Saturated fatty acids regulate retinoic acid signalling and suppress tumorigenesis by targeting fatty acid-binding protein 5

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Long chain fatty acids (LCFA) serve as energy sources, components of cell membranes and precursors for signalling molecules. Here we show that these biological compounds also regulate gene expression and that they do so by controlling the transcriptional activities of the retinoic acid (RA)-activated nuclear receptors RAR and PPARβ/δ. The data indicate that these activities of LCFA are mediated by FABP5, which delivers ligands from the cytosol to nuclear PPARβ/δ. Both saturated and unsaturated LCFA (SLCFA, ULCFA) bind to FABP5, thereby displacing RA and diverting it to RAR. However, while SLCFA inhibit, ULCFA activate the FABP5/PPARβ/δ pathway. We show further that, by concomitantly promoting the activation of RAR and inhibiting the activation of PPARβ/δ, SLCFA suppress the oncogenic properties of FABP5-expressing carcinoma cells in cultured cells and in vivo. The observations suggest that compounds that inhibit FABP5 may constitute a new class of drugs for therapy of certain types of cancer.
 saturated and unsaturated long chain fatty acids (SLCFA, ULCFA) share common roles as energy sources and key membrane components, but also display distinct biological activities. Hence, while high concentrations of SLCFA trigger acute endoplasmic reticulum stress, induce apoptosis and can lead to insulin resistance and diabetes, ULCFA can protect cells from apoptosis and improve glucose uptake1–7. It was also reported that SLCFA can inhibit, whereas ULCFA can induce proliferation of cancer cells8–11. The molecular mechanisms that underlie distinct activities of different types of LCFA remain poorly understood. In cells, LCFA are associated with fatty acid-binding proteins (FABPs), members of the intracellular lipid-binding proteins (iLBPs) family, which also includes proteins that bind retinol or its transcriptionally active metabolite retinoic acid (RA)12–14. Some iLBPs are known to cooperate with specific nuclear receptors. Hence, cellular RA-binding protein 2 (CRBP2) and FABP5, respectively, deliver RA to the two nuclear receptors activated by this hormone: the classical RA receptor RAR and the alternative RA receptor PPARβ/δ (refs 15–17). Consequently, RA activates RAR in cells that express a low FABP5/CRBP2 ratio, but is targeted to PPARβ/δ in cells in which this ratio is high15–21. As RAR and PPARβ/δ control different sets of genes, RA can exert distinct and sometimes opposing activities. For example, RA inhibits the growth of carcinoma cells where it functions preferentially through CRBP2 and RAR but promotes proliferation where, due to a high level of FABP5, it activates PPARβ/δ (refs 15,16). Indeed, FABP5 displays oncogenic activities22–29. FABP5 is upregulated in multiple types of human cancers22–25 as well as in tumours that arise in the MMTV–neu mouse model of breast cancer20. In these mice, tumour development is inhibited by genetically decreasing the FABP5/CRBP2 ratio15,16,28. Notably, while FABP5 can bind many lipophilic compounds15,31, it is mobilized to the nucleus in specific response to PPARβ/δ agonists such as RA and ULCFA, but not on binding of non-PPAR ligands such as SLCFA15,32,33.

Here we show that SLCFA and ULCFA differentially regulate the transcriptional activities of RAR and PPARβ/δ, and that FABP5 is a critical mediator of these responses. Both LCFA types displace RA from FABP5 and thereby divert the hormone to RAR and activate this receptor. However, while SLCFA block FABP5 and inhibit PPARβ/δ, ULCFA are delivered by FABP5 to PPARβ/δ to induce its activation. We show further that, by concomitantly activating RAR and inhibiting PPARβ/δ, SLCFA suppress the growth of FABP5-expressing carcinomas. These findings define novel physiological functions for LCFA, provide a rationale for understanding distinct biological activities of SLCFA and ULCFA, and suggest that FABP5 inhibitors may comprise a new class of anticarcinogenic drugs.

Results

LCFA regulate transcriptional activation by RAR and PPARβ/δ. The activation status of RAR and PPARβ/δ in vivo was examined using mice that globally express β-galactosidase (lacZ) under the control of an RAR response element (RARE–lacZ reporter mice)34, and mice that globally express luciferase under the control of a PPAR response element (PPRE–luc reporter mice)35. Treatment with RA activated the reporter in multiple tissues of RARE–lacZ mice (Fig. 1a, Supplementary Fig. 1a). Co-treatment with RA and with the pan–RAR antagonist AGN193109 attenuated the activation of RAR, verifying the specificity of the response (Supplementary Fig. 1b). Examination of responses in PPRE–luc mice revealed that, similarly to the effect of the PPARβ/δ-selective ligand GW1516 (GW), RA upregulated luciferase expression in these mice (Fig. 1b, Supplementary Fig. 1c). The data thus demonstrate that RA activates both RAR and PPARβ/δ in vivo. Reporter mice were then crossed with FABP5−/− mice. Ablation of FABP5 enhanced lacZ expression in RARE reporter mice (Fig. 1c), and markedly decreased luciferase expression in PPRE–luc reporters (Fig. 1d, Supplementary Fig. 1d,e). As FABP5 does not deliver ligands to other PPAR isoforms36, the effect of its ablation in PPRE–luc mice must have specifically stemmed from alterations in the activation of PPARβ/δ. The data thus show that FABP5 suppresses the activation of RAR and promotes the activation of PPARβ/δ in vivo. Importantly, RA failed to upregulate luciferase expression in PPRE–luc mice lacking FABP5 (Fig. 1d, Supplementary Fig. 1f), demonstrating that this protein is critical for RA-induced activation of PPARβ/δ.

FABP5 can bind multiple ligands, including RA and LCFA. The equilibrium dissociation constants (Kd) for the association of FABP5 with the SLCFA palmitate (16:0) and stearate (18:0), and the ULCFA linoleate (18:2) and olate (18:1) were measured by fluorescence competition titrations37 using bacterially expressed recombinant FABP5 (Supplementary Fig. 1g). Binding of the fluorescent lipid 1-anilino-8-naphthalene-8-sulfonic acid (ANS) to the protein was examined by fluorescence titrations (Supplementary Fig. 1h), which yielded a Kd of 70 ± 6.4 nM. The affinities of LCFA for FABP5 were then assessed by monitoring their ability to displace ANS from the protein (Fig. 1e). Kds for binding of 16:0, 18:0, 18:2, and 18:1 to FABP5 were found to be 20.4 ± 4.2, 15.3 ± 2.4, 19.3 ± 3.3, and 18.5 ± 4.1 nM (data are mean ± s.d., n = 3), respectively, a somewhat stronger affinity than that of RA (42.3 ± 6.4 nM (ref. 28)). Human keratinocyte HaCaT cells, which express high levels of FABP515, were used to examine whether FABP5 links cellular responses to its different ligands. Cells were cultured in charcoal-treated medium to deplete them of retinoids and transactivation assays were carried out. Cells were co-transfected either with a vector encoding an RARE-driven luciferase and an expression vector for RARz, or with a PPRE-driven luciferase and an expression vector for PPARβ/δ, treated with LCFA, and luciferase activity was measured. In the absence of RA, neither SLCFA nor ULCFA affected the activity of RAR (Fig. 1g,i). SLCAF also did not activate PPARβ/δ (Fig. 1h) but, as previously reported32,38, ULCFAs functioned as agonists for this receptor (Fig. 1j, Supplementary Fig. 1j). Strikingly, in the presence of RA, treatment with <10 μM concentrations of all LCFA modulated the transcriptional activities of both receptors. Both SLCFA- and ULCFA-activated RAR (Fig. 1g,i). PPARβ/δ was inhibited by SLCFA (Fig. 1h) but activated by ULCFA (Fig. 1j).

A HaCaT cell line in which the expression of FABP5 is stably decreased was then generated (Fig. 1f). Lowering the level of FABP5 abrogated the ability of both 16:0 and 18:2 to activate RAR in the presence of RA (Fig. 1g,i). Reducing FABP5 expression decreased the activity of PPARβ/δ even in the absence of RA, indicating that cells contain other endogenous PPARβ/δ ligands that rely on FABP5 for their nuclear delivery (Fig. 1h,j). Decreasing the expression of FABP5 also diminished the ability of both SLCFA and ULCFA to regulate RA-dependent PPARβ/δ activity (Fig. 1h,j).

Modulation of the transcriptional activities of RAR and PPARβ/δ by LCFA was further examined by monitoring their effects on expression of endogenous target genes for these receptors in NaF mammary carcinoma cells. NaF cells are derived from tumours that arise in the MMTV–neu mouse model of breast cancer30 and they express a high level of FABP5 (ref. 16). Similarly to their effects in transcriptional activation assays, both SLCFA and ULCFA induced the expression of the RAR target genes Rarb and Cyp26a (Fig. 2a,b, and Supplementary Fig. 2a–c). Also in accordance with transactivation assays, SLCFA decreased
Figure 1 | LCFAs regulate the transcriptional activity of RAR and PPARβ/δ. (a) X-gal staining of organs (top) and tissue sections (bottom) of RARE–LacZ mice treated with vehicle (−) or RA (1 mg). Representative images out of three mice per group are shown. WAT, white adipose tissue; T, trachea. Magnifications: brain 400×, intestine 200×. Scale bars represent 50 μm in brain sections and 100 μm in intestine sections. (b) Left: Images of PPRE–luc mice injected with vehicle, GW1516 (GW), or RA (4 mg). Right: quantitation of data from three mice per group. (c) X-gal staining of organs of RARE–LacZ+/−/FPABP5−/− (RLZ5−/−) and RARE–LacZ+/−/FPABP5−/− (RLZ5+/+) mice. Representative images out of six mice per group are shown. (d) Representative images of PPRE–luc+/−/FPABP5−/− (PLCS−/−, n = 3) and PPRE–luc+/−/FPABP5−/− (PLCS+/+) mice injected with vehicle or RA. (e) Fluorescence competition titrations demonstrating binding of LCFA to FABP5. (f) ImmunobLOTS demonstrating reduced level of FABP5 in HaCaT cells stably expressing FABP5 short hairpin RNA. (g-j) Transactivation assays in HaCaT cells stably expressing shGFP or shFABP5 co-transfected with vectors encoding RARs and RARE-driven luciferase (g,i), or PPARβ/δ and a PPRE-driven luciferase (h,j). Cells were treated with 16:0 (g,h) or 18:2 (i,j) in the presence or absence of RA. Data are mean ± s.d. of three independent experiments. **P < 0.01 versus non-treated cells, calculated by unpaired t-test.

(FIG. 2c, and Supplementary FIG. 2a,b), and ULCPF5 increased (FIG. 2d, Supplementary FIG. 2c) the expression of PPARβ/δ target genes Pdpk1 and Plin2. Cells were then treated with Triacsin (TriC), an inhibitor of fatty acyl CoA ligase, the enzyme that catalyses the first step in fatty acid metabolism.39 TriC elevated the level of total free fatty acids in the cells by about twofold (Supplementary FIG. 2d) and augmented the respective activities of both SLCFA and ULCPF5 (FIG. 2e–h, and Supplementary FIG. 2e,f). The data thus demonstrate that these effects are exerted by the LCPF5A themselves and not by their metabolic products. Notably, TriC upregulated the expression of RAR target genes even in the absence of ectopic administration of LCFA (FIG. 2e,f), indicating that RA signalling is controlled by alterations in endogenous LCFA levels. TriC treatment per se did not significantly affect the expression of PPARβ/δ targets (FIG. 2g,h), likely reflecting that TriC elevates the levels of both SLCFA, which inhibit, and ULCPF5, which activate PPARβ/δ, resulting in an overall neutral effect.

FABP5 and RA are critical for LCPF5A function. NaF cells express FABP3 and FABP5 but the latter displays a markedly higher level (Supplementary FIG. 2g). Decreasing FABP5 expression in NaF cells (Supplementary FIG. 2h) upregulated the RAR target gene Rarb (Supplementary FIG. 2i), and suppressed the PPARβ/δ target gene Pdpk1 (Supplementary FIG. 2j). The pan–RAR antagonist LE540 abolished the ability of 16:0 to induce RAR targets (Supplementary FIG. 3a) but had no effect on the responsiveness of PPARβ/δ target genes (Supplementary FIG. 3b). These data demonstrate that induction of RAR target genes by LCFA does not stem from an RAR-independent function of these compounds. These observations also show that RAR is not involved in the modulation of PPARβ/δ activity by 16:0. To examine whether RA is necessary for these effects, cells were depleted of retinoids by culturing in charcoal-treated medium. The depletion decreased the expression of both RAR and PPARβ/δ target genes (FIG. 2i,j). 16:0 did not induce the expression of RAR target genes in the absence of retinoids, and the response was restored following replenishment with RA (FIG. 2i). Unlike the absolute RA dependence of the responsiveness of RAR targets, 16:0 downregulated the expression of PPARβ/δ targets even in the absence of retinoids (FIG. 2j). These observations likely reflect that, in contrast with CRABP2 and RA, which are specifically activated by RA, FABP5 and PPARβ/δ can be activated by other endogenous ligands. Hence, 16:0 displaces all PPARβ/δ ligands from FABP5.

RARE–lacZ and PPRE–luc reporter mice were separated into three groups, which were fed a regular chow, a diet enriched in 16:0, or a diet enriched with safflower oil in which the predominant fatty acid is 18:2 (SF/18:2). Feeding RARE–lacZ mice with diets enriched with either 16:0 or SF/18:2 markedly enhanced X-gal staining, demonstrating the activation of RAR (FIG. 2k, Supplementary FIG. 4a). In PPRE–luc reporter mice, 16:0–enriched diet decreased, and SF/18:2–enriched diet increased
PPAR activation (Fig. 2l, Supplementary Fig. 4b). Strikingly, PPRE–luc mice fed 16:0-enriched diet displayed a markedly reduced response to RA-induced PPAR activation (Fig. 2m, Supplementary Fig. 4c). Hence, dietary SLCFA and ULCFA differentially regulate RA signalling in vivo.

**LCFA differentially modulate cancer cell growth.** Considering the pro-proliferative activities of the FABP5/PPARβ/δ pathway, the opposing effects of SLFCA and ULCFAs on PPARβ/δ activation suggest that they may differentially modulate cancer cell growth. Indeed, SLFCA suppressed, while ULCFA facilitated NaF cell proliferation (Fig. 3a,b). Normal human mammary epithelial cells (HMEC) and MCF-7 mammary carcinoma cells, which express low levels of FABP5, NaF and MBA-MD-231 (231) mammary carcinoma cells and PC3M prostate cancer cells, which highly express the binding protein (Fig. 3c) were used to examine the involvement of FABP5 in these opposing effects of the two types of LCFA. 16:0 downregulated the expression of the PPAR target gene Rarb and upregulated the PPARβ/δ target Pdpk1 in PC3M and 231 cells but not in MCF-7 cells (Fig. 3d). RA facilitated proliferation of 231 and PC3M cells, but not of HMEC and MCF-7 cells (Fig. 3e). Strikingly, SLFCA potently inhibited the growth of 231 and PC3M cells, but had no effect on proliferation of either HMEC or MCF-7 (Fig. 3f, Supplementary Fig. 5a). This activity was abolished on depletion of retinoids and restored on replenishing depleted media with RA (Fig. 3g). 18:2 promoted the growth of the FABP5-expressing 231 and PC3M cells but, similarly to SLFCA, had no effect on growth of MCF-7 and HMEC cells (Fig. 3h). Both RA and 18:2 promoted cell proliferation when administered alone, and the activity was additive when these two PPARβ/δ ligands were added together (Fig. 3i). Treatment of cells with the PPARβ/δ antagonist, PT-S58 (ref. 40), inhibited cell proliferation with an additive effect observed on co-treatment with LCFA (Fig. 3j,k). Moreover, despite the inhibition of PPARβ/δ in the presence of PT-S58, 18:2 suppressed cell proliferation (Fig. 3k). Considering that PT-S58 inhibits PPARβ/δ (ref. 40) but does not associate with FABP5, the activities of LCFA in the presence of the inhibitor likely stemmed from the activation of RAR. Taken together, the data indicate that, by binding to FABP5, both SLFCA and ULCFA activate RAR by shifting RA towards this nuclear receptor.

In accordance with the pronounced pro-carcinogenic activities of FABP5 (refs 15,28), decreasing the expression of the protein in NaF cells (Supplementary Fig. 2h) inhibited proliferation (Fig. 4a), Supplementary Fig. 5b), converted RA from a pro- to an anti-proliferative agent (Fig. 4b), hampered formation of colonies in soft agar (Fig. 4c, Supplementary Fig. 5c) and suppressed tumour development in a xenograft mouse model (Fig. 4d). Treatment of NaF cells with 16:0 inhibited their growth (Fig. 4e, Supplementary Fig. 5b), triggered apoptosis (Fig. 4f,g) and markedly suppressed colony formation (Fig. 4h). These anti-oncogenic activities of 16:0 were markedly diminished in cells in which FABP5 expression was decreased (Fig. 4e–g, Supplementary Fig. 5b). Ectopic expression of FABP5 in MCF-7 cells induced cell proliferation (Fig. 4i) and suppressed apoptosis (Fig. 4j). Strikingly, unlike parental MCF-7 cells, overexpression of FABP5 rendered these cells sensitive to 16:0-induced growth arrest and apoptosis.
expression of the endoplasmic reticulum stress markers Chop and concentrations of 16:0 reduced the expression levels of both Grp78 and FABP5 mRNA in normal human mammary epithelial cells (HMEC), NaF, MDA-MB-231 and MCF-7 mammary carcinoma cells, and PC3M prostate carcinoma cells. (d) Fdps1 and Rarb mRNA in denoted cells treated with 16:0 (10 μM, 6 h). (e) Cells were treated with vehicle (−) or RA (1 μM) for 4 days and proliferation assessed by MTT assays. (f) Effect of 16:0 (4 days) on proliferation of denoted cells. (g) Cells were cultured in CT-FBS, treated with 16:0 in the absence or presence of RA (0.2 μM, 4 days), and proliferation assessed by MTT assays. **P < 0.01, by unpaired t-test versus cells cultured in CT-FBS replinished with RA. (h) Effect of 18:2 (4 days) on proliferation of denoted cells assessed by MTT assays. (i) Cells were cultured in CT-FBS, co-treated with 18:2 and RA (0.2 μM) for 4 days and proliferation assessed by MTT assays. (j) Effect of 16:0 (j) or 18:2 (k) on proliferation of NaF cell in the presence or absence of the PPARβ/δ antagonist PT-S58 (5 μM). #P < 0.01 versus untreated control, *P < 0.05 versus PT-S58 treatment without LCFA. Data in all panels are Mean ± s.d. *P < 0.05, **P < 0.01 versus the respective control. P-values were calculated by unpaired t-test.

16:0 induces genome-wide regulation of cancer-related genes. NaF cells, cultured in delipidated medium, were treated with the PPARβ/δ-selective agonist GW1516 (GW), the pan–RAR agonist 4-[[(E)-2-(5,5,8,8-tetramethyl-6,7-dihydrocyclopenta[b]furan-2-yl)prop-1-enyl]benzoic acid (TTNPB) or RA (4 h., 1 μM each). Transcriptome analyses (Affymetrix Mouse Gene 2.1 ST Arrays) revealed that 1,047 and 1,474 genes were commonly regulated by RA and GW1516 and RA and TTNPB, respectively (Supplementary Fig. 6a). Notably, more genes were regulated by RA (3,960 genes) than by either GW (1,979 genes) or TTNPB (2,598 genes). Transcriptome analyses were also carried out in cells treated with 16:0 (10 μM, 4 h) in the presence of retinoins. The analysis identified 258 and 446 genes commonly regulated by 16:0 and GW1516 and 16:0 and TTNPB, respectively (Fig. 5a,b). Hierarchical clustering identified 69 genes whose expression was upregulated by GW1516 and downregulated by 16:0 (Fig. 5c, Supplementary Data 1), and 51 genes that were upregulated by both TTNPB and 16:0 (Fig. 5d, Supplementary Data 2). The data thus show that the expression of a substantial cohort of PPARβ/δ target genes is negatively regulated by 16:0 while many RAR target genes are induced by this LCFA. Strikingly, expression of all 120 genes regulated by 16:0 and GW1516 and by 16:0 and TTNPB were upregulated by RA (Fig. 5e, Supplementary Data 1, and Supplementary Data 2).

Validating the transcriptome analysis, quantitative real-time PCR showed that the PPARβ/δ target genes Cad47 and Tgfb2 were downregulated, and expression of the RAR target genes Skt3 and Cereblon (Crbn) increased on treatment with 16:0 (Fig. 4f). Functional analyses of the 120 genes regulated by 16:0 in common with GW1516 and TTNPB (Ingenuity Pathway Analysis) indicated that the most significantly enriched network is 'cellular development, cancer and embryonic development' (P = 0.0002). Notably, most genes in this network were
downregulated by 16:0 treatment (Fig. 5g, green), implying an anti-oncogenic activity.

16:0 suppresses tumor growth in vivo. Female NCr athymic mice were separated into three groups and fed a regular chow diet, a diet enriched with 16:0, or a SF/18:2-enriched diet. Following a week of feeding, NaF cells were injected into the mammary fat pad and tumor growth was monitored. Consumption of either LCFA-enriched diet elevated serum levels of free fatty acids (Fig. 5h). Mice in the three groups consumed similar amounts of food (Supplementary Fig. 6b), and displayed similar weight gain (Fig. 5i). Total FFA levels in tumours that arose in mice fed 16:0-enriched and SF/18:2-enriched diets, respectively, were similar in cultured cells, tumours that arose in mice fed 16:0-enriched and SF/18:2-enriched diets, respectively, were similar in chow-fed animals (Fig. 6c,e). Tumours in mice fed 16:0 or SF/18:2 were produced higher levels of the RAR target genes Rarb, Caspase 9, Skt3 and Crbn, although the effect of 16:0 was more pronounced than that of SF/18:2 (Fig. 6d,e, Supplementary Fig. 6d). In addition, expression of cyclin D1, which is suppressed by RAR, was markedly lower in tumours of 16:0 fed mice (Fig. 6d). Notably, expression of the proliferation marker Ki67 was lower and apoptosis was more pronounced in tumours of 16:0 fed mice (Fig. 6d). Expression of the endoplasmic reticulum stress markers Chop and Grp78 were similar in tumours of mice fed the three diets (Supplementary Fig. 6e), indicating that the growth-inhibitory activity of 16:0 did not originate from initiation of endoplasmic reticulum stress.

Discussion

These studies establish that RA activates both RAR and PPARβ/δ in vivo, and that the iLBP FABP5 is a critical regulator of both receptors. The observations further reveal that, through targeting FABP5, the important dietary components LCFA can regulate gene expression by governing the transcriptional activities of both...
Figure 5 | 16:0-regulated cancer-related PPARβ/δ and RAR target genes. (a–g) NaF cells were treated with GW1516 (GW; 1 μM), 16:0 (10 μM), RA (1 μM), or TTNPB (T; 1 μM) for 4 h. Changes in gene expression were analysed using Affymetrix Mouse Gene 2.1 ST Arrays (Affymetrix, USA). (a) Venn diagram showing genes co-regulated by GW and 16:0. (b) Venn diagram showing overlap of genes regulated by TTNPB (T) and 16:0. (c) Hierarchical clustering of genes regulated by GW and 16:0. Boxed: genes upregulated by GW and downregulated by 16:0. (d) Hierarchical clustering of genes regulated by TTNPB and 16:0. Boxed: genes upregulated by both TTNPB and 16:0. (e) Heatmap representing expression of genes boxed in c and d regulated by 16:0, TTNPB, GW and RA. (f) Validation of microarray data. Heatmap showing mRNA levels of denoted genes, measured by qPCR. Data is presented as log2 of the 2⁻ΔΔCT ratio and the correlation (R) to the array signal intensity is indicated. Mean ± s.e.m. (n = 4). (g) Predicted network of genes with known function in cellular development, cancer and embryonic development (P = 2.2 × 10⁻⁶) affected by 16:0 treatment in NaF cells. Arrows indicate activation. Lines ending in short perpendicular lines indicate repression. Red shapes and green shapes indicate upregulated and downregulated genes, respectively. Solid and dashed lines indicate direct and indirect interactions, respectively. (h) Concentrations of free FAs in serum of mice fed chow or diets enriched with 16:0 or SF/18:2 for 4 weeks. Mean ± s.e.m. (n = 6). **P < 0.01 calculated by unpaired t-test. (i) Body weights of mice fed denoted diets. Mean ± s.e.m. (n = 6).

RA-responsive nuclear receptors. We thus define a novel function for these fundamental biological building blocks. Taken together, the observations suggest the model depicted in Fig. 6g. SLCFA displace RA and other PPARβ/δ ligands from FABP5 and thereby block the delivery of such ligands to this receptor and inhibit its activation. Displacement of RA from FABP5 diverts the hormone to RAR, and as SLCFA do not induce either the nuclear import of FABP5 (ref. 32) or the transcriptional activity of PPARβ/δ (ref. 15), these LCFA concomitantly activate RAR and inhibit PPARβ/δ. On the other hand, binding of ULCA to FABP5 similarly reroutes RA to RAR, but these compounds trigger translocation of FABP5 to the nucleus where they are delivered to PPARβ/δ and induce its activation. ULCA thus activates both RAR and PPARβ/δ. Importantly, in vivo, LCFA shifted RA signalling when provided as dietary components, and, in cultured cells, they exerted such an effect at remarkably low concentrations. Hence, while SLCSFA induce lipotoxicity and endoplasmic reticulum stress at concentrations of ~500 μM (ref. 5), these compounds regulate the transcriptional activities of RAR and PPARβ/δ at <10 μM levels. Moreover, even in the absence of ectopic administration, short-term inhibition of fatty acid metabolism was sufficient to markedly induce expression of RAR target genes. The data thus show that the transcriptional activity of RAR is exquisitely sensitive to small alterations in cellular LCFA concentrations. Notably, while LCFA-induced activation of RAR critically depended on the presence of RA, PPARβ/δ was active and its activity was inhibited by 16:0 even in the absence of RA. These observations likely reflect that cells contain multiple endogenous ligands for PPARβ/δ and reveal that SLCSFA suppress the activation of this receptor regardless of the nature of the agonist it uses under particular circumstances.

The ability of LCFA to modulate gene expression by governing RA signalling is expected to have profound and wide-ranging consequences for cell function. One example addressed here is their involvement in regulating carcinoma cell growth. We show that, in FABP5-expressing carcinoma cells, SLCSFA inhibit and ULCA induce cell proliferation. We show further that, in such carcinomas, SLCSFA convert RA from a pro-proliferative to a growth-inhibitory agent, induce apoptosis, suppress oncogenic properties and inhibit tumour development in vivo. SLCSFA induced the expression of multiple RAR targets and reduce the levels of multiple PPARβ/δ target genes, many of which have a known function in cancer. Taken together with the reports that expression of FABP5 is upregulated and is associated with poor survival in several types of human cancers[22–24,26,28], the data indicate that compounds that inhibit FABP5 may constitute a promising new class of drugs for therapy of certain types of cancer. While SLCSFA function as potent FABP5 inhibitors, their rapid metabolism and potentially detrimental activities at pharmacological concentrations preclude their use as efficacious...
drugs. However, the data presented here provide precise criteria for developing FABPs inhibitors and a strong proof-of-principle for the efficacy of such compounds in suppressing carcinoma cell growth.

Methods

Reagents. All-trans RA, 16:0, 18:3, 18:1 and 18:2, KFe(CN)₆, NaFe(CN)₆, 3H₂O and MgCl₂ were purchased from Sigma. GW1516 (GW), TTNPB and antagonists for RARs, RARG and RARβ (BMS 195614, LE 135 and MM 11253, respectively) were purchased from Tocris Biosciences. The pan-RAR antagonist AGN193109 (BMS 195614, LE 135 and MM 11253, respectively) was a gift from Hiyori Kagechika (University of Tokyo). X-gal was from Fisher Scientific.

Cells. NaF, COS-7, MDA-MB-23, MCF-7 and HaCaT cells were cultured in DMEM supplemented with 10% fetal calf serum (Invitrogen Life Sciences). HMEC cells were cultured in HMEC ready medium (Invitrogen Life Sciences). NaF cells were a gift from PhilipLeder (HarvardMedicalSchool, Boston, MA). HMEC cells were purchased from Thermo Fisher Scientific (A10565), COS-7, MDA-MB-23, MCF-7 and HaCaT cell lines were purchased from ATCC. For generation of NaF and HaCaT cell lines, lentiviral vectors pLKO.1 puro encoding respective short hairpin RNA targeting mouse and human FABPs respectively, (Open Biosystems, AL, USA) were packaged in HEK293T cells by co-transfecting with pCMV and pMD2G. Virus was harvested 1–2 days after transfection and used to infect the cells. Single clones were selected using 10 μg ml⁻¹ puromycin. MCF-7 cells were transduced with human FABP5 subcloned into pEGFP-N3 expression vector52 using TransIT-BrCa reagent (Mirus Bio, LLC). The small interfering RNAs (Dharmacon) were transfected into NaF cells using Lipofectamine 2000 (Life Technologies).

Endoplasmic reticulum stress assay. For treatments with high 16:0 concentrations, the FA was complexed with bovine serum albumin (BSA). BSA (affinity) was delipidated using activated charcoal. 16:0 was dissolved in ethanol and added to the BSA solution to create a stock solution of 1 mM 16:0. The solution was incubated at 55°C and for 30 min, then filtered and diluted in cell media to establish the desired concentrations.

Mice. PPRE–luc mice (RepTOP PPRE–Luc)35 were obtained from Charles River Laboratories. RARE–lacZ mice, which globally express β-galactosidase (lacz) gene under the control of the RARE of the RA target gene Rarb34 were purchased from Jackson Laboratories. FABP5⁻/⁻ mice45 on a C57BL/6J background were provided by Golshan Hotamisligil (Harvard School of Public Health). To generate the experimental groups RARE–LacZ ⁺/⁺/FABP5⁻/⁻ (n = 6) and PPRE–luc ⁺/⁺/FABP5⁻/⁻ (n = 6), each of the reporter lines was crossed with FABP5⁺/⁺ mice. For xenografts experiments, female NCr nude mice were obtained from the Athymic Animal and Xenograft Core Facility at the Case Western Reserve Cancer Center. All mice experiments were performed under the American Association for Accreditation of Laboratory Animal Care guidelines. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University (protocol 2013-0040). Mice were maintained on a 12 h light and dark cycle and had access to water and diet ad libitum.

Mouse experiments. RARE–lacZ and PPRE–luc males and females (4–6 weeks old, n = 3 each) were treated with RA (1 or 4 mg). PPRE–luc mice (4–6 weeks old, n = 3) were used for GW (4 mg) treatments. Lindig solutions were prepared immediately before use in a vehicle of sterile saline:polyethylene glycol:Tween 80 (80:10:10 v/v) and injected intraperitoneally. For AGN193109 treatments, a total of six RARE–lacZ males were used (three mice treated and three controls). AGN193109 solution was prepared to a final concentration of 1 mg kg⁻¹ and administered by intraperitoneal injections. Mice were treated with AGN193109 and then co-injected with RA the following day.

RARE–lacZ and PPRE–luc males and females (4–6 weeks old, n = 6), were used. Mice were fed ad libitum with a denoted diet and the reporter activity was measured a week later either by X-gal staining or imaging. At the end point, PPRE–luc mice were treated with RA, and imaged again 6h later. Mice diets (BioServ, Frenchtown, NJ) contained 18.1% protein, 4.8% fibre and 2.2% ash. Regular chow contained 7.1% fat, 59.3% carbohydrate and a total of 3.74 kcal g⁻¹.

Figure 6 | 16:0 shifts RA signalling and suppresses mammary tumour growth in vivo. (a) Concentrations of 16:0 in tumours of mice fed denoted diets. Mean ± s.d. (n = 6). **P<0.01 by unpaired t-test versus chow control. (b) NaF cells (2 × 10⁵) were transplanted into the mammary fat pad of NCr athymic female mice fed denoted diets and tumour growth was monitored. Mean ± s.d. (n = 6). **P<0.01 by unpaired t-test versus chow control. (c,d) mRNAs for PPARβ/δ (e) or RAR target genes (f) in mice fed denoted diets. Mean ± s.d. (n = 6). *P<0.05, **P<0.01 calculated by unpaired t-test.
Antibodies against FABP5 and PDPK1 were purchased form R&D Systems (AF1476 and AF3077, respectively) and BD Transduction Laboratories (611070, respectively). All antibodies were treated in a 1:1,000 dilution. Uncropped scans of western blots shown in Figs 4g,i,m and 6e are presented in Supplementary Fig. 7.

**Colony formation assays.** A layer of 0.8% agarose in cell media was cast in a six-well plate and set in room temperature to solidify. Cells were suspended in 0.25% agarose in media and 1 ml from this mixture containing 5,000 cells was added to each well. Cells were cultured for 21 days. Media were replenished every 3 days. Colonies were visualized by staining with 0.005% crystal violet and counted under a light microscope.

**Palmitic acid analysis.** Palmitic acid was quantified using stable isotope dilution liquid chromatography mass spectrometry (LC/MS) after conversion to the corresponding methyl ester. Methyl esters were synthesized by refluxing palmitic acid in methanol in the presence of methanolic sodium methoxide. Methyl esters were then converted to the trimethylsilyl derivative using BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) and then analyzed by LC/MS. The LC/MS system consisted of a Waters Alliance 2795 HPLC and a Finnigan TSQ-7000 triple quadrupole mass spectrometer equipped with an electrospray ionization source. The mobile phase was a linear gradient between 100% water and 100% methanol over 15 min followed by washing with methanol. Data was acquired using the method previously described (1).

**Statistical significance was assessed using an independent sample *t*-test versus respective untreated controls.**

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**Immunoblotting.** Total cell protein was extracted using RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS). Proteins were resolved by electrophoresis on SDS-PAGE gels and transferred onto nitrocellulose membranes. membranes were blocked with primary antibodies, followed by washes with Tween-TBS, and incubation with horse-radish-peroxidase-conjugated antibodies. Protein expression was detected by exposure to enhanced chemiluminescence (ECL) and exposed to XR-B X-ray film. Band intensities were determined using Origin 8 software.
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Acknowledgements
We are grateful to Hideyoshi Kagechika (University of Tokyo) for providing LE450. This work was supported by National Institute of Health grants DK060684 to N.N., and HL076491 to S.L.H. Mass spectrometry studies were performed on instruments housed within a facility supported in part by a Center of Innovations Award by AB SCIEX.

Author contributions
L.L. and N.N. designed the study and wrote the manuscript. L.L. and M.K.D carried out the work was supported by National Institute of Health grants DK060684 to N.N., and HL076491 to S.L.H. Mass spectrometry studies were performed on instruments housed within a facility supported in part by a Center of Innovations Award by AB SCIEX.

Additional information
Accession codes. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession code GSE73186.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Levi, L. et al. Saturated fatty acids regulate retinoic acid signalling and suppress tumorigenesis by targeting fatty acid-binding protein 5. Nat. Commun. 6:7894 doi: 10.1038/ncomms7974 (2015).