatory role in energy metabolism among the NR superfamily. They bind to the peroxisome proliferator-response element (PPRE) on the gene promoter as a heterodimer with retinoid X receptors (21). Among three PPAR subtypes [ $\alpha$ ,  $\gamma$ , and  $\delta$  (also referred to as  $\beta$ )], PPAR $\alpha$ , highly expressed in the liver, binds fatty acids or lipid-lowering fibrates (22) and controls the expression of target genes related to fatty acid  $\beta$ -oxidation and transport. PPAR $\alpha$ -deficient mice develop hepatic steatosis during fasting or high-fat diet feeding. Contrastingly, PPAR $\gamma$ , highly expressed in the adipose tissue as a master regulator of adipogenesis, is a target molecule of the insulin sensitizer thiazolidinedione (23). PPAR $\delta$ , the most recently studied PPAR subtype, is expressed in various tissues including gastrointestinal tissues as compared to PPAR $\alpha$  and PPAR $\gamma$  and regulates fatty acid and glucose metabolism in metabolic tissues (19, 24–26). PPAR $\delta$  activation by its specific agonist GW1516 increases expression of genes related to fatty acid  $\beta$ -oxidation (e.g., *CPT1A* and *HMGCS2*). PPAR $\delta$  also regulates cholesterol and lipoprotein metabolism in intestine (27–29). These findings show that PPAR $\delta$  plays a crucial role in intestinal energy metabolism. In addition to normal physiology, a role of PPAR $\delta$  on colon cancer has been reported (25, 30, 31). Although several studies showed that PPAR $\delta$  is protumorigenic, protective effects of PPAR $\delta$  were also reported, suggesting a complex and diverse role of PPAR $\delta$  in colon cancer.

The present study explored the intestinal NRs activated by lactic acid bacteria–derived fatty acids. Using cell-based reporter assays, we found that two GLA (18:3n-3)-derived fatty acids, 13-hydroxy-*cis*-6,*cis*-9-octadecadienoic acid ( $\gamma$ HYD) and 13-oxo-*cis*-6,*cis*-9-octadecadienoic acid ( $\gamma$ KetoD), activate human PPAR $\delta$  potently. Additionally,  $\gamma$ HYD and  $\gamma$ KetoD stimulated fatty acid  $\beta$ -oxidation in human intestinal organoids. Our findings suggest that certain fatty acids produced by gut microbiota might regulate lipid metabolism in the human intestine and improve host energy metabolism.

## Results

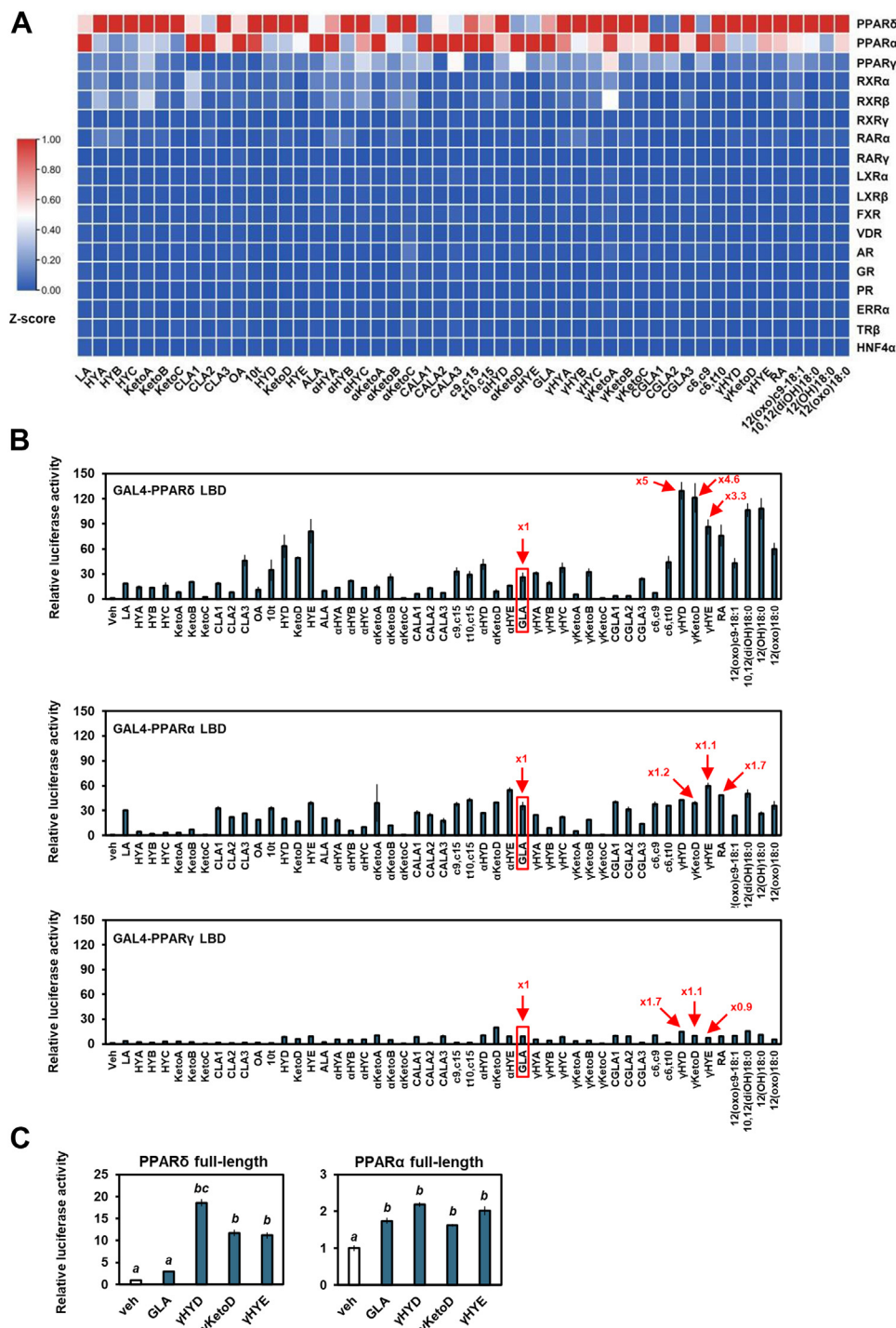
### Identification of intestinal NRs activated by lactic acid bacteria–derived fatty acids

We performed cell-based luciferase reporter assays to identify intestinal NR activators and screened 50 fatty acids produced by gut lactic acid bacteria (13). For screening, chimeric receptors containing the LBD of various NRs that are highly expressed in the intestine (19) and the DNA-binding domain of the yeast transcription factor GAL4 (GAL4-NRs) were used (32). Luciferase reporter assays using HEK293 cells showed that lactic acid bacteria–derived fatty acids preferentially activates PPARs, especially PPAR $\alpha$  and PPAR $\delta$  (Fig. 1A). Other NRs were not stimulated by these fatty acids. Interestingly, some saturated metabolites of GLA, namely  $\gamma$ HYD,  $\gamma$ KetoD, and 10,13-dihydroxy-*cis*-6-octadecenoic acid ( $\gamma$ HYE), had a more robust PPAR $\delta$  activation than their original compound, GLA (Fig. 1B). These activations were not seen in PPAR $\alpha$ , indicating a PPAR subtype-selective fatty acid sensing. Similar activation of PPAR $\delta$  by saturated metabolites of linoleic acid (18:2n-6, LA) and  $\alpha$ -linolenic acid (18:3n-3, ALA) were also observed. Because activation of PPAR $\alpha$  and PPAR $\gamma$  by lactic acid bacteria–derived metabolites of LA and ALA were studied previously (33–35), our present study focused on PPAR $\delta$  and GLA.

To investigate the specificity of GLA, the activation of PPARs by other 18-carbon PUFAs, that is, LA and ALA (14) was analyzed (Fig. 1B). Modification of LA (HYD, KetoD, and HYE) and ALA ( $\alpha$ HYD,  $\alpha$ KetoD, and  $\alpha$ HYE) had little or no effect on the ligand-induced activation of PPAR $\alpha$ . Interestingly, 13-hydroxylation of LA and ALA, as well as GLA, potently increased PPAR $\delta$  activation. 13-oxo and 13-dihydroxy LA, but not ALA, also activate PPAR $\delta$ . We next investigated transactivation of full-length PPARs by fatty acids. The expression plasmid for PPARs was transfected into HEK293 cells with a luciferase reporter plasmid containing PPRE, and then luciferase activity was measured. Consistent with the GAL4-NR-related results, GLA-derived fatty acids ( $\gamma$ HYD,  $\gamma$ KetoD, and  $\gamma$ HYE) but not GLA activated PPAR $\delta$  potently (Fig. 1C). In contrast, PPAR $\alpha$  was activated almost equally by GLA and GLA-derived fatty acids. These results indicate that GLA saturation selectively activates PPAR $\delta$ .

### $\gamma$ HYD and $\gamma$ KetoD are potential activators of PPAR $\delta$

We also examined the activation of PPARs by other dietary PUFAs, namely docosahexaenoic acid (22:6n-3, DHA) and



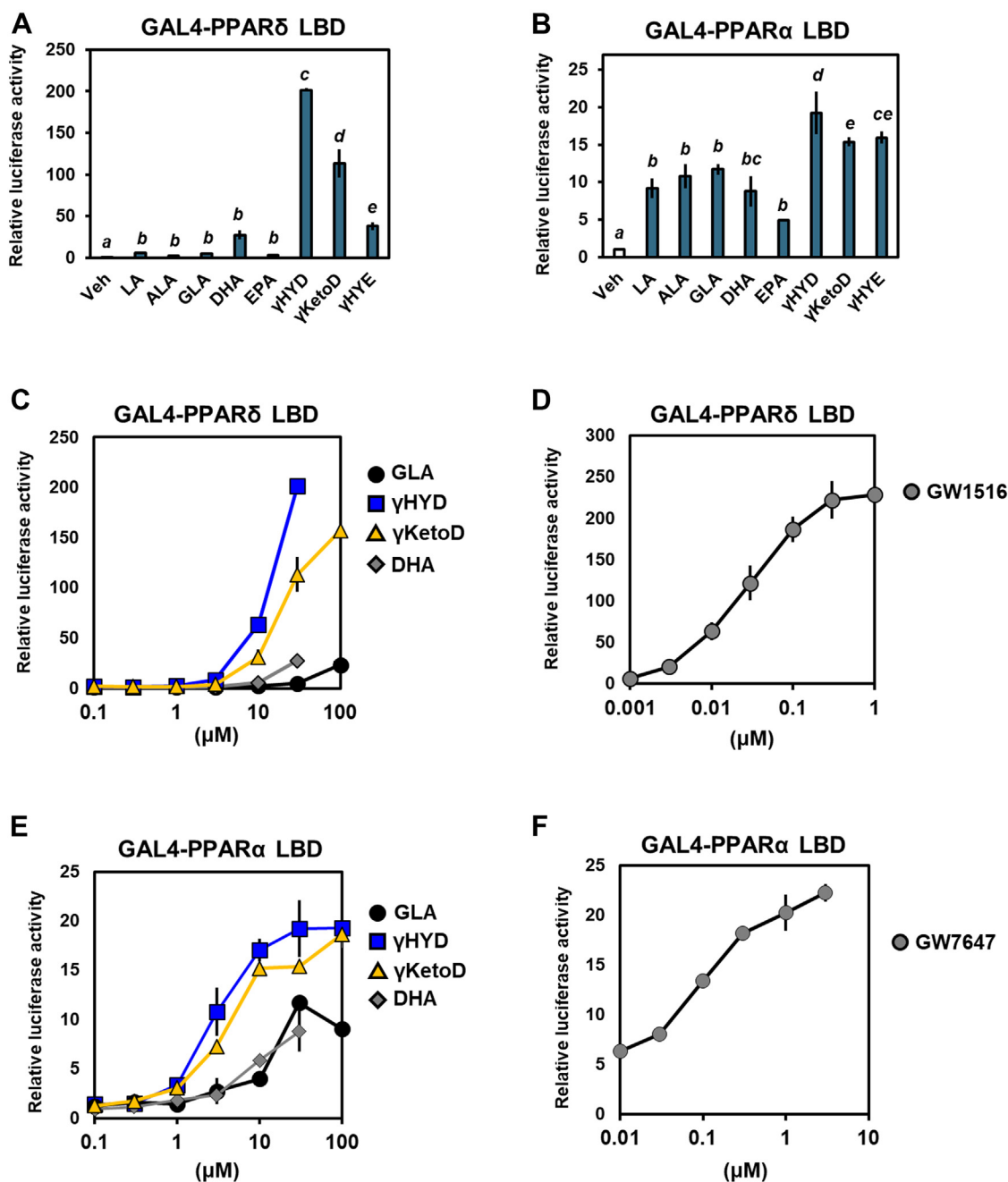
**Figure 1. Gut microbial  $\gamma$ -linolenic acid metabolites show selective activation of PPAR $\delta$  and PPAR $\alpha$ .** A and B, representative data of the ligand screening of GAL4-receptor luciferase assays. HEK293 cells were cotransfected with the GAL4-NRs LBD expression plasmids and a luciferase reporter gene and treated with 100  $\mu$ M fatty acids. C, effects of fatty acids on full-length PPARs in a luciferase reporter assay in which HEK293 cells were used. Data represent means  $\pm$  SEM (n = 3), and Tukey's test was used for analysis. Different letters indicate significant differences between groups ( $p < 0.05$ ). Veh, vehicle; ALA,  $\alpha$ -linolenic acid; GLA,  $\gamma$ -linolenic acid; LA, linoleic acid; LBD, ligand-binding domain; NR, nuclear hormone receptor; PPAR, peroxisome proliferator-activated receptor.

eicosapentaenoic acid (20:5n-3), which have been reported as natural occurring ligands for PPARs (36, 37). GLA-derived fatty acids, especially  $\gamma$ HYD and  $\gamma$ KetoD, activated PPAR $\delta$  more robustly than DHA and eicosapentaenoic acid as well as GLA, whereas the activation of PPAR $\alpha$  by these PUFAs was similar (Fig. 2, A and B); thus, GLA-derived fatty acids are

potential naturally occurring activators of PPAR $\delta$ . In a luciferase reporter assay in which GAL4-PPARs were used, PPAR activations by fatty acids and PPAR agonists were dose-dependent (Fig. 2, C-F). As matched with Figure 1, PPAR $\delta$  activation by  $\gamma$ HYD and  $\gamma$ KetoD was potent [effective concentration for half-maximal response ( $EC_{50}$ ) = 10.6 and



## GLA metabolites are PPAR $\delta$ ligands



**Figure 2. GLA-derived fatty acids are potential naturally occurring PPAR $\delta$  activators.** A and B, effects of GLA metabolites and PUFAs on PPARs in GAL4-receptor luciferase assays. HEK293 cells were cotransfected with the GAL4-PPARs LBD expression plasmids and a luciferase reporter gene and treated with 30  $\mu$ M fatty acids. C–F, comparative dose-response relationships of PPAR $\delta$  (C and D) and PPAR $\alpha$  (E and F). EC<sub>50</sub>s were calculated using the results in (C–F), as shown in Table 1. Data represent means  $\pm$  SEM (n = 3), and Tukey's test was used for analysis. Different letters indicate significant differences between groups ( $p < 0.05$ ). PUFA, polyunsaturated fatty acid; PPAR, peroxisome proliferator-activated receptor; GLA,  $\gamma$ -linolenic acid; LBD, ligand-binding domain.

20.3  $\mu$ M, respectively, Table 1]. These activation efficacies were much higher than that of GLA (EC<sub>50</sub> = 509  $\mu$ M), indicating that  $\gamma$ HYD and  $\gamma$ KetoD are potential activators of PPAR $\delta$ .

### GLA-derived fatty acids regulate the recruitment of coactivators and corepressors

We performed mammalian two-hybrid assays to determine the mechanism underlying the activation of PPARs by

GLA derivatives. NRs, including PPARs, interact with co-repressors, such as NCoR without ligands. Upon ligand binding, NRs release the corepressors and recruit coactivators such as SRC-1, inducing transcription (17). Expression plasmids for GAL4-SRC-1 contain GAL4 and the receptor-interacting domain of the coactivator SRC-1 and VP16-PPARs, which contain PPARs and the trans-activation domain of the herpes virus VP16 protein and were used. As reported previously, PPAR activation by specific agonists increased the ligand-dependent

**Table 1**  
EC50 values of GLA-derived fatty acids in relation to PPAR $\delta$  (A) and PPAR $\alpha$  (B)

Ligand/fatty acids	EC <sub>50</sub> ( $\mu$ M)
A. GAL4-PPAR $\delta$ LBD	
GW1516	0.025
DHA	44.559
GLA	509.000
$\gamma$ HYD	10.585
$\gamma$ KetoD	20.324
B. GAL4-PPAR $\alpha$ LBD	
GW7647	0.106
DHA	9.324
GLA	11.003
$\gamma$ HYD	2.842
$\gamma$ KetoD	4.136

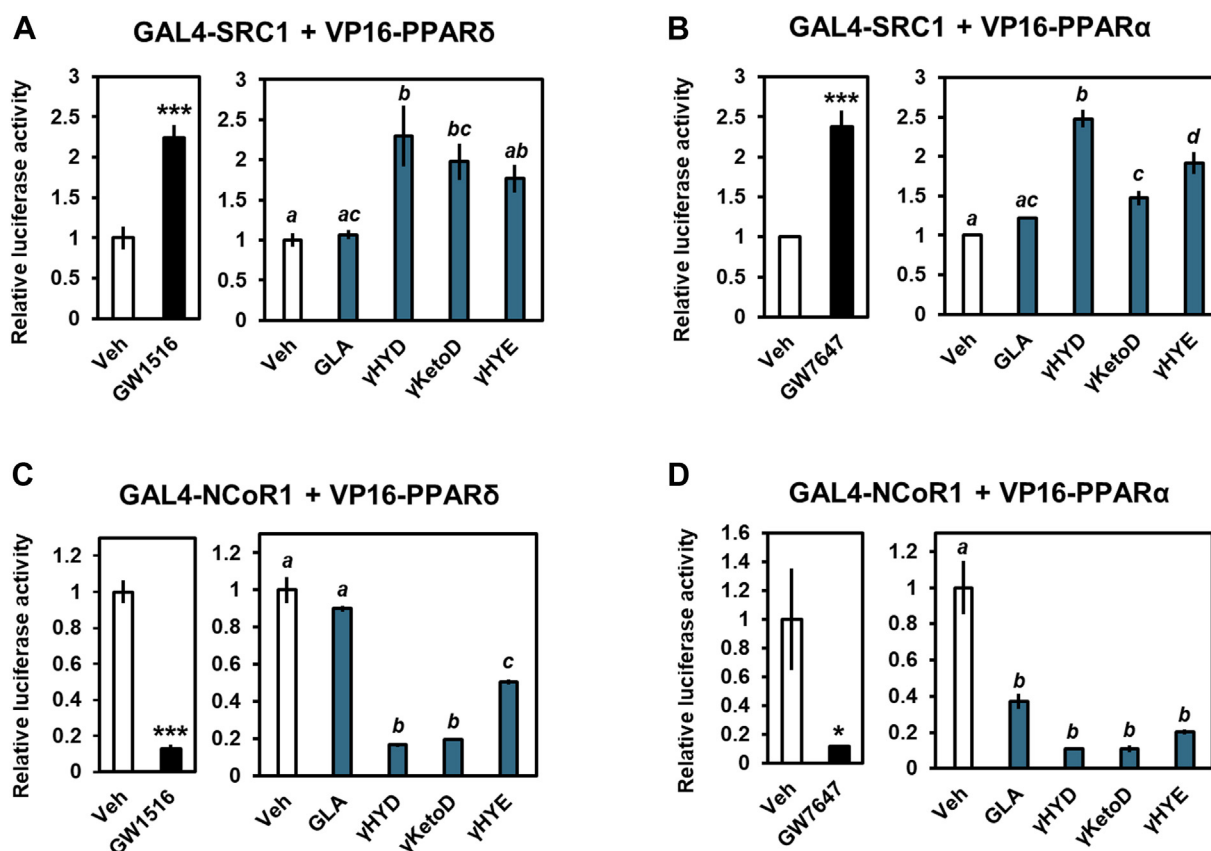
Values were calculated from luciferase activity using a Quest Graph EC50 Calculator.

recruitment of SRC-1 to PPARs (Fig. 3, A and B). Additionally,  $\gamma$ HYD,  $\gamma$ KetoD, and  $\gamma$ HYE but not GLA induced coactivator-recruitment to PPARs. Importantly, these data are consistent with the activation of PPARs (Fig. 1B). Similar to the SRC-1-related results,  $\gamma$ HYD,  $\gamma$ KetoD, and  $\gamma$ HYE reduced the interaction between PPARs and corepressor NCoR (Fig. 3, C and D). Thus, GLA-derived fatty acids activate PPARs through the association/dissociation of coactivators and corepressors.

### GLA-derived fatty acids bind directly to human PPAR $\delta$

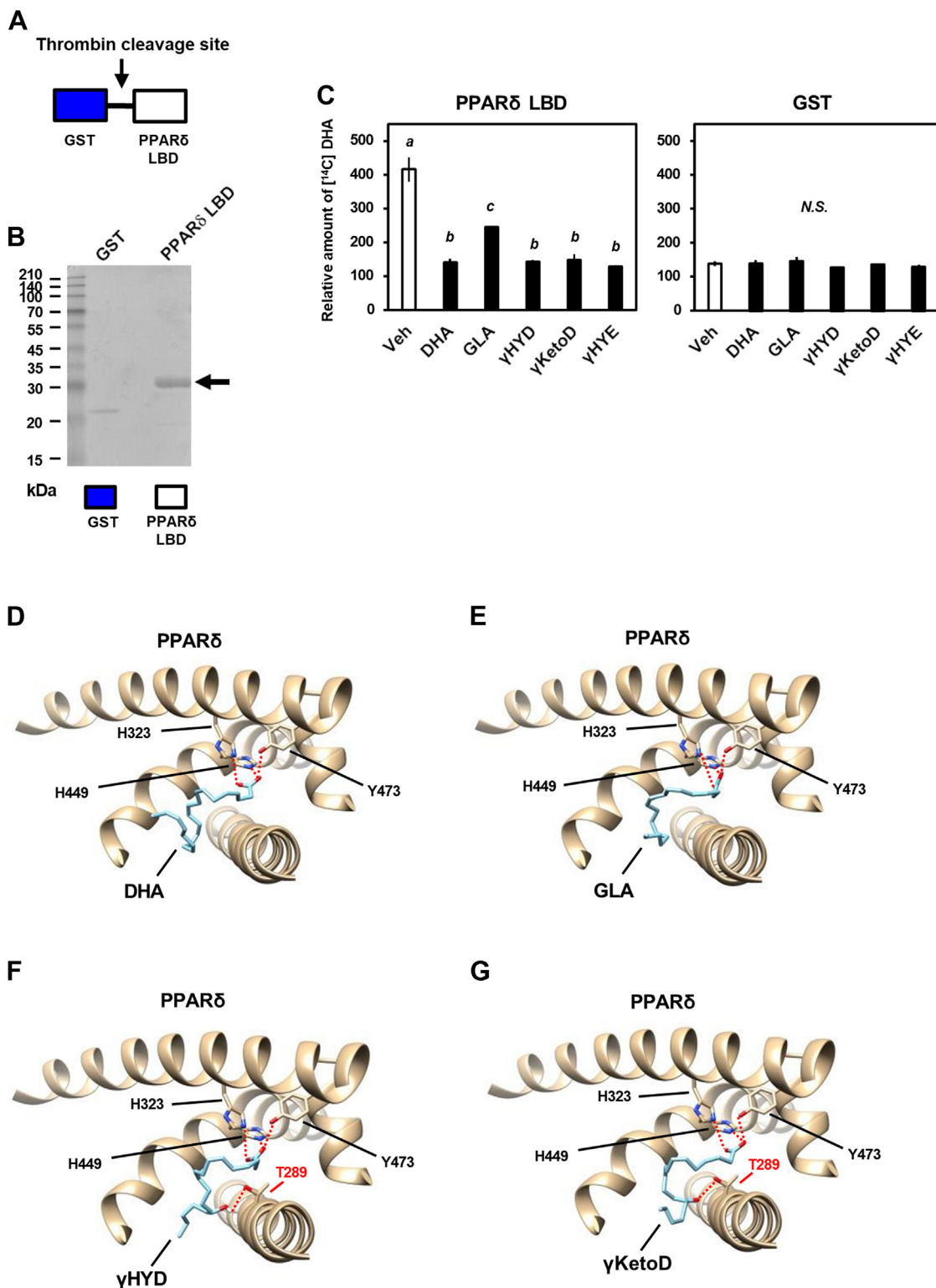
To identify the mechanism underlying the activation of PPAR $\delta$  by GLA-derived fatty acids, we performed a [<sup>14</sup>C] DHA competitive ligand-binding assay describing the displacement of ligands from receptors with recombinant human PPAR $\delta$  LBD protein produced by a bacterial expression system (Figs. 4, A and B and S2). PPAR $\delta$  LBD or glutathione-S-transferase (GST) recombinant protein was incubated with both [<sup>14</sup>C] DHA and nonradioactive competitors including GLA derivatives. A competitive inhibition of [<sup>14</sup>C] DHA binding was analyzed as a direct binding to PPAR $\delta$  LBD (see Experimental procedures). Similar to the DHA-related results, GLA derivatives ( $\gamma$ HYD,  $\gamma$ KetoD, and  $\gamma$ HYE) displaced [<sup>14</sup>C] DHA binding to PPAR $\delta$  LBD competitively (Fig. 4C). Consistent with transactivation, the only weak competition was observed with GLA, which activates PPARs moderately, and no competition was observed with GST protein. These results suggest that GLA-derived fatty acids bind directly to human PPAR $\delta$ .

Next, we performed a docking simulation using AutoDock Vina (38) to construct binding models of human PPAR $\delta$  LBD with GLA-derived fatty acids. Consistent with a previous study (39), human PPAR $\delta$  bound to a ligand (DHA)



**Figure 3. GLA-derived fatty acids regulate coactivator/corepressor exchange.** A and B, mammalian two-hybrid assay of PPARs and the coactivator SRC-1. HEK293 cells were cotransfected with VP16-PPAR $\delta$  (A) or VP16-PPAR $\alpha$  (B). C and D, mammalian two-hybrid assay of PPARs and the corepressor NCoR. HEK293 cells were cotransfected with VP16-PPAR $\delta$  (C) or VP16-PPAR $\alpha$  (D). The ligands used were 100 nM GW1516 (PPAR $\delta$  agonist), 1  $\mu$ M GW7647 (PPAR $\alpha$  agonist), or 30  $\mu$ M fatty acids. Data represent means  $\pm$  SEM (n = 3), and Student's *t* test or Tukey's test was used for analysis. \**p* < 0.05 and \*\*\**p* < 0.001 or different letters indicate significant differences between groups (*p* < 0.05). Veh, vehicle; GLA,  $\gamma$ -linolenic acid; NCoR, nuclear receptor corepressor; SRC-1, steroid receptor coactivator-1; PPAR, peroxisome proliferator-activated receptor.

## GLA metabolites are PPAR $\delta$ ligands

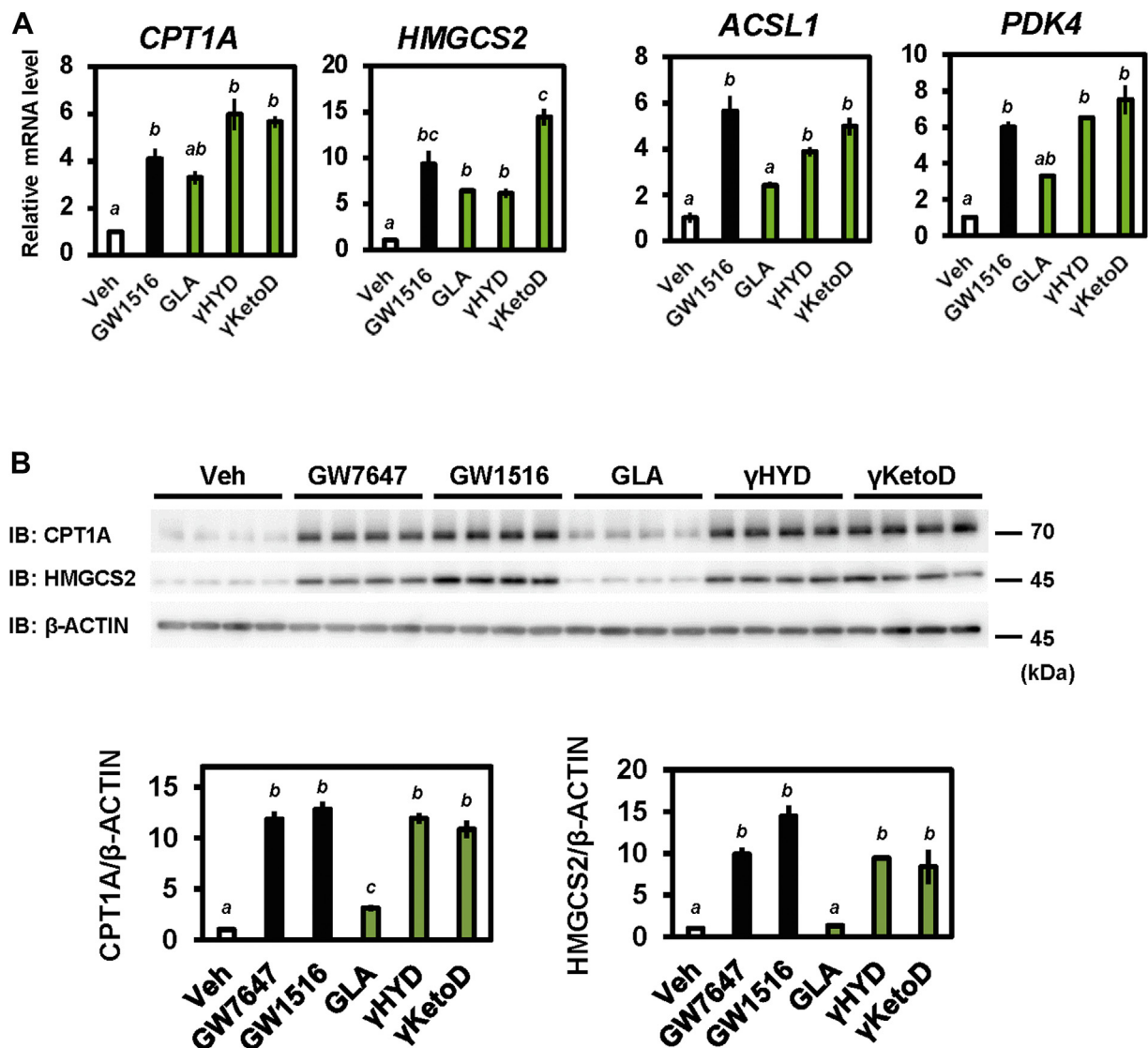


**Figure 4. Binding of GLA-derived fatty acids to PPAR $\delta$ .** A, a construction of GST-human PPAR $\delta$  LBD protein. B, purified human PPAR $\delta$  LBD recombinant protein used in the binding assay was analyzed using Coomassie Brilliant Blue staining. C, competitive binding of GLA-derived fatty acids to purified human PPAR $\delta$  LBD proteins in the presence of 100 nM [ $^{14}$ C] DHA and 1  $\mu$ M nonlabeled fatty acids. (D–G), binding model of DHA (D), GLA (E),  $\gamma$ HYD (F), and  $\gamma$ KetoD (G) to human PPAR $\delta$  (PDB: 3gwxa), as analyzed using AutoDock Vina. Data represent means  $\pm$  SEM ( $n = 3$ ), and Tukey's test was used for analysis. Different letters indicate significant differences between groups ( $p < 0.05$ ). CBB, Coomassie Brilliant Blue; PPAR, peroxisome proliferator-activated receptor; GLA,  $\gamma$ -linolenic acid; DHA, docosahexaenoic acid; LBD, ligand-binding domain;  $\gamma$ HYD, 13-hydroxy-*cis*-6,*cis*-9-octadecadienoic acid;  $\gamma$ KetoD, 13-oxo-*cis*-6,*cis*-9-octadecadienoic acid.

through hydrogen bonds with polar residues (H323, H449, and Y473) (Fig. 4D). Furthermore, the docking results showed that both GLA and GLA-derived fatty acids also associate with these polar residues (Fig. 4, E–G). Interestingly, the additional polar residue (T289) formed hydrogen bonds selectively with GLA-derived fatty acids but not GLA (Fig. 4, F and G). H323, H449, and Y473 were associated with the carboxy-terminus of fatty acids, whereas T289 donated a hydrogen bond to C13 of  $\gamma$ HYD or  $\gamma$ KetoD. T289 of human PPAR $\delta$  is conserved among mammals and this threonine residue is replaced by serine in PPAR $\alpha$  and PPAR $\gamma$  (Fig. S3), the activations of which were not affected by the saturation of GLA. These results suggest that saturation at C13 of GLA by lactic acid bacteria may serve to donate hydrogen bonds to T289 of PPAR $\delta$  and stabilize receptor-ligand docking, in a subtype-selective manner.

#### Human intestinal PPAR $\delta$ is activated by GLA metabolites

In studies of human intestinal biology, human intestinal epithelial organoids are considered powerful tools because they represent structure and functions *in vivo*. To investigate the physiological role of GLA-derived fatty acids in human tissues, we used human-induced pluripotent stem cell–derived intestinal organoids (40). First, we confirmed that endogenous PPAR $\delta$  gene expression was indistinguishable between human intestinal organoids and differentiated Caco-2 cells, that is, the human cell line which expresses endogenous PPAR $\delta$  (Fig. S4A) (41, 42). In addition, PPAR $\delta$  protein levels in human intestinal organoids are comparable to mouse tissues where PPAR $\delta$  is highly expressed (Fig. S4B). Treatment with the PPAR $\delta$  agonist GW1516 upregulated the mRNA expression of target genes in the organoids (*i.e.*, CPT1A, HMGCS2, PDK4, and ACSL1; Fig. 5A). In agreement with the GW1516-based results, the



**Figure 5. GLA-derived fatty acids increase the expression of PPAR $\delta$  target genes in human intestinal organoids.** A and B, human intestinal organoids were treated with 1  $\mu$ M GW1516 (PPAR $\delta$  agonist) or 30  $\mu$ M GLA-derived fatty acids for 24 h. Relative mRNA levels were determined using RT-qPCR (A). CPT1A, HMGCS2, and  $\beta$ -ACTIN protein levels were measured using Western blotting (B). Data represent means  $\pm$  SEM ( $n = 3$  or 4), and Tukey's test was used for analysis. Different letters indicate significant differences between groups ( $p < 0.05$ ). IB, immunoblotting; Veh, vehicle. PPAR, peroxisome proliferator-activated receptor; CPT1A, carnitine palmitoyltransferase 1A; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; ACSL1, Acyl-CoA Synthetase Long Chain Family Member 1; PDK4, pyruvate dehydrogenase kinase 4; GLA,  $\gamma$ -linolenic acid.



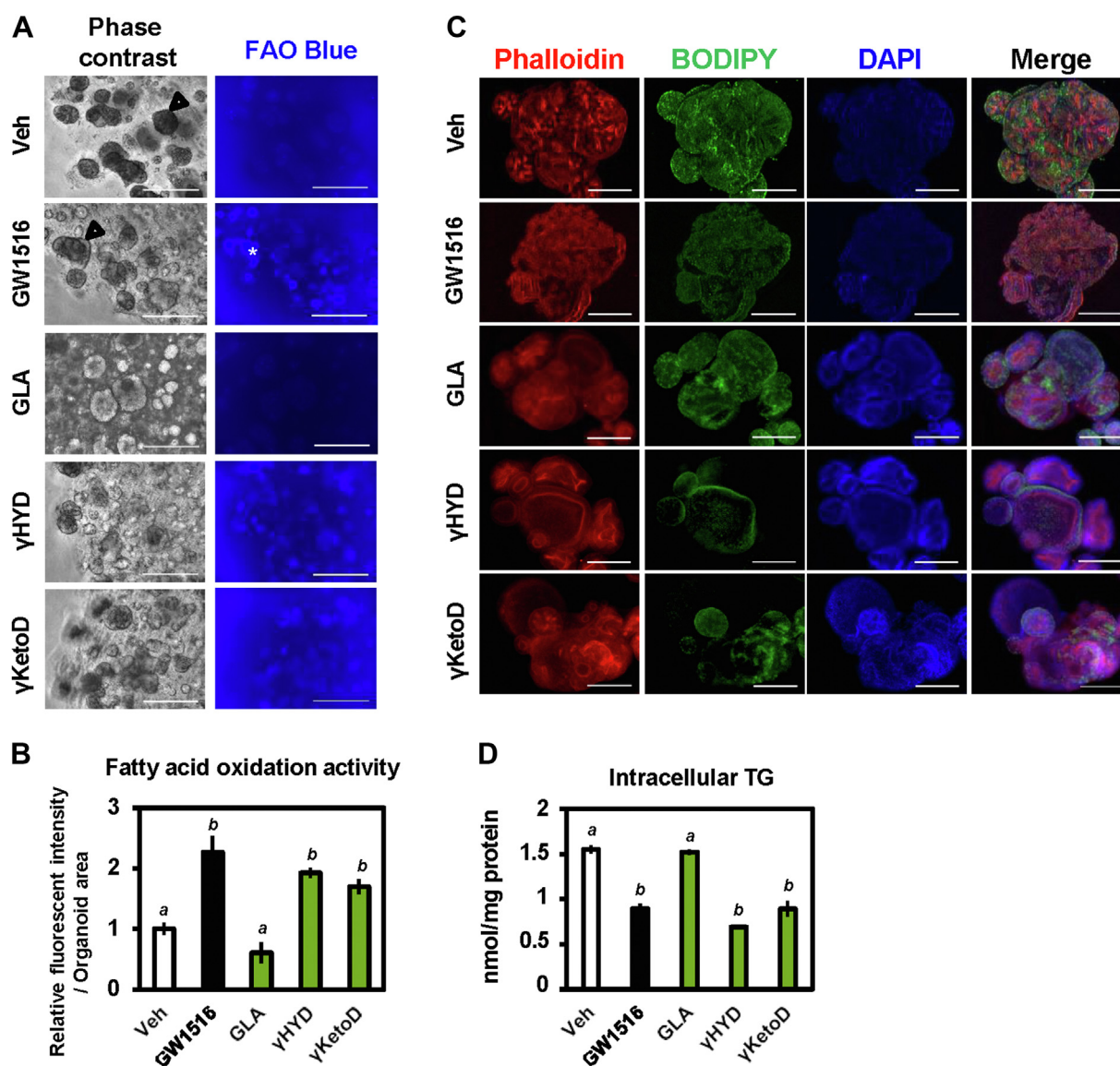
## GLA metabolites are PPAR $\delta$ ligands

expression of PPAR $\delta$  target genes was increased significantly by treatment with GLA-derived fatty acids relative to treatment with GLA (Fig. 5A). Similar results occurred when primary intestine tissue-derived human organoids were used (Fig. S4C). In addition, protein expression levels of CPT1A and HMGCS2 were significantly increased (Fig. 5B). These findings indicate that GLA-derived fatty acids activate PPAR $\delta$  in human intestinal epithelial cells.

### GLA-derived fatty acids reduce lipid accumulation in human intestinal organoids

Because activation of PPAR $\delta$  leads to fat burning through induction of fatty acid  $\beta$ -oxidation-related target genes, we

examined the regulation of lipid metabolism by GLA derivatives in human intestinal organoids. Activation of fatty acid  $\beta$ -oxidation in living cells was detected using the fluorescence dye FAOBlue (43). Consistent with the observed increase in mRNA levels, the PPAR $\delta$  agonist and GLA-derived fatty acids but not GLA significantly increased the activity of fatty acid  $\beta$ -oxidation (Fig. 6, A and B). Next, we analyzed intracellular triglyceride accumulation using BODIPY staining of neutral lipid. Consistent with the activities of fatty acid  $\beta$ -oxidation, intracellular triglyceride levels were reduced in the presence of GLA-derived fatty acids (Fig. 6C). Quantitative analysis revealed that GLA-derived fatty acids but not GLA significantly reduced intracellular triglyceride levels and the PPAR $\delta$



**Figure 6. GLA-derived fatty acids promote fatty acid  $\beta$ -oxidation and reduce lipid accumulation in human intestinal organoids.** A and B, fluorescence detection of fatty acid  $\beta$ -oxidation activity in living organoids using FAOBlue. Organoids were incubated with 30  $\mu$ M FAOBlue at 37  $^{\circ}$ C for 30 min. The scale bar represents 500  $\mu$ m (A). Relative fluorescence intensities were determined and normalized to the organoid surface area (B). C, triglyceride accumulation in human intestinal organoids was analyzed using BODIPY staining. Organoids were stained with Phalloidin (red: F-actin), BODIPY 493/503 (green: lipid droplets), and DAPI (blue: nucleus). The scale bar represents 200  $\mu$ m. D, quantification of intracellular triglyceride. Organoids pretreated with 500  $\mu$ M of oleic acid for 48 h were then treated with 1  $\mu$ M GW1516 (PPAR $\delta$  agonist) and 30  $\mu$ M GLA-derived fatty acids for 24 h. Fluorescence imaging and fluorescence intensity were analyzed using a BZ-X800 analyzer (Keyence). Data are means  $\pm$  SEM (n = 3), and Tukey's test was used for analysis. Different letters indicate significant differences between groups ( $p < 0.05$ ). PPAR, peroxisome proliferator-activated receptor; GLA,  $\gamma$ -linolenic acid.



agonist (Fig. 6D). The PPAR $\delta$  agonist and GLA-derived fatty acids do not affect cell viability (Fig. S5). Thus, GLA-derived fatty acids may reduce lipid accumulation through activation of fatty acid  $\beta$ -oxidation in human intestinal epithelial cells.

## Discussion

Human gut microbiota produces numerous molecules through the metabolism of dietary components, such as dietary fibers, bile acids, and amino acids. These microbiota-derived metabolites play crucial roles in regulating host metabolism (44, 45). SCFAs, one of the major microbiota-derived metabolites, are produced *via* the fermentation of dietary fibers and are the most well-studied metabolites in host–microbiota interactions by which host energy metabolism is regulated through the activation of SCFA-sensing GPCRs (*e.g.*, GPR41 and GPR43) (8, 10). Some bacteria metabolize LCFAs, especially PUFAs, to generate hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and partially saturated *trans*-fatty acids (13, 14). The biological signal of LCFAs is mainly mediated through membrane LCFA-sensing GPCRs (*e.g.*, GPR40 and GPR120) and nuclear fatty acid receptor PPARs. Previous studies showed that microbiota-derived PUFAs stimulate GPCRs and PPARs (33, 35, 46–48). Although intestinal GPCRs for microbiota-derived LCFAs in the intestine have been reported, nuclear mediators have yet to be identified. Fatty acids and fatty acid–derived molecules such as eicosanoids and endocannabinoids regulate the activities of PPARs and other NRs including retinoid X receptor, hepatocyte nuclear factor-4 $\alpha$ , retinoic acid receptor, and liver X receptor (49–51). These NRs are highly expressed in the intestine (19), suggesting they are potential mediators of microbiota-derived fatty acid derivatives. The present study showed that GLA and GLA metabolites activate PPARs, especially PPAR $\alpha$  and PPAR $\delta$ , selectively. Fatty acids activate all PPARs as dietary ligands. Endogenous ligands for PPAR $\alpha$  (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine) and PPAR $\gamma$  (15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>) have been reported (36, 52–54); prostacyclin and carbaprostacyclin were reported as endogenous ligands for PPAR $\delta$  (55, 56), although they also activated other PPARs. Our study found that GLA metabolites activate PPAR $\delta$  potently and to a greater extent than that achieved by other fatty acids. Lactic acid bacteria–derived fatty acids are present in the intestine, the primary site of fatty acid absorption. Although bacterial fatty acid metabolites exist at lower levels than original compounds, these metabolites produced by lactic acid bacteria were detected in the intestine (13, 46), implying that substantial levels of these metabolites are present in host tissues. These findings suggest that lactic acid bacteria–derived GLA metabolites function as ligands for PPAR $\delta$ .

Similar to a previous study (57), our data showed that GLA preferentially activates PPAR $\alpha$  and PPAR $\delta$  rather than PPAR $\gamma$ . Consistent with another study (39), our docking simulation indicated that  $\gamma$ HYD and  $\gamma$ KetoD are capable of binding to human PPAR $\delta$  through hydrogen bonds between the fatty acids and the polar residues of human PPAR $\delta$  (His323, His449,

and Tyr473), which are located in arm I of the LBD. The binding ability of fatty acids to PPAR $\delta$  is generally proportional to the length of their hydrocarbon chains, indicating the strength of hydrophobic interactions (57). However, in the current study,  $\gamma$ HYD and  $\gamma$ KetoD, both C18 fatty acids, showed comparable ligand-binding abilities to that of the C22 fatty acid DHA (Fig. 4B). Our docking model showed that  $\gamma$ HYD and  $\gamma$ KetoD but not GLA formed additional hydrogen bonds with Thr289. GLA saturation selectively activates PPAR $\delta$  transactivation but not other PPARs (Fig. 1B). Saturations of LA (HYD, KetoD, and HYE) and ALA ( $\alpha$ HYD) are also effective (Fig. 1B), suggesting that fatty acid saturation may be a common mechanism underlying the PPAR $\delta$  activation. His323, His449, and Tyr473 of human PPAR $\delta$  were important residues in hydrogen bonding with other fatty acids (39). These amino acid residues and Thr289 are located in arm I of the LBD, indicating the helix 12, which contributes to the ligand-dependent recruitment of the coactivator complex. Although the LBD of PPARs share 60%–70% sequence identity (58), Thr289 of human PPAR $\delta$  is not conserved and replaced with the serine residue in PPAR $\alpha$  and PPAR $\gamma$  (Fig. S3). Threonine and serine are similar amino acids with polar uncharged side chains, and the serine residues of PPAR $\alpha$  (Ser280) and PPAR $\gamma$  (Ser289) are reported to contribute to bind other fatty acids (39). Because the ligand-binding assays of PPAR $\alpha$  and PPAR $\gamma$  was not performed, further studies are required to determine how the threonine residue selectively contributes to the binding and the activation of PPAR $\delta$  by GLA metabolites. In this study, the mammalian two-hybrid assay using GAL4-coactivator/corepressor and VP16-NRs was used to detect a recruitment of coactivator/corepressor to NRs in response to ligands or fatty acids. Because this model relies on the VP16 transactivation domain of a yeast protein for activity, further experiments including chromatin immunoprecipitation assay should be required to verify an endogenous interaction between them.

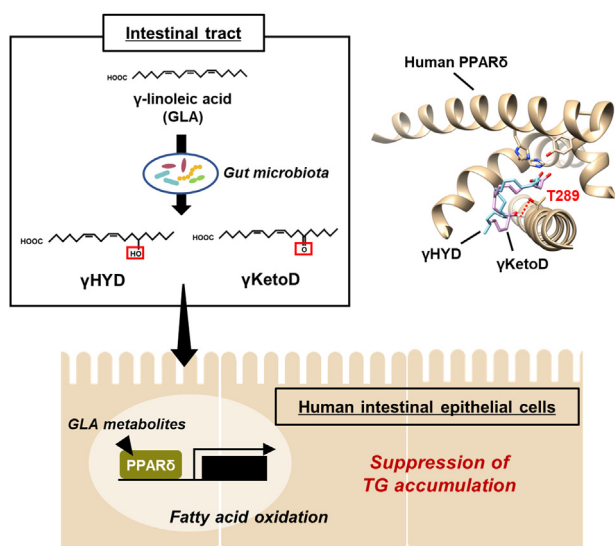
PPAR $\delta$  is reported to play an important role as a regulator in lipid metabolism in multiple tissues, including the liver and skeletal muscle, through the regulation of fatty acid  $\beta$ -oxidation–related gene expression. In addition, intestinal PPAR $\delta$  protects against diet-induced obesity and dyslipidemia (27). Cardiovascular disease is a major cause of death in worldwide, and postprandial hyperlipidemia is an important risk factor for cardiovascular disease (59). An absorbed dietary lipid is assembled into a chylomicron in the intestine for secretion into the lymph system in the postprandial state. Excess dietary lipids are temporally stored as cytosolic lipid droplets (CLDs) (60). In obese mice, intestinal CLDs are enlarged. They contain the distinctive expression profile of CLD-localized proteins, suggesting that the changes mentioned above prevent absorbed lipids from being mobilized and utilized effectively (61–63). In clinical and experimental studies, PUFA intake improves postprandial hyperlipidemia (64, 65). In the present study, GLA metabolites reduced triglyceride accumulation in human intestinal epithelial cells by activating fatty acid  $\beta$ -oxidation. Lipid transport by fatty acid transport proteins (FABP1 and FABP2) is critical for fatty acid catabolism in

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enterocytes. FABP1 is involved in fatty acid oxidation in the intestine, whereas FABP2 directs fatty acids to triacylglyceride synthesis (66). Consistent with these studies, we found that the expression of FABP1 but not FABP2 was increased by GLA metabolites (Fig. S4D), suggesting that these metabolites direct fatty acids to degradation in the intestine. Interestingly, GLA metabolites also increased the expression of PPAR $\delta$  target genes in human hepatocytes (Fig. S6), indicating improved lipid metabolism in multiple tissues through PPAR $\delta$  activation.

Organoids are recognized as *in vitro* organ models with highly physiological characteristics (67). Human intestinal cell lines, such as Caco-2 cells, lack some important pathways, including lipoprotein metabolism, observed *in vivo* (68, 69). In addition, PPARs exhibit marked species differences in response to ligand activation (70, 71). We found that GLA-derived fatty acids activate PPAR $\delta$  and reduce lipid accumulation in human intestinal organoids. Considering these findings with evidence that microbiota-derived metabolites are present in the intestine, we suggest that GLA metabolites could improve lipid metabolism *via* PPAR $\delta$  activation in the human intestine. Although intestinal organoids possess high physiological properties, *in vivo* study may need to describe whether GLA metabolites are *bona fide* physiological ligands for PPAR $\delta$ .

In conclusion, the GLA-derived fatty acids,  $\gamma$ HYD and  $\gamma$ KetoD, produced by lactic acid bacteria are ligands for PPAR $\delta$ . GLA saturation selectively enhances PPAR $\delta$  transactivation through an additional hydrogen bond between Thr289 and fatty acids. Moreover, the GLA-derived fatty acids reduce CLD levels in human intestinal epithelial cells (Fig. 7). These findings will likely contribute to the development of effective preventative treatments for metabolic disorders, including obesity, because the aforementioned fatty acid metabolites exhibited high bioactivity in reducing lipid storage in human intestinal epithelial cells.



**Figure 7.** Models of the mechanism by which lactic acid bacteria-derived GLA metabolites activate PPAR $\delta$  and modulate lipid metabolism in human intestine. GLA,  $\gamma$ -linolenic acid; PPAR $\delta$ , peroxisome proliferator-activated receptor delta.

## Experimental procedures

### Materials

Lactic acid bacteria-derived fatty acids were produced as described previously (Fig. S1 and Table S1) (13). GW7647 and GW1516 were purchased from Sigma-Aldrich. The following antibodies were purchased from commercial sources: anti-HMGCS2 and anti-CPT1A (ab137043 and ab128568); anti- $\beta$ -actin (A5441). All other chemicals of analytical grade were purchased from Sigma-Aldrich, FUJIFILM Wako Pure Chemical, or nakalai tesque.

### Animals

C57BL/6J mice were purchased from Nihon Crea. Mice were housed with a 12:12-h light–dark cycle and provided free access to water and standard diet (Labo MR Stock; Nosan Corporation Bio-Department). All animal experiments were conducted according to the guidelines of the Animal Usage Committee of the University of Tokyo. The experiments using animal studies were approved by the Animal Usage Committee of The University of Tokyo.

### Cell culture

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. As described previously, human intestinal organoid cultures and passages were conducted (40). Briefly, organoids were cultured with a 25% L-WRNH-conditioned medium embedded in Matrigel Matrix (Corning). The entire medium was changed every 2 to 3 days, and passages were performed every 5 to 6 days. Following passages, cell dissociation was performed using TrypLE express (Corning) at a general ratio of 1:8 embedded in Matrigel Matrix on a Nunclon Delta 4-well dish (Thermo Scientific). All cells were cultured at 37  $^{\circ}$ C in a 5% CO $_2$  atmosphere. The experiments using primary human organoids complied with the Declaration of Helsinki and were approved by the human ethical committee of The University of Tokyo and Osaka University. All tissues were sampled with informed consent.

### Cotransfection and luciferase assay

HEK293 cells were plated in 96-well plates (Greiner Bio-One) and cultured on DMEM medium supplemented with 10% charcoal-treated fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin for 24 h. After that, they were transfected with 50 ng of one of the reporter plasmids, 40 ng of one of each of the expression plasmids, and 10 ng of pCMV- $\beta$ -Gal (an expression plasmid for  $\beta$ -galactosidase) using the calcium phosphate method. At 24 h after transfection, vehicle, fatty acids, or ligands were added. After incubation for an additional 24 h, luciferase activity was measured using a SpectraMax M2 (Molecular Devices). Luciferase activities were normalized to  $\beta$ -galactosidase activities. Plasmids were generous gifts from Dr Makoto Makishima (GAL4-SRC1 and GAL4-NCOR), Dr David Mangelsdorf and Dr Steven Kliewer

(pCMX, pCMX-GAL4-NR LBD, TK-MH100 $\times$ 4-luc, pCMX TK-PPREx3-luc, and TK-luc), and Dr Kazuhiko Umesono (pCMX-PPAR $\delta$ ).

### RNA isolation and real-time quantitative PCR

Total RNA was extracted from cells or organoids using ISOGEN (Nippon gene) per the manufacturer's instructions. cDNA was synthesized and amplified from total RNA using a High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Real-time quantitative PCR was performed using an Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) and FastStart Universal SYBR Green Master (Roche Applied Science) per the manufacturer's protocols. All relative mRNA expression levels were normalized to that of *18S*. Primer sequences are listed in Table S2.

### Western blot analysis

Cells or organoids were treated as described in the figure legends. After treatment, protein extraction was prepared using a cell suspension in RIPA buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 1% NP-40, and 0.25% sodium deoxycholate] supplemented with protease inhibitors. All samples were treated with 6 $\times$  Laemmli buffer [1 mM Tris-HCl (pH 6.8), 30% glycerol, 10% SDS, 600 mM DTT, and 0.03% bromophenol blue]. The protein samples were analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies and visualized by Amersham Bioscience ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences) or Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore).

### Recombinant protein expression and purification

Human PPAR $\delta$  LBD (140–442) construct was cloned into a pGEX-4T1 vector (GE Healthcare Life Sciences) at the restriction enzyme site of *Sma*I and *Xho*I. The protein was expressed in *Escherichia coli* BL21 (DE3) pLys cells (Novagen) as a C-terminal fusion to a GST tag with a thrombin cleavage site. A single colony was cultured in 2 ml of LB media supplemented with 20  $\mu$ g/ml of ampicillin and 30  $\mu$ g/ml of chloramphenicol at 37  $^{\circ}$ C and 200 rpm for 18 h. The pre-culture was then transferred to 1 l of LB media until the A600 reached 0.6 to 0.8. IPTG was added to produce a final concentration of 0.4 mM, and expression was continued at 24  $^{\circ}$ C and 150 rpm for 18 h. Cells were collected using centrifugation, and pellets were resuspended in lysis buffer comprising 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM DTT, 1 mM EDTA, and 10% glycerol before sonication was performed. The supernatant was then collected using centrifugation at 100,000g and 4  $^{\circ}$ C for 70 min, after which it was loaded onto GST Accept (nacalai tesque) and equilibrated using the lysis buffer. Subsequently, the column was washed using five column volumes of the lysis buffer and thrombin cleavage buffer comprising 20 mM Tris-HCl (pH 8.4), 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, and 1 mM DTT, respectively. The GST tag was removed *via* digestion using biotinylated thrombin (Merck) for 18 h at 4  $^{\circ}$ C. The

biotinylated thrombin was removed using Dynabeads M-280 streptavidin (Veritas). The purity of the target proteins was verified using SDS-PAGE.

### Ligand-binding assay

A ligand-binding assay was performed as described previously with minor modifications (72). Briefly, purified recombinant human PPAR $\delta$  LBD protein was incubated with 100 nM (10 Ci/mmol) [<sup>14</sup>C] DHA in GP buffer (137 mM NaCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.68 mM KCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 1% sodium azide, and 0.1% gelatin) overnight at 4  $^{\circ}$ C with or without various competitors (1  $\mu$ M), including DHA and GLA-derived fatty acids. Unbound ligands were removed by adsorption to 3% dextran-coated charcoal, and the supernatant was collected. The PPAR $\delta$ -bound [<sup>14</sup>C] DHA was measured using a scintillation counter (PerkinElmer).

### Docking simulation

A docking simulation was performed using the AutoDock Vina computational program (38) to predict the binding models of human PPAR $\delta$  LBD protein with GLA-derived fatty acids. The PPAR $\delta$  LBD segment of the PPAR $\delta$  X-ray crystallography structure (PDB: 3GWX, 2AWH, 2BAW, and 2B50) was obtained and used as a receptor.

### Fluorescence detection of fatty acid $\beta$ -oxidation

Fatty acid  $\beta$ -oxidation activity in living cells was detected using FAOBlue (Funakoshi) (43) per the manufacturer's instructions. Human intestinal organoids pretreated with 500  $\mu$ M of oleic acid for 48 h were then treated with 1  $\mu$ M GW1516 (PPAR $\delta$  agonist) or 30  $\mu$ M GLA-derived fatty acids together with oleic acid for 24 h. Embedded human intestinal organoids were incubated with 30  $\mu$ M FAOBlue at 37  $^{\circ}$ C for 30 min. The fluorescence signal derived from fatty acid  $\beta$ -oxidation activity was detected using fluorescence microscopy (BZ-X800, Keyence).

### Intracellular triglyceride measurements

Intracellular triglyceride was measured as described previously (73). Briefly, human intestinal organoids pretreated with 500  $\mu$ M of oleic acid for 48 h were then treated with 1  $\mu$ M GW1516 (PPAR $\delta$  agonist) or 30  $\mu$ M GLA-derived fatty acids together with oleic acid for 24 h. Organoids were harvested using Cell Recovery Solution (Corning) from Matrigel. Intracellular triglyceride was extracted using hexane in 2-propanol (3:2, v/v), and its levels were determined using the Triglyceride Assay Kit Quantification (abcam) and normalized to the levels of total cellular protein, per the manufacturer's instructions.

### Neutral lipid staining

Human intestinal organoids were harvested using Cell Recovery Solution (Corning) from Matrigel. The organoids were treated with a blocking solution (3% bovine serum albumin solution in PBS). They were then fixed and permeabilized



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using Fixation/Permeabilization Kit (BD Biosciences), after which they were stained with BODIPY 493/503 (Thermo Fischer Scientific), Alexa Fluor 568 Phalloidin (Thermo Fischer Scientific), and DAPI (Dojindo) according to the manufacturers' instructions. BODIPY, Phalloidin, and DAPI were used to counterstain neutral lipids, F-actin, and DNA, respectively.

### Statistical analysis

All data are presented as the mean  $\pm$  SEM. Data were analyzed statistically using one-way ANOVA followed by Tukey's post hoc test or Dunnett's test. Statistical differences were considered significant at  $p < 0.05$ .

### Data availability

All data are contained within the article.

**Supporting information**—This article contains supporting information.

**Acknowledgments**—We thank Dr Makoto Makishima (Nihon University School of Medicine), Dr David Mangelsdorf and Dr Steven Kliewer (both University of Texas Southwestern Medical Center) for providing plasmids, and Dr Michihisa Umetani (University of Houston) for helpful advice related to the ligand binding assay.

**Author contributions**—M. N., M. S., and R. S. conceptualization; M. N., M. S., P. L., Y. T., and K. N. investigation; M. N., M. S., Y. T., L. P., Y. Y., K. N., and R. S. methodology; M. N., M. S., P. L., Y. T., Y. Y., S. S., H. K., S. K., J. O., K. N., and R. S. resources; M. N., M. S., P. L., and K. N. formal analysis; M. N., M. S., and R. S. writing—original draft; M. N., M. S., P. L., Y. T., Y. Y., S. S., H. K., S. K., J. O., K. N., and R. S. writing—review and editing; M. N. and M. S. validation; M. S., J. O., and R. S. funding acquisition; M. S. and R. S. project administration; M. S. and R. S. supervision; M. S. visualization; P. L., Y. T., Y. Y., and K. N. data curation.

**Funding and additional information**—This work was supported by JSPS KAKENHI Grant-in-Aid for Scientific Research (B) 19H02906 (to M. S.), Grant-in-Aid for Scientific Research (A) 20H00408 (to R. S.), the New Energy and Industrial Technology Development Organization (NEDO) P20011 (to J. O.), the Ministry of Health and Welfare of Japan and Public/Private R&D Investment Strategic Expansion Program: PRISM (to J. O.), JST-Mirai Program JPMJMI21C1 (to J. O.), and the Japan Agency for Medical Research and Development (AMED) 21ek0410062h0003 (to J. O.).

**Conflict of interest**—The authors declare that they have no conflicts of interest related to the content of this article.

**Abbreviations**—The abbreviations used are: ALA,  $\alpha$ -linolenic acid; CLD, cytosolic lipid droplet; DHA, docosahexaenoic acid; GLA,  $\gamma$ -linolenic acid; GPCR, G protein-coupled receptor; LA, linoleic acid; LBD, ligand-binding domain; LCFA, long-chain fatty acid; NCoR, nuclear receptor corepressor; NR, Nuclear hormone receptor; PPAR $\delta$ , peroxisome proliferator-activated receptor delta; PPRE, peroxisome proliferator-response element; PUFA, polyunsaturated fatty acid; SCEFA, short-chain fatty acid; SRC-1, steroid receptor coactivator-1;  $\gamma$ HYD, 13-hydroxy-*cis*-6,*cis*-9-octadecadienoic acid;

$\gamma$ HYE, 10,13-dihydroxy-*cis*-6-octadecenoic acid;  $\gamma$ KetoD, 13-oxo-*cis*-6,*cis*-9-octadecadienoic acid.

### References

1. Vrieze, A., Van Nood, E., Holleman, F., Salojärvi, J., Kootte, R. S., Bartsman, J. F., *et al.* (2012) Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* **143**, 913–916.e917
2. Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., *et al.* (2011) Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J.* **5**, 220–230
3. Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., *et al.* (2005) Diversity of the human intestinal microbial flora. *Science* **308**, 1635–1638
4. Gerritsen, J., Smidt, H., Rijkers, G. T., and de Vos, W. M. (2011) Intestinal microbiota in human health and disease: the impact of probiotics. *Genes Nutr.* **6**, 209–240
5. Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006) Microbial ecology: human gut microbes associated with obesity. *Nature* **444**, 1022–1023
6. Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027–1031
7. Keenan, M. J., Zhou, J., Hegsted, M., Pelkman, C., Durham, H. A., Coulon, D. B., *et al.* (2015) Role of resistant starch in improving gut health, adiposity, and insulin resistance. *Adv. Nutr.* **6**, 198–205
8. Kaczmarczyk, M. M., Miller, M. J., and Freund, G. G. (2012) The health benefits of dietary fiber: beyond the usual suspects of type 2 diabetes mellitus, cardiovascular disease and colon cancer. *Metabolism* **61**, 1058–1066
9. Lu, Y., Fan, C., Li, P., Chang, X., and Qi, K. (2016) Short chain fatty acids prevent high-fat-diet-induced obesity in mice by regulating G protein-coupled receptors and gut microbiota. *Sci. Rep.* **6**, 37589
10. Kimura, I., Inoue, D., Maeda, T., Hara, T., Ichimura, A., Miyauchi, S., *et al.* (2011) Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). *Proc. Natl. Acad. Sci. U. S. A.* **108**, 8030–8035
11. Kimura, I., Ozawa, K., Inoue, D., Imamura, T., Kimura, K., Maeda, T., *et al.* (2013) The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. *Nat. Commun.* **4**, 1829
12. Hara, T., Kashiwara, D., Ichimura, A., Kimura, I., Tsujimoto, G., and Hirasawa, A. (2014) Role of free fatty acid receptors in the regulation of energy metabolism. *Biochim. Biophys. Acta* **1841**, 1292–1300
13. Kishino, S., Takeuchi, M., Park, S. B., Hirata, A., Kitamura, N., Kunisawa, J., *et al.* (2013) Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 17808–17813
14. Hirata, A., Kishino, S., Park, S. B., Takeuchi, M., Kitamura, N., and Ogawa, J. (2015) A novel unsaturated fatty acid hydratase toward C16 to C22 fatty acids from *Lactobacillus acidophilus*. *J. Lipid Res.* **56**, 1340–1350
15. Committee, N. R. N. (1999) A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97**, 161–163
16. De Bosscher, K., Desmet, S. J., Clarisse, D., Estébanez-Perpiña, E., and Brunsveld, L. (2020) Nuclear receptor crosstalk - defining the mechanisms for therapeutic innovation. *Nat. Rev. Endocrinol.* **16**, 363–377
17. Kang, Z., and Fan, R. (2020) PPAR $\alpha$  and NCOR/SMRT corepressor network in liver metabolic regulation. *FASEB J.* **34**, 8796–8809
18. Klepsch, V., Moschen, A. R., Tilg, H., Baier, G., and Hermann-Kleiter, N. (2019) Nuclear receptors regulate intestinal inflammation in the context of IBD. *Front. Immunol.* **10**, 1070
19. Bookout, A. L., Jeong, Y., Downes, M., Yu, R. T., Evans, R. M., and Mangelsdorf, D. J. (2006) Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell* **126**, 789–799



20. Schmidt, D. R., and Mangelsdorf, D. J. (2008) Nuclear receptors of the enteric tract: guarding the frontier. *Nutr. Rev.* **66**, S88–97
21. Evans, R. M., and Mangelsdorf, D. J. (2014) Nuclear receptors, RXR, and the big bang. *Cell* **157**, 255–266
22. Kersten, S., Desvergne, B., and Wahli, W. (2000) Roles of PPARs in health and disease. *Nature* **405**, 421–424
23. Chiarelli, F., and Di Marzio, D. (2008) Peroxisome proliferator-activated receptor-gamma agonists and diabetes: current evidence and future perspectives. *Vasc. Health Risk Manag.* **4**, 297–304
24. Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., *et al.* (1994) Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7355–7359
25. Modica, S., Gofflot, F., Murzilli, S., D'Orazio, A., Salvatore, L., Pellegrini, F., *et al.* (2010) The intestinal nuclear receptor signature with epithelial localization patterns and expression modulation in tumors. *Gastroenterology* **138**, 636–648
26. Girroir, E. E., Hollingshead, H. E., He, P., Zhu, B., Perdew, G. H., and Peters, J. M. (2008) Quantitative expression patterns of peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta) protein in mice. *Biochem. Biophys. Res. Commun.* **371**, 456–461
27. Doktorova, M., Zwarts, L., Zutphen, T. V., Dijk, T. H. V., Bloks, V. W., Harkema, L., *et al.* (2017) Intestinal PPAR $\delta$  protects against diet-induced obesity, insulin resistance and dyslipidemia. *Sci. Rep.* **7**, 846
28. Vrins, C. L., van der Velde, A. E., van den Oever, K., Levels, J. H., Huet, S., Oude Elferink, R. P., *et al.* (2009) Peroxisome proliferator-activated receptor delta activation leads to increased transintestinal cholesterol efflux. *J. Lipid Res.* **50**, 2046–2054
29. Brunham, L. R., Kruit, J. K., Iqbal, J., Fievet, C., Timmins, J. M., Pape, T. D., *et al.* (2006) Intestinal ABCA1 directly contributes to HDL biogenesis *in vivo*. *J. Clin. Invest.* **116**, 1052–1062
30. Peters, J. M., Walter, V., Patterson, A. D., and Gonzalez, F. J. (2019) Unraveling the role of peroxisome proliferator-activated receptor- $\beta/\delta$  (PPAR $\beta/\delta$ ) expression in colon carcinogenesis. *NPJ Precis Oncol.* **3**, 26
31. Giordano Attianese, G. M., and Desvergne, B. (2015) Integrative and systemic approaches for evaluating PPAR $\beta/\delta$  (PPAR $\delta$ ) function. *Nucl. Recept Signal.* **13**, e001
32. Willy, P. J., Umesono, K., Ong, E. S., Evans, R. M., Heyman, R. A., and Mangelsdorf, D. J. (1995) LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* **9**, 1033–1045
33. Goto, T., Kim, Y. I., Furuzono, T., Takahashi, N., Yamakuni, K., Yang, H. E., *et al.* (2015) 10-oxo-12(Z)-octadecenoic acid, a linoleic acid metabolite produced by gut lactic acid bacteria, potently activates PPAR $\gamma$  and stimulates adipogenesis. *Biochem. Biophys. Res. Commun.* **459**, 597–603
34. Nagatake, T., Kishino, S., Urano, E., Murakami, H., Kitamura, N., Konishi, K., *et al.* (2022) Intestinal microbe-dependent  $\omega$ 3 lipid metabolite  $\alpha$ ketoA prevents inflammatory diseases in mice and cynomolgus macaques. *Mucosal Immunol.* **15**, 289–300
35. Ohue-Kitano, R., Yasuoka, Y., Goto, T., Kitamura, N., Park, S. B., Kishino, S., *et al.* (2018)  $\alpha$ -Linolenic acid-derived metabolites from gut lactic acid bacteria induce differentiation of anti-inflammatory M2 macrophages through G protein-coupled receptor 40. *FASEB J.* **32**, 304–318
36. Forman, B. M., Chen, J., and Evans, R. M. (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4312–4317
37. Krey, G., Braissant, O., L'Horsset, F., Kalkhoven, E., Perroud, M., Parker, M. G., *et al.* (1997) Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. *Mol. Endocrinol.* **11**, 779–791
38. Trott, O., and Olson, A. J. (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **31**, 455–461
39. Fyffe, S. A., Alphey, M. S., Buetow, L., Smith, T. K., Ferguson, M. A., Sorensen, M. D., *et al.* (2006) Recombinant human PPAR-beta/delta ligand-binding domain is locked in an activated conformation by endogenous fatty acids. *J. Mol. Biol.* **356**, 1005–1013
40. Takahashi, Y., Sato, S., Kurashima, Y., Yamamoto, T., Kurokawa, S., Yuki, Y., *et al.* (2018) A refined culture system for human induced pluripotent stem cell-derived intestinal epithelial organoids. *Stem Cell Rep.* **10**, 314–328
41. Mathur, S. N., Watt, K. R., and Field, F. J. (2007) Regulation of intestinal NPC1L1 expression by dietary fish oil and docosahexaenoic acid. *J. Lipid Res.* **48**, 395–404
42. van der Veen, J. N., Kruit, J. K., Havinga, R., Baller, J. F., Chimini, G., Lestavel, S., *et al.* (2005) Reduced cholesterol absorption upon PPARdelta activation coincides with decreased intestinal expression of NPC1L1. *J. Lipid Res.* **46**, 526–534
43. Uchinomiya, S., Matsunaga, N., Kamoda, K., Kawagoe, R., Tsuruta, A., Ohdo, S., *et al.* (2020) Fluorescence detection of metabolic activity of the fatty acid beta oxidation pathway in living cells. *Chem. Commun. (Camb)* **56**, 3023–3026
44. Sonnenburg, J. L., and Bäckhed, F. (2016) Diet-microbiota interactions as moderators of human metabolism. *Nature* **535**, 56–64
45. McCarville, J. L., Chen, G. Y., Cuevas, V. D., Troha, K., and Ayres, J. S. (2020) Microbiota metabolites in health and disease. *Annu. Rev. Immunol.* **38**, 147–170
46. Miyamoto, J., Igarashi, M., Watanabe, K., Karaki, S. I., Mukouyama, H., Kishino, S., *et al.* (2019) Gut microbiota confers host resistance to obesity by metabolizing dietary polyunsaturated fatty acids. *Nat. Commun.* **10**, 4007
47. Miyamoto, J., Mizukure, T., Park, S. B., Kishino, S., Kimura, I., Hirano, K., *et al.* (2015) A gut microbial metabolite of linoleic acid, 10-hydroxy-cis-12-octadecenoic acid, ameliorates intestinal epithelial barrier impairment partially via GPR40-MEK-ERK pathway. *J. Biol. Chem.* **290**, 2902–2918
48. Hira, T., Ogasawara, S., Yahagi, A., Kamachi, M., Li, J., Nishimura, S., *et al.* (2018) Novel mechanism of fatty acid sensing in enteroendocrine cells: specific structures in oxo-fatty acids produced by gut bacteria are responsible for CCK secretion in STC-1 cells via GPR40. *Mol. Nutr. Food Res.* **62**, e1800146
49. Sampath, H., and Ntambi, J. M. (2005) Polyunsaturated fatty acid regulation of genes of lipid metabolism. *Annu. Rev. Nutr.* **25**, 317–340
50. de Urquiza, A. M., Liu, S., Sjöberg, M., Zetterström, R. H., Griffiths, W., Sjövall, J., *et al.* (2000) Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science* **290**, 2140–2144
51. Levi, L., Wang, Z., Doud, M. K., Hazen, S. L., and Noy, N. (2015) Saturated fatty acids regulate retinoic acid signalling and suppress tumorigenesis by targeting fatty acid-binding protein 5. *Nat. Commun.* **6**, 8794
52. Chakravarthy, M. V., Lodhi, I. J., Yin, L., Malapaka, R. R., Xu, H. E., Turk, J., *et al.* (2009) Identification of a physiologically relevant endogenous ligand for PPARalpha in liver. *Cell* **138**, 476–488
53. Kliewer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* **83**, 813–819
54. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* **83**, 803–812
55. Lim, H., Gupta, R. A., Ma, W. G., Paria, B. C., Moller, D. E., Morrow, J. D., *et al.* (1999) Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. *Genes Dev.* **13**, 1561–1574
56. Hatae, T., Wada, M., Yokoyama, C., Shimonishi, M., and Tanabe, T. (2001) Prostacyclin-dependent apoptosis mediated by PPAR delta. *J. Biol. Chem.* **276**, 46260–46267
57. Xu, H. E., Lambert, M. H., Montana, V. G., Parks, D. J., Blanchard, S. G., Brown, P. J., *et al.* (1999) Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol. Cell* **3**, 397–403
58. Khuchua, Z., Glukhov, A. I., Strauss, A. W., and Javadov, S. (2018) Elucidating the beneficial role of PPAR agonists in cardiac diseases. *Int. J. Mol. Sci.* **19**, 3464
59. Nordestgaard, B. G., Benn, M., Schnohr, P., and Tybjaerg-Hansen, A. (2007) Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. *JAMA* **298**, 299–308

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60. Xiao, C., Stahel, P., and Lewis, G. F. (2019) Regulation of chylomicron secretion: focus on post-assembly mechanisms. *Cell Mol. Gastroenterol. Hepatol.* **7**, 487–501
61. Zembroski, A. S., D'Aquila, T., and Buhman, K. K. (2021) Characterization of cytoplasmic lipid droplets in each region of the small intestine of lean and diet-induced obese mice in response to dietary fat. *Am. J. Physiol. Gastrointest. Liver Physiol.* **321**, G75–G86
62. D'Aquila, T., Zembroski, A. S., and Buhman, K. K. (2019) Diet induced obesity alters intestinal cytoplasmic lipid droplet morphology and proteome in the postprandial response to dietary fat. *Front. Physiol.* **10**, 180
63. Bouchoux, J., Beilstein, F., Pauquai, T., Guerrera, I. C., Chateau, D., Ly, N., *et al.* (2011) The proteome of cytosolic lipid droplets isolated from differentiated Caco-2/TC7 enterocytes reveals cell-specific characteristics. *Biol. Cell* **103**, 499–517
64. Drouin-Chartier, J. P., Tremblay, A. J., Lépine, M. C., Lemelin, V., Lamarche, B., and Couture, P. (2018) Substitution of dietary  $\omega$ -6 polyunsaturated fatty acids for saturated fatty acids decreases LDL apolipoprotein B-100 production rate in men with dyslipidemia associated with insulin resistance: a randomized controlled trial. *Am. J. Clin. Nutr.* **107**, 26–34
65. Fernandez, M. L., and West, K. L. (2005) Mechanisms by which dietary fatty acids modulate plasma lipids. *J. Nutr.* **135**, 2075–2078
66. Lagakos, W. S., Gajda, A. M., Agellon, L., Binas, B., Choi, V., Mandap, B., *et al.* (2011) Different functions of intestinal and liver-type fatty acid-binding proteins in intestine and in whole body energy homeostasis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **300**, G803–G814
67. Rossi, G., Manfrin, A., and Lutolf, M. P. (2018) Progress and potential in organoid research. *Nat. Rev. Genet.* **19**, 671–687
68. Levy, E., Mehran, M., and Seidman, E. (1995) Caco-2 cells as a model for intestinal lipoprotein synthesis and secretion. *FASEB J.* **9**, 626–635
69. Takahashi, Y., Noguchi, M., Inoue, Y., Sato, S., Shimizu, M., Kojima, H., *et al.* (2022) Organoid-derived intestinal epithelial cells are a suitable model for preclinical toxicology and pharmacokinetic studies. *iScience* **25**, 104542
70. Lawrence, J. W., Li, Y., Chen, S., DeLuca, J. G., Berger, J. P., Umbenhauer, D. R., *et al.* (2001) Differential gene regulation in human *versus* rodent hepatocytes by peroxisome proliferator-activated receptor (PPAR) alpha. PPAR alpha fails to induce peroxisome proliferation-associated genes in human cells independently of the level of receptor expression. *J. Biol. Chem.* **276**, 31521–31527
71. Neels, J. G., and Grimaldi, P. A. (2014) Physiological functions of peroxisome proliferator-activated receptor  $\beta$ . *Physiol. Rev.* **94**, 795–858
72. Umetani, M., Domoto, H., Gormley, A. K., Yuhanna, I. S., Cummins, C. L., Javitt, N. B., *et al.* (2007) 27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen. *Nat. Med.* **13**, 1185–1192
73. Sasaki, T., Watanabe, Y., Kuboyama, A., Oikawa, A., Shimizu, M., Yamauchi, Y., *et al.* (2021) Muscle-specific TGR5 overexpression improves glucose clearance in glucose-intolerant mice. *J. Biol. Chem.* **296**, 100131