

CELL BIOLOGY

ω -3 polyunsaturated fatty acids direct differentiation of the membrane phenotype in mesenchymal stem cells to potentiate osteogenesis

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Mammalian cells produce hundreds of dynamically regulated lipid species that are actively turned over and trafficked to produce functional membranes. These lipid repertoires are susceptible to perturbations from dietary sources, with potentially profound physiological consequences. However, neither the lipid repertoires of various cellular membranes, their modulation by dietary fats, nor their effects on cellular phenotypes have been widely explored. We report that differentiation of human mesenchymal stem cells (MSCs) into osteoblasts or adipocytes results in extensive remodeling of the plasma membrane (PM), producing cell-specific membrane compositions and biophysical properties. The distinct features of osteoblast PMs enabled rational engineering of membrane phenotypes to modulate differentiation in MSCs. Specifically, supplementation with docosahexaenoic acid (DHA), a lipid component characteristic of osteoblast membranes, induced broad lipidomic remodeling in MSCs that reproduced compositional and structural aspects of the osteoblastic PM phenotype. The PM changes induced by DHA supplementation potentiated osteogenic differentiation of MSCs concurrent with enhanced Akt activation at the PM. These observations prompt a model wherein the DHA-induced lipidome leads to more stable membrane microdomains, which serve to increase Akt activity and thereby enhance osteogenic differentiation. More broadly, our investigations suggest a general mechanism by which dietary fats affect cellular physiology through remodeling of membrane lipidomes, biophysical properties, and signaling.

INTRODUCTION

The plasma membrane (PM) is the interface between a cell and its environment and is therefore responsible for a plethora of tightly regulated parallel tasks. To achieve this functional complexity, mammalian cells produce hundreds of distinct lipid species (1–3) that are actively turned over and trafficked to produce spatial and temporal lipid gradients between cellular compartments (4–6). In addition to the regulatory roles performed by individual lipid molecules, membrane physiology is strictly dependent on the biophysical phenotypes (membrane fluidity, lateral organization, etc.) arising from the collective behaviors of lipids and proteins (7). In particular, PMs are spatially compartmentalized into lateral microdomains that actively regulate signal transduction (8) and membrane trafficking (9), among many other cell functions (10). Despite accumulating evidence for the existence of lateral heterogeneity in cellular membranes (11), the mechanistic relationships between lipid compositions, biophysical properties, and functional outputs remain largely unknown.

The intricate biochemical and biophysical homeostasis of mammalian membranes must be maintained under constant challenge from external lipid inputs (for example, from dietary fats), with perturbations of lipid profiles potentially inducing profound physiological and pathological consequences. Most notable among these are the deleterious consequences (diabetes, cardiovascular disease, and lipotoxicity) associated

with the dietary overconsumption of saturated fats. Inversely, a number of beneficial effects have been ascribed to dietary ω -3 polyunsaturated fatty acids (PUFAs), specifically docosahexaenoic acid (DHA). DHA is critical for neural development (12, 13) and has also been implicated as a protective agent against a variety of adverse inflammatory (14) and cardiovascular (15) conditions. There is also a substantial literature suggesting that ω -3 PUFAs are beneficial for bone health (16–20), including randomized clinical studies (18). The cellular and molecular mechanisms underlying these associations remain unclear (21), but the susceptibility of membrane composition to both pharmacological interventions (for example, statins) (22) and dietary lipid inputs (cholesterol and fatty acids) (23, 24) suggests the intriguing possibility that dietary fats are integrated into membrane lipids, change membrane properties, and thereby affect signal transduction and cell function. However, the influence of dietary fats on membrane physiology has not been widely explored.

Eukaryotic cells are composed of many different membranes, with the PM being the major site of extracellular signal transduction. Further, as a result of being exposed to the extracellular space, PMs are subject to large variations in their environment. Although this variation is self-evident for single-celled organisms, PMs of different cell lineages in multicellular organisms can experience markedly different conditions. For example, the apical PM of a renal epithelial cell is subject to low pH and high urea, as well as potentially significant fluctuations in pH, other ions, and osmolarity. Whether the PM phenotypes of various cell types reflect those cells' environmental and functional variations is not known. This knowledge gap persists because most of the research into the composition and organization of mammalian PMs has relied on erythrocytes as model PMs, as these cells do not contain internal membrane-bound organelles (25). More recently, a number of approaches have been developed to enrich PMs from tissues and cultured cells (26, 27), but there has yet been no systematic analysis of variations in PM

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compositions between cell types, or how these variations contribute to cell identity and functionality.

Here, we used electrospray ionization–tandem mass spectrometry (ESI-MS/MS) to show extensive, cell-autonomous remodeling of PM lipidomes resulting from differentiation of human mesenchymal stem cells (MSCs) into two distinct cell types, adipocytes and osteoblasts. MSC differentiation resulted in distinct, cell-specific biophysical and biochemical phenotypes, which could be modulated by supplementation with exogenous fatty acids, namely, the fish oil fatty acid ω -3 DHA. Membrane remodeling by both DHA incorporation and differentiation was associated with alterations of PM signaling, most notably the activation of Akt. Ultimately, these lipidomic, biophysical, and signaling changes potentiated osteogenic differentiation of MSCs. These results demonstrate that membrane phenotypes are central regulators of cell behavior that are susceptible to remodeling by exogenous fatty acids, suggesting a novel mechanism by which dietary fats could influence cell function.

RESULTS AND DISCUSSION

Differentiation of PM biophysical properties

Because membrane composition and organization are key determinants of cell physiology, we hypothesized that different cell lineages should have distinct membrane phenotypes. We evaluated this hypothesis by measuring the compositional, biophysical, and signaling characteristics of MSC membranes as a function of *in vitro* differentiation into adipocytes or osteoblasts. MSCs facilitate the investigation of cell-autonomous regulation of the membrane phenotypes while controlling for confounding factors such as cellular origin, isolation, and culture conditions. Although it is not clear whether *in vitro* differentiation results in terminally, functionally differentiated cells, we observed expected lineage-specific morphological, histological, and protein expression markers of osteoblastic and adipocytic phenotypes (fig. S1), confirming cellular differentiation (28). For conciseness, we will refer to these phenotypes as “osteoblasts” and “adipocytes,” although these terms are not intended to imply equivalence to the analogous cell types *in vivo*.

To evaluate differentiation of membrane phenotypes, we isolated intact PMs as Giant PM Vesicles (GPMVs) from undifferentiated MSCs and compared them to differentiated lineages. The separation of GPMVs into microscopic, liquid-ordered, and liquid-disordered phases (fig. S2) has been used extensively to investigate membrane microdomains (29–32) and the partitioning of components between them (33, 34). The characteristic temperature dependence of this phenomenon (Fig. 1A) reveals the miscibility temperature (T_{misc} ; the temperature at which 50% of GPMVs show microscopic phase separation), which is hypothesized to be related to the size and lifetime of membrane nanodomains *in vivo* (35). *In vitro* differentiation of MSCs induced a clear divergence of T_{misc} , revealing that microdomain stability in GPMVs was increased in osteoblasts and decreased in adipocytes (Fig. 1, A and B). This effect was highly significant across MSCs independently derived from seven different human donors (Fig. 1B). The magnitudes of the observed effects are quantitatively similar to other major membrane perturbations (29, 36, 37), suggesting that cellular differentiation results in considerable alteration to the stability of membrane microdomains. We also probed the differentiation in PM fluidity by quantifying the generalized polarization (GP) of C-laurdan, a solvatochromic dye sensitive to membrane packing (38). As for T_{misc} , MSC differentiation led to a divergence in the packing of the membranes, with significantly more ordered, packed PMs resulting from osteogenic differentiation and less ordered

PMs in adipocytes (Fig. 1C), again consistent among human donors (Fig. 1D).

Note that isolated GPMVs are not identical to PMs in live cells (39) and therefore that measurements of microdomain stability in these should not be equated with rafts in live cells. Potentially significant differences include the lack of phosphoinositides and cytoskeletal lamination in GPMVs (40), as well as the loss of strict phosphatidylserine (PS) leaflet asymmetry (31), and the fact that GPMVs are at equilibrium. Despite these concerns, GPMVs provide a robust and coherent model system to investigate the biophysical and compositional properties of isolated mammalian PMs, as supported by several recent studies (34, 41–44) that converge on the conclusion that microscopic domains in GPMVs are related to nanoscopic entities in live cell membranes that influence cellular physiology.

Differentiation of MSC membrane lipidomes

In simple synthetic model membranes, biophysical properties are determined by lipid composition (45, 46). To investigate the compositional determinants of the biophysical changes induced by MSC differentiation, we quantified the comprehensive lipidomes of MSC PMs. ESI-MS/MS shotgun lipidomics allowed direct identification and quantitation of ~600 molecular species in each sample (all lipidomic data sets are included in the Supplementary Materials). We validated our PM preparation by observing that both sphingolipids and cholesterol were approximately twofold enriched in PMs relative to crude membranes (CMs; fig. S3A), with a concomitant depletion of the major glycerophospholipids (GPLs). In addition, storage lipids [triacylglycerides (TAGs)] were largely excluded from the PM preparations, despite comprising nearly a third of the lipids detected in adipocytes (fig. S3C). These enrichments were fully consistent with expectations for PMs (5, 47). Principal components analysis (PCA) (fig. S3D) and Western blotting against organelle markers (fig. S3E) further validated the PM preparation.

We then measured changes in membrane lipidomes induced by *in vitro* differentiation of MSCs (Fig. 1E). For lipid classes defined by the hydrophilic headgroup, only the plasmalogen forms of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were significantly increased in osteoblast PMs relative to undifferentiated MSCs (Fig. 1F), with no significant changes observed in adipocytes (trends similar in CMs; fig. S4A). In contrast, significant differences between cell lineages were observed in the acyl chain composition of GPLs. There was a clear differentiation of lipid length (that is, the combined number of carbons in the two lipid acyl chains), with osteogenesis resulting in longer lipids in both PMs (Fig. 1G) and CMs (fig. S4, B and C). A similar divergence was observed for lipid unsaturation; osteoblasts produced strikingly more polyunsaturated lipids (those with three or more double bonds) at the expense of monounsaturated lipids (Fig. 1H and fig. S4, D and E). The differentiation of both lipid length and unsaturation was highly significant, with 34% more long lipids (≥ 36 carbon acyl chains) and 61% more polyunsaturated lipids in osteoblast PMs compared to undifferentiated MSCs (Fig. 1, G and H, insets). The results were notably consistent between three human donors, as evidenced by PCA of the lipidomes. Samples from three individual donors formed tight cell type–dependent clusters, clearly distinct from the other two cell types (Fig. 1I). PC1 defined 45% of the variation and encompassed much of the variation in lipid length and unsaturation, with the “osteoblastic lipids” cluster defined by GPLs with three or more unsaturations and those containing long acyl chains (Fig. 1I and table S2). PC1 showed the same differentiation-associated divergence as the biophysical phenotypes in Fig. 1 (A to D) (that is, osteoblasts >

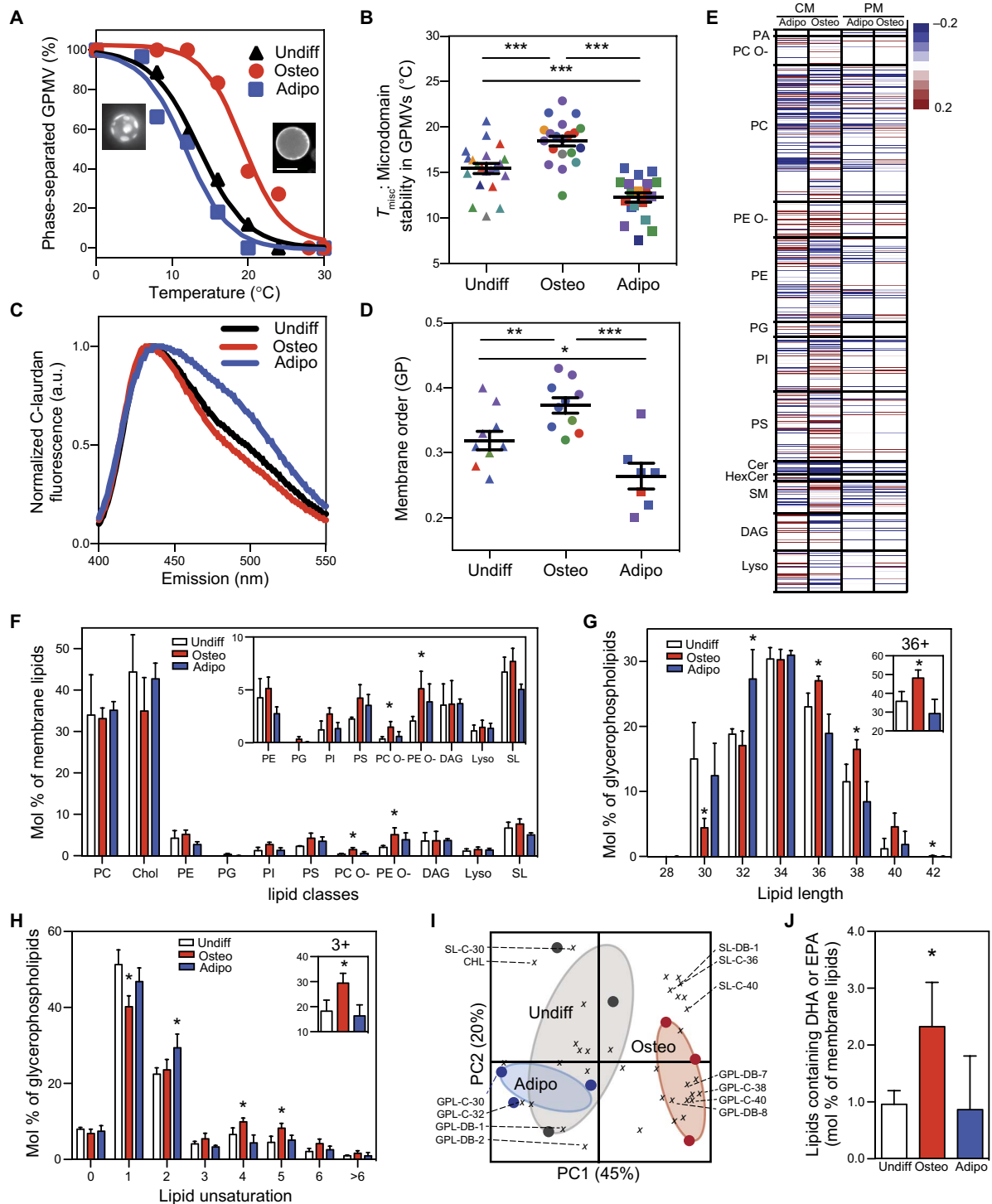


Fig. 1. Differentiation of physical properties and comprehensive lipidomes of hMSC PMs. (A) Sigmoidal fits (solid lines) to phase separation data (50 to 100 vesicles per point) for quantification of phase separation temperature (T_{misc}). (B) Quantification of T_{misc} after 14 days of MSC differentiation; colors denote seven individual human donors. (C) Normalized C-laurdan emission spectra of GPMVs obtained from differentiated MSCs. a.u., arbitrary units. (D) Quantification of membrane order by C-laurdan GP; colors denote four individual human donors. (E) Differentiation-induced changes [$\log_{10}(\text{Diff}/\text{Undiff})$], with red and blue values indicating an increase and decrease in individual lipid species, respectively. (F) Comparison of lipid classes in PMs after 14 days of MSC differentiation; inset shows expanded y axis for clarification. (G) PM GPL length (combined number of carbons in the two acyl chains) after MSC differentiation; inset shows mole percent (mol %) of lipids ≥ 36 carbons in each cell type. (H) PM GPL unsaturation after MSC differentiation; inset shows mol % of lipids with three or more unsaturations. (I) PCA of lipid structural classes and features in the three cell lineages with the loading plot (summary of variables) superimposed on the score plot for the samples. Filled circles are individual human donors, and "x" are individual variables contributing to the principal components. Table S2 shows the variable loadings defining PC1 and PC2. (J) Mol % of membrane lipids containing ω -3 PUFA [DHA or eicosapentaenoic acid (EPA)] as one of the acyl chains. All data in (F) to (H) and (J) are means $\pm$ SD for three to four human donors. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ determined by Student's t test compared to Undiff in (F) to (J).

undifferentiated > adipocytes), suggesting that lipids with longer PUFAs are determinants of the osteoblastic membrane phenotype.

On the basis of the above evidence, we conclude that biophysical and biochemical membrane phenotypes are clearly cell type-specific, even between closely related cell lineages. These differences in membrane properties between the three cell types may reflect their distinct functions. Most notably, osteoblasts are responsible for constitutive secretion of large architectural extracellular matrix (ECM) components, likely necessitating unique membrane features.

Remodeling of the membrane phenotype by ω -3 DHA

To confirm the inference that osteoblastic membrane properties are driven by long, polyunsaturated lipids, we focused on the longest, most polyunsaturated constituents of mammalian lipids: ω -3 PUFAs. Lipids containing ω -3 PUFAs (that is, a 20:5 or 22:6 acyl chain) were over-represented in osteoblast PMs by $\sim$ 2.5-fold relative to undifferentiated MSCs (Fig. 1J), marking one of the largest relative lipidomic differences between the PMs of the three cell types. These observations suggested a rational strategy for inducing the osteoblastic PM phenotype by feeding MSCs a lipid component characteristic of differentiated osteoblasts. Supplementing the culture medium of undifferentiated MSCs with DHA at a physiologically appropriate concentration (20 μ M; see Materials and Methods) led to a marked accumulation of ω -3 PUFA-containing lipids in all cell types (Fig. 2A and fig. S5, A and B). The detailed lipidomic data allowed us to compare the distribution of ω -3 PUFAs between the various lipid classes before and after DHA supplementation. Before supplementation, undifferentiated MSCs showed a major ($\sim$ 85%) accumulation of DHA in PE lipids, despite these comprising only $\sim$ 10% of the total lipidome (Fig. 1F), with a further specific enrichment ($\sim$ 65%) in PE plasmalogen (Fig. 2B). This segregation was wholly specific for ω -3 PUFAs because the distribution of ω -6 PUFAs (that is, lipids bearing 20:4 or 22:4 acyl chain) generally followed the relative abundance of the GPL classes (fig. S6). These observations revealed an unexpectedly precise channeling of ω -3 PUFAs into a specific lipid class (PE plasmalogen). Following DHA supplementation, ω -3 PUFAs were more broadly distributed among different lipid classes than in undifferentiated MSCs, resembling the distribution observed after osteoblast differentiation (Fig. 2B). However, even after supplementation, ω -3 PUFAs were completely excluded from phosphatidic acid (PA), phosphatidylglycerol (PG), and sphingolipids, confirming the selectivity of PUFA incorporation.

Beyond its incorporation, DHA supplementation also led to whole-sale lipidome remodeling (Fig. 2, C to F). There was the expected substitution of ω -3 DHA in place of ω -6 PUFAs, evidenced by a reduction in polyunsaturated lipids not containing ω -3 fatty acids (Fig. 2D). More surprising were the effects on monounsaturated and fully saturated lipids: both increased significantly (Fig. 2D), largely attributable to a nearly 20% increase in lipid-incorporated saturated acyl chains (Fig. 2D, inset, and fig. S5C). Similarly, cholesterol content was significantly enhanced by DHA supplementation (Fig. 2E). These effects may reflect compensatory mechanisms for the fluidizing/disordering effect of the PUFAs, in line with the homeoviscous adaptation theory of biological membranes (48).

DHA-mediated remodeling of the MSC lipidome significantly and specifically [relative to monounsaturated oleic acid (OA), which does not induce lipidomic remodeling (49)] increased phase separation temperature in undifferentiated isolated PMs (Fig. 2, G and H) to a level commensurate with osteoblast PMs. We propose that the physico-chemical mechanisms for DHA-enhanced stability of microdomains

in isolated PMs are as follows: (i) DHA is incorporated into membrane lipids; (ii) the resulting PUFA-containing lipids preferentially partition into the liquid-disordered domain (50, 51); (iii) the disordered domain is fluidized by the presence of PUFA-containing lipids (Fig. 2, I and J), (iv) enhancing the difference between the ordered and disordered domains (fig. S7) (51, 52) and stabilizing microdomains in GPMVs (Fig. 2, G and H), as previously observed (37, 49).

In summary, DHA supplementation of undifferentiated MSCs promoted the osteoblastic PM phenotype: longer, more polyunsaturated lipids (Fig. 1, G and H, and fig. S4, B to E); more ω -3 PUFAs (Fig. 1I and fig. S4F) broadly distributed among different lipid classes (Fig. 2B); and more stable membrane domains in GPMVs (Figs. 1, A and B, and 2, G and H). These observations were confirmed by PCA of the membrane lipidomes. DHA supplementation led to similar effects in all three cell lineages (higher on PC1 and much lower on PC2; dashed lines in Fig. 2F), with these changes inducing an osteoblast-like lipidomic profile in DHA-treated undifferentiated MSCs (Fig. 2F).

DHA potentiates osteogenic differentiation

Because osteoblasts had a distinct PM phenotype, and because this phenotype could be mimicked in undifferentiated MSCs by DHA supplementation, we hypothesized that DHA would also affect osteogenic differentiation. We temporally quantified osteogenic differentiation by an automated image processing protocol to detect deposition of calcified ECM nodules (53) and observed that supplementation of differentiation medium with DHA significantly enhanced matrix deposition (Fig. 3, A and B). This effect was specific for DHA, as matrix deposition was unaffected by monounsaturated OA. DHA-mediated potentiation of differentiation was confirmed by a histological assay (Alizarin Red staining; Fig. 3, C and D), Western blotting for a key transcription factor in osteoblast differentiation (RUNX2; Fig. 3E), and quantitative polymerase chain reaction (qPCR) of the osteoblastic markers bone sialoprotein 2 (BSP2) and alkaline phosphatase (ALP; Fig. 3, F and G). Notably, DHA supplementation had no significant effect on adipogenesis (fig. S8), although slightly reduced lipid droplet content (fig. S8C) and adipogenic gene expression (fig. S8E) were observed; a discussion regarding this lack of effect of DHA-mediated lipidomic remodeling on adipocyte differentiation is included in the legend to fig. S8.

DHA has been proposed to affect cells via a variety of potential mechanisms (21), including direct agonism of G protein-coupled receptors or nuclear receptors, alteration of eicosanoid production, and modulation of membrane phenotypes via incorporation into lipids (as proposed here). To determine whether DHA-mediated potentiation of osteogenic matrix deposition was due primarily to its effect on the PM phenotype, we mimicked the biophysical effects of DHA by treating cells with deoxycholic acid (DCA). Subtoxic concentrations of this bile acid have been shown to phenocopy certain aspects of DHA-mediated PM remodeling (37, 49), namely, stabilization of membrane microdomains in isolated GPMVs by fluidizing liquid-disordered domains. Treating MSCs with DCA concentrations that reproduced the biophysical effects of DHA (100 μ M; compare fig. S9A to Fig. 2, I and J) also reproduced DHA's effect of enhancing osteogenic matrix deposition (fig. S9, B and C), thus supporting the hypothesis that DHA promotes osteogenic lineage specification by stabilizing membrane microdomains.

DHA enhances Akt phosphorylation to potentiate osteogenic differentiation

How does the PM phenotype regulate differentiation? A direct connection between membrane organization and cell physiology is signal

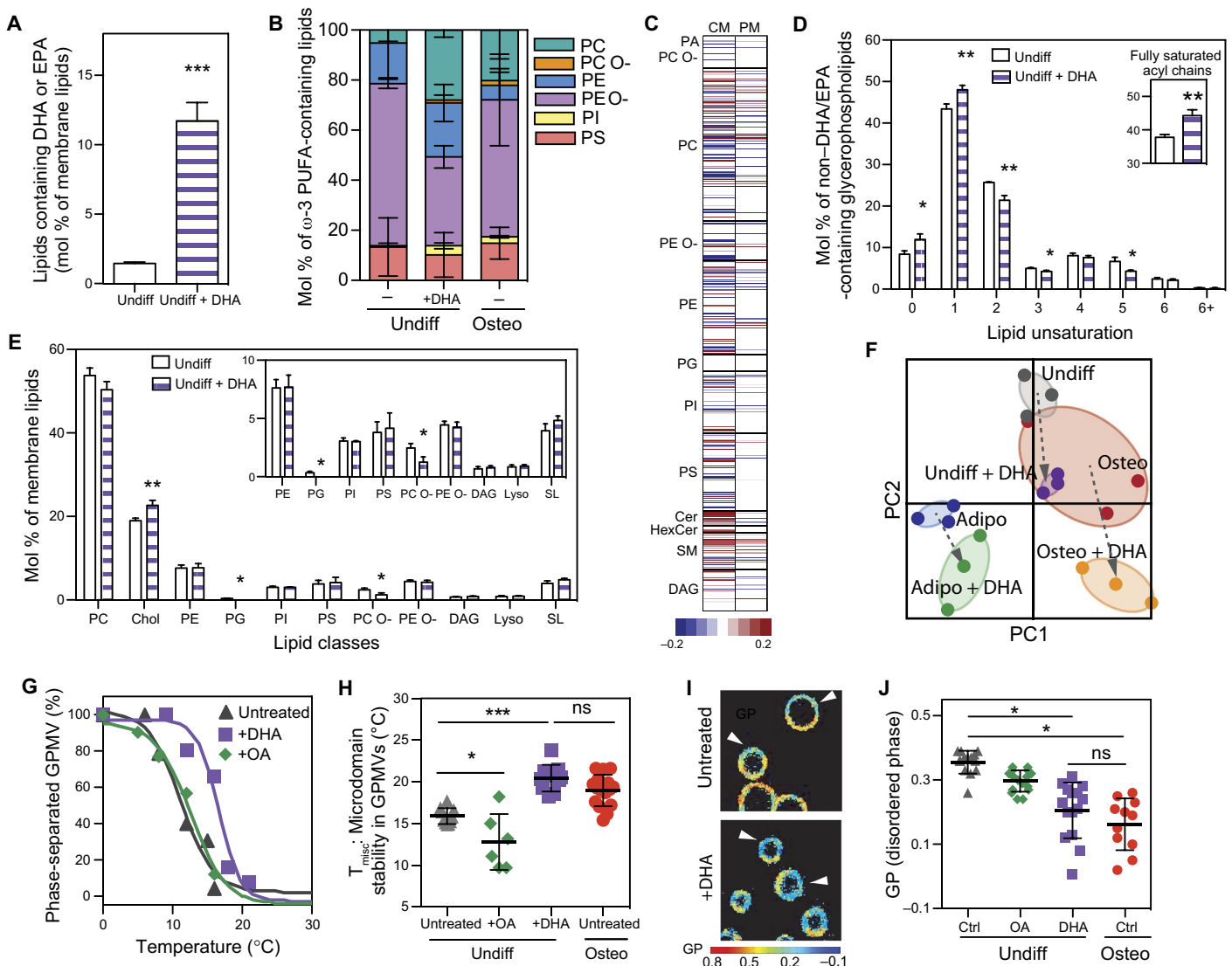


Fig. 2. ω -3 DHA induces biophysical and biochemical remodeling of MSC membranes. (A) Lipids containing DHA (22:6) or EPA (20:5) are significantly increased in membranes from MSCs supplemented with 20 μ M DHA. (B) Distribution of the GPL classes of ω -3-containing lipids. (C) DHA-induced changes [$\log_{10}(\text{DHA-treated}/\text{untreated})$], with red and blue values indicating an increase and decrease of each individual lipid species, respectively. (D) DHA-induced changes to GPL unsaturation as a mol % of all non-DHA/EPA-containing GPLs. Inset shows mol % of saturated acyl chains in non-DHA/EPA-containing lipids. (E) Effect of DHA supplementation on lipid classes in MSC membranes. (F) Unbiased PCA of the lipid structural class and features in the three cell lineages (untreated and DHA-supplemented). Circles represent individual human donors. Table S3 shows the variable loadings defining PC1 and PC2. The dashed lines represent the effects of DHA. (G) Temperature-dependent phase separation in GPMVs isolated from MSCs as a function of supplementation with DHA or OA. (H) Comparison between T_{misc} for fatty acid-supplemented undifferentiated MSCs and those induced to differentiate into osteoblasts. ns, not significant. (I) C-laurdan GP images of isolated GPMVs from untreated and DHA-supplemented MSCs. (J) Quantification of the GP of the disordered microdomains. Data in (A) to (F) are means $\pm$ SD for three human donors. Data in (G) to (H) are means $\pm$ SD for at least five human donors. Data plotted in (J) are means $\pm$ SD of individual vesicles from one representative experiment of three independent trials. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

transduction at the PM (8). To probe the mechanisms underlying the promotion of osteogenesis by DHA-induced PM remodeling, we performed unbiased systems-level transcriptomic and proteomic analysis. Genome-wide transcriptional analysis revealed a significant positive correlation (Pearson's $r = 0.51$; $P < 0.001$) between gene regulation triggered by DHA supplementation and osteogenesis (Fig. 4A). This result was remarkable because there was no a priori expectation that these two unrelated stimuli would have similar effects on gene expression. The slope of the correlation (DHA/osteogenesis = 0.32) reveals that, although the transcriptional signatures of the two stimuli are qualitatively

similar, the effects of DHA supplementation were $\sim 30\%$ of those induced by osteogenic differentiation, as might be expected if DHA is potentiating osteogenic signaling rather than directly inducing it. Notably, peroxisome proliferator-activated receptor (PPAR)-mediated gene expression was unaffected by either perturbation, suggesting that DHA-mediated agonism of this nuclear receptor family is unlikely to account for our observations (table S4).

To identify the DHA-affected genes associated with the promotion of osteogenic differentiation, we focused on those with $\geq 30\%$ change in expression in the same direction (that is, up- or down-regulated by both

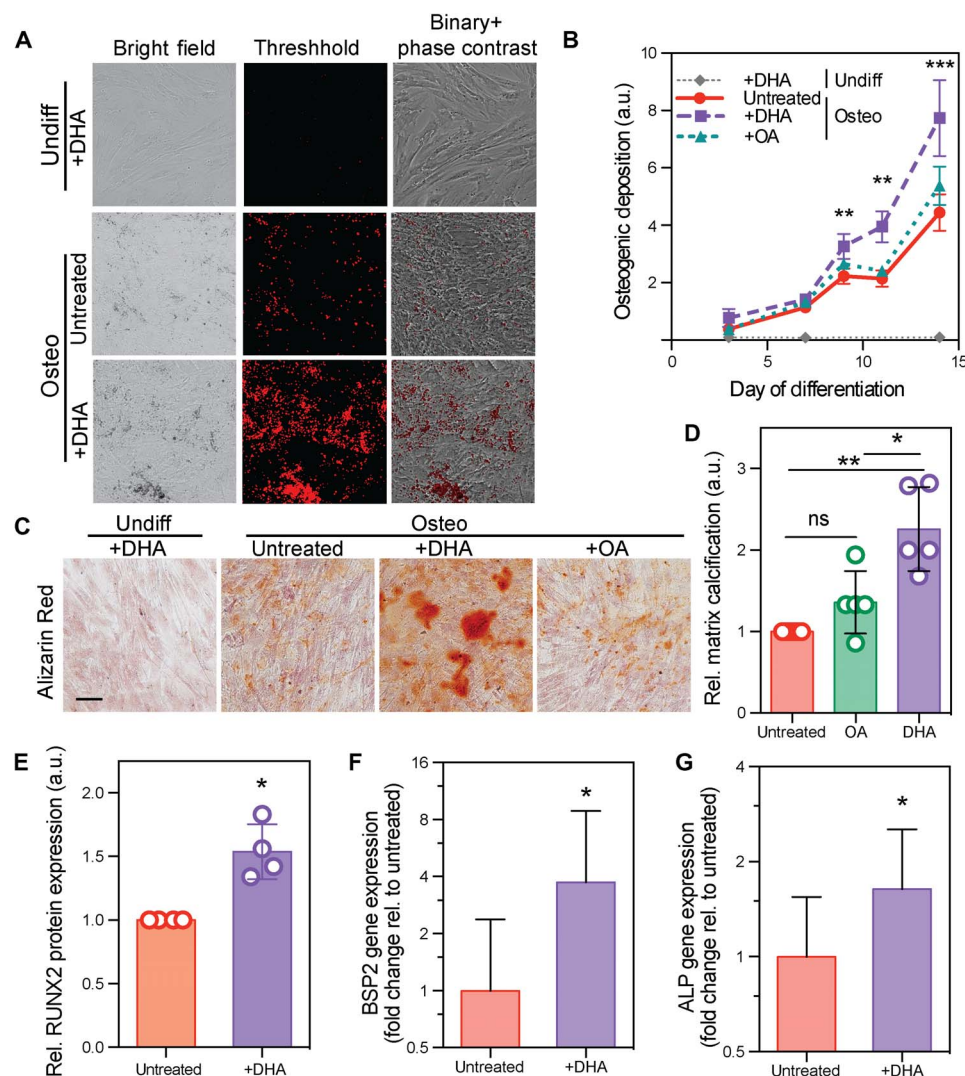


Fig. 3. Supplementation with DHA potentiates osteogenic differentiation. (A and B) The extent of osteogenesis was followed during differentiation by automated image processing of ossified matrix deposition. (A) Bright-field images (left) were automatically thresholded (middle) to calculate the area covered by deposited osteogenic matrix (red overlay). (B) Effect of fatty acid supplementation on osteogenic deposition by osteoblast-differentiated MSCs as a function of differentiation time. Significances shown are unpaired *t* tests for individual time points (+DHA compared to untreated). (C) Representative images of MSCs stained with Alizarin Red S to visualize insoluble calcium deposits after 14 days of osteogenic differentiation with or without fatty acid supplementation. (D) Automated quantification of Alizarin Red staining as a function of fatty acid supplementation. (E) Protein expression of the osteogenic master regulator transcription factor RUNX2 is enhanced by DHA supplementation; Western blotting of protein expression 3 days after induction of differentiation with or without concomitant DHA supplementation. (F and G) Transcription of osteoblast markers (F) BSP2 and (G) ALP is enhanced by DHA, as measured by qPCR at 14 days after induction of differentiation. (B and D) Data are means $\pm$ SD of $n > 4$ independent experiments for at least three human donors. (E) Data are means $\pm$ SD of $n = 3$ experiments on two human donors. (F and G) Data are means $\pm$ 95% confidence interval of $n = 5$ independent experiments on three human donors. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

DHA and osteogenic differentiation). Of the ~47,000 probes, ~1400 changed by at least 30% in DHA feeding and ~3800 by osteogenic differentiation. Among these, 816 were found in both groups (Fig. 4B), 4.5-fold more than would be expected from random overlap. We analyzed the biological processes associated with these mutually varying genes and noted significant enrichment not only in categories associated with cell differentiation and bone development (consistent with DHA-mediated potentiation of osteogenic differentiation) but also several related to signaling at the PM, including integrin signaling, actin organization, and general signal transduction. Finally, gene set enrichment analysis (GSEA) of genes up-regulated by DHA early in osteogenic differentiation (day 3 after induction) revealed nodes associated with

(i) osteogenesis, including ECM remodeling and TGF β signaling, and (ii) signaling at the PM via integrins, growth factor receptors, GTPases, MAPK, and Akt (Fig. 4C).

To identify the cell signaling modules responsible for the enhancement of osteogenesis by DHA and confirm the pathways identified by GSEA, we used reverse phase protein array (RPPA) to probe the expression and activation state of >200 proteins in early osteogenesis (day 3 after induction). As with transcription, osteogenesis- and DHA-mediated effects on MSC protein expression were correlated across the entire data set (Pearson's $r = 0.44$; fig. S10), further affirming the relationship between DHA supplementation and osteogenic differentiation. Figure 4D (and fig. S14) shows a clustered heatmap of proteins whose abundance

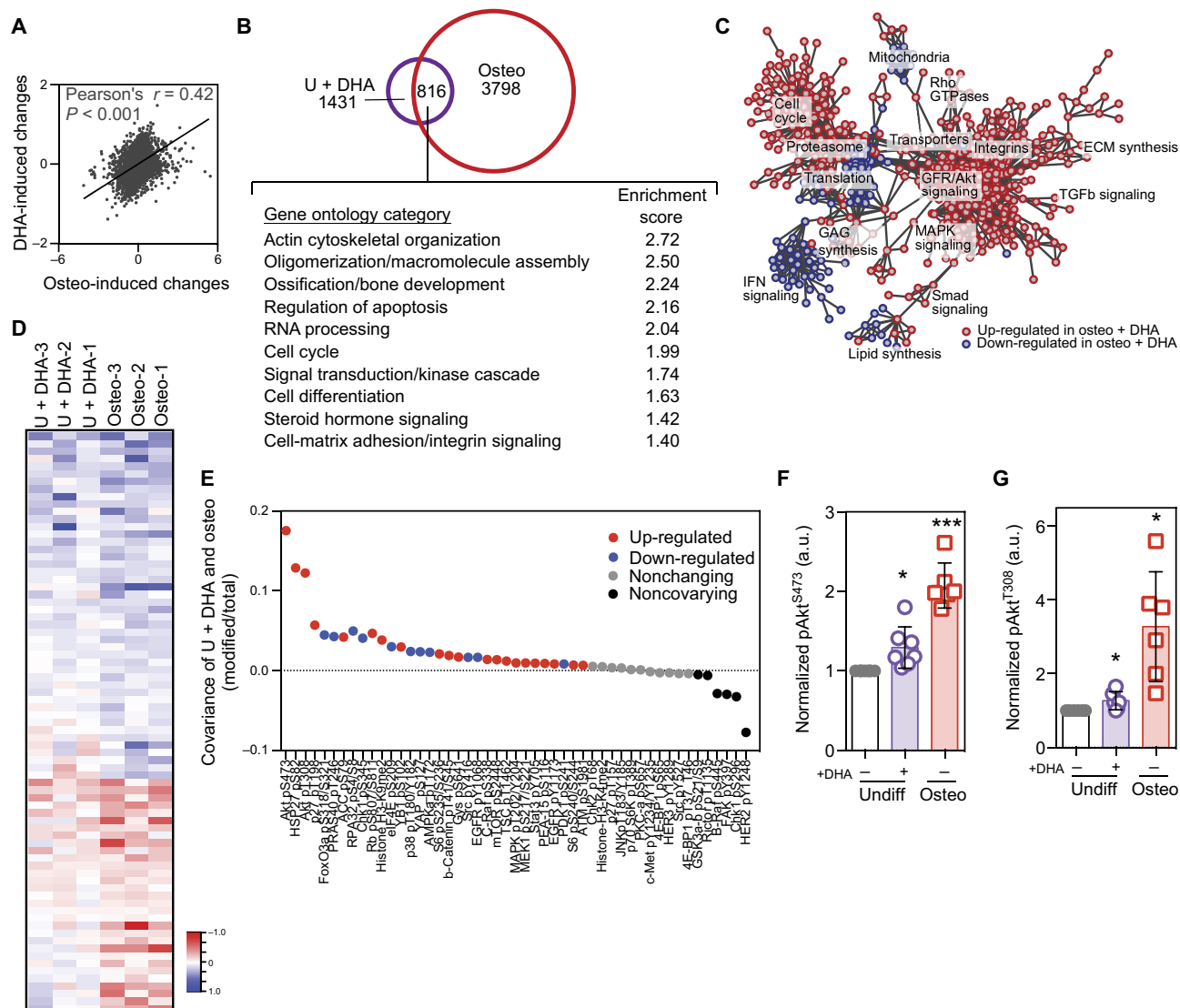


Fig. 4. Supplementation with DHA induces osteogenic gene and protein expression profile via alterations of PM signaling, notably via Akt. (A) Scatterplot of genome-wide changes in gene expression induced by DHA supplementation compared to osteogenic differentiation. The strong transcriptional correlation between these two unrelated stimuli suggests that DHA induces osteogenic signaling in MSCs. **(B)** Genes varying by more than 30% due to either DHA supplementation or induction of osteoblastic differentiation in MSCs show significant overlap. Ontological categorization of these reveals not only enrichments in differentiation and ossification but also signaling and organization of the PM. **(C)** GSEA visualization of gene sets significantly up-regulated (red) or down-regulated (blue) by DHA supplementation in early osteogenic differentiation (day 3). Nodes represent gene sets, and lines represent overlap between gene sets. The labels summarize the pathways or cellular functions that comprise each of the hubs within the network. TGF β , transforming growth factor- β ; GTPases, guanosine triphosphatases; MAPK, mitogen-activated protein kinase; IFN, interferon; GFR, growth factor receptor; GAG, glycosaminoglycan. **(D)** RPPA was performed on lysates from MSCs early in osteogenesis (day 3 of induction) or treated with DHA for 3 days. Fold changes over untreated ($\log_2$) are shown for all antibodies showing significantly ($P < 0.05$) different responses with osteogenic differentiation. Osteogenic changes parallel those induced by DHA across three human donors, illustrating the robustness of the result. **(E)** Covariance (see Materials and Methods for calculation details) between DHA- and osteogenesis-induced posttranslational modifications to signaling proteins (modified/total protein). Red and blue dots indicate signaling modifications that are up- or down-regulated, respectively, by both DHA and osteogenesis. Gray dots indicate those not changing with either treatment. Black dots represent those varying in opposing directions (for example, up-regulated in osteogenic and down-regulated in DHA). **(F and G)** Quantification of Western blots for phosphorylated Akt [Ser⁴⁷³ (F) and Thr³⁰⁸ (G); normalized to total Akt] 3 days after DHA supplementation or osteogenic induction. Data are means $\pm$ SD of at least four independent experiments on three human donors.

was significantly ($P < 0.05$) affected by induction of osteogenic differentiation, highlighting the similarity to the effect of DHA on undifferentiated MSCs.

The RPPA includes a number of probes specific for posttranslationally modified signaling proteins, allowing the analysis of signaling pathway activation. Remarkably, as for gene and protein expression data,

there was a broad similarity between signals activated by DHA supplementation and osteogenic differentiation, as revealed by the positive covariance between these treatments for most evaluated probes (Fig. 4E). The most strongly covarying protein modification was up-regulation of Akt phosphorylation at both activation sites (S473 and T308). Western blotting of phosphorylated Akt (pS473 and pT308) relative to total

Akt validated the observations from the RPPA analysis, because DHA feeding and osteogenesis both significantly enhanced Akt phosphorylation (Fig. 4, F and G, and fig. S11, B and C). It is somewhat surprising that the enhanced Akt phosphorylation induced by DHA supplementation did not affect adipogenic differentiation (fig. S8), given the important role of Akt in regulating adipocyte differentiation (54). We speculate that the differential effects of membrane remodeling on MSC differentiation will be highly context-dependent; in our experiments, the “context” is set largely by the introduction of differentiation media (please see extended discussion in the legend to fig. S8).

Enhanced Akt activation is correlated with DHA-stabilized membrane microdomains

Together, these transcriptomic, proteomic, and biochemical analyses revealed broad similarities between DHA-mediated membrane remodeling and osteogenic MSC differentiation, particularly prominent in their mutual up-regulation of Akt phosphorylation. These results suggested that the DHA-induced membrane phenotype may enhance osteogenesis by up-regulating Akt activation, consistent with the indispensable role for Akt signaling in MSC osteogenesis (54, 55). An obligate step in Akt activation by phosphorylation is its recruitment to the PM (56, 57) and oligomerization (58). To determine whether DHA feeding affected Akt at the PM, we used quantitative transmission electron microscopy (TEM) to evaluate the abundance and nanoscopic or-

ganization of an Akt probe in intact cell PMs. Specifically, PM sheets from baby hamster kidney (BHK) cells expressing the green fluorescent protein (GFP)-tagged PH domain of Akt (PH-Akt) were labeled with anti-GFP antibodies coupled to gold nanoparticles (switching cell types was necessitated by technical limitations with efficiently transfecting primary MSCs; we confirmed DHA-mediated microdomain stabilization in PMs isolated from BHKs; fig. S13A). TEM imaging revealed that DHA induced a significant increase in the abundance of PM-localized PH-Akt (Fig. 5A and fig. S13B). Further, the organization of Akt in the PM was determined using spatial statistics to analyze the immunogold point patterns, with the maximum value of the univariate K -function ($L_{\max}$) used to quantify the extent of clustering. Independent of the increased abundance, DHA increased the nanoscale clustering of PH-Akt in the PM (Fig. 5, B and C), consistent with previous observations suggesting that Akt clustering is associated with enhanced activation (58). Enhanced PH-Akt clustering correlates with our observations of DHA-stabilized membrane microdomains in isolated PMs (Fig. 2, G and H), suggesting that these two effects may be related, that is, enhanced phase separation in GPMVs is reflective of more stable nanoscopic domains in live cells, which serve to concentrate Akt. This inference is supported by the fact that we observed higher Akt activity in raft microdomains (see below and Fig. 5D). However, it should be emphasized that the relationship between the stability of phase separation in GPMVs and the stability of nanodomains in live cells remains hypothetical (59).

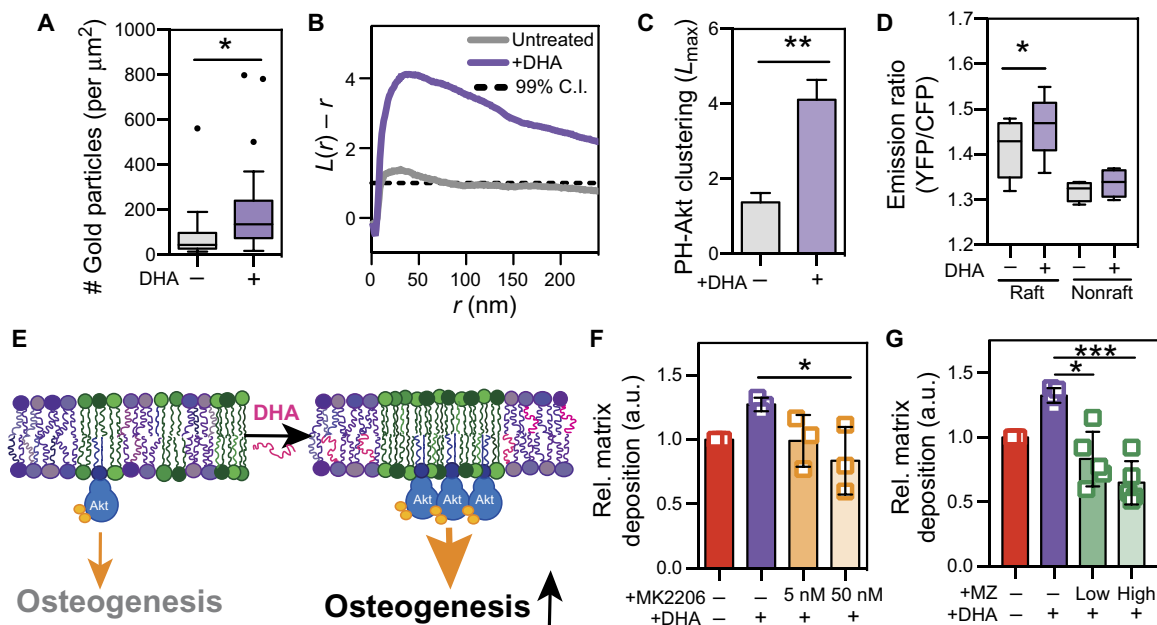


Fig. 5. DHA enhances Akt activation at the PM. (A to C) PM sheets of BHK cells expressing GFP-PH-Akt were labeled with anti-GFP gold nanoparticles and imaged by TEM. (A) The abundance of Akt on the PM is shown by box-and-whisker plots of gold particles per square micrometer. (B and C) Spatial mapping of GFP-PH-Akt distribution on the same PM sheets. (B) Representative weighted mean K -function $L(r) - r$, where values above the 99% confidence interval (C.I.) (dashed line) indicate nonrandom clustering of the lipid probe. (C) Peak $L(r) - r$ values, $L_{\max}$, derived from K -function curves [as in (B)] confirm enhanced nanoclustering of PH-Akt induced by DHA. (D) BHK cells expressing Lyn-AktAR (Raft) and Kras-AktAR (Nonraft) treated with 20 μ M DHA for 3 days and imaged by confocal microscopy with an excitation of 405 nm and emission in the range of 465 to 500 nm [cyan fluorescent protein (CFP)] and 515 to 550 nm [yellow fluorescent protein (YFP)]. YFP/CFP emission ratio (reflective of extent of FRET and thus Akt activation) for at least 25 cells per condition is shown. Data are means $\pm$ SD of four independent experiments. (E) Model for DHA-mediated promotion of osteogenesis. DHA induces membrane remodeling and stabilized raft microdomains, which leads to increased Akt abundance and clustering at the PM, thus enhancing Akt activation to potentiate osteogenic differentiation. (F) Relative osteogenic matrix deposition after 7 days of MSC differentiation in the presence of DHA and/or MK2206 (a specific Akt inhibitor), as in Fig. 3 (A and B). Data are means $\pm$ SD of three independent experiments on two human donors. (G) Relative osteogenic matrix deposition after 7 days of MSC differentiation in the presence of DHA and/or myriocin + Zaragozic acid (MZ) (low, 10 μ M myriocin/5 μ M Zaragozic acid; high, 25 μ M myriocin/5 μ M Zaragozic acid). Data are means $\pm$ SD of four independent experiments on two human donors. Significances in (A) and (C) are unpaired t tests, in (D) are paired t tests compared to untreated cells, and in (E) and (F) are paired t tests compared to DHA-treated cells. *** P < 0.001, ** P < 0.01, * P < 0.05.

To support the connection between enhanced microdomain stability in GPMVs and DHA-mediated enhancement of Akt activation and osteogenic differentiation in MSCs, we tested the effects of DCA on Akt activation. As previously noted, DCA enhanced microdomain stability in GPMVs to a level similar to DHA (see fig. S9A). This effect also correlated with similar effects on Akt activation because both RPPA (fig. S11A) and Western blotting (figs. S11, B and C, and S12) revealed significant up-regulation of Akt phosphorylation by DCA in both undifferentiated MSCs and differentiated osteoblasts. As noted above, DCA treatment also significantly enhanced osteogenic differentiation (fig. S9), supporting the inference that both DCA and DHA may promote osteogenic differentiation by stabilizing membrane microdomains and thereby promoting Akt activation.

The above observations are consistent with previous demonstrations that Akt membrane recruitment, oligomerization, and activation are regulated by raft membrane microdomains across a variety of cell types (60–63). To directly evaluate the effect of DHA on Akt activity in the PMs of living cells, we transfected BHK cells with fluorescence resonance energy transfer (FRET)-based Akt activity reporters (AktARs). By targeting AktAR to either raft (Lyn-AktAR) or non-raft (Kras-AktAR) domains, as previously described (61), we could independently investigate the effects of membrane remodeling on Akt activity in distinct membrane subdomains. We observed greater phosphorylation of the AktAR in raft microdomains [consistent with a previous report by Gao and Zhang (61)], and this effect was further enhanced by DHA supplementation (Fig. 5D).

These observations suggest a model (Fig. 5E) wherein DHA-mediated membrane remodeling stabilizes raft microdomains, which enhance Akt activation, which in turn promotes osteogenic differentiation. We tested this model by chemical inhibition of either Akt activity or lipids associated with membrane microdomains. The specific Akt inhibitor MK2206 abolished the effect of DHA on increased osteogenic matrix deposition in a dose-dependent fashion (Fig. 5F), confirming that Akt activity is essential for DHA-mediated potentiation of osteogenesis. Raft microdomains were disrupted by a combination of small-molecule inhibitors designed to inhibit the formation of their core lipid components; namely, myriocin was used to inhibit sphingolipid synthesis and Zaragozic acid to inhibit cholesterol synthesis. This combinatorial treatment was previously demonstrated to disrupt domain formation without affecting cellular phospholipid composition (62, 64). We confirmed that this treatment reduced the phase separation temperature in GPMVs (fig. S13A) and significantly reduced Akt activity in raft domains (fig. S13C), both consistent with the conclusion that it disrupted raft domains. Finally, the combined myriocin + Zaragozic acid (MZ) treatment abrogated DHA-mediated enhancement of osteogenic differentiation in a dose-dependent manner (Fig. 5G), suggesting enhanced Akt activation by microdomain stabilization as a possible mechanism for DHA-mediated promotion of osteogenic differentiation.

Conclusion

In summary, the membrane phenotype of MSCs is highly flexible and susceptible to both cell-autonomous and exogenous regulation. MSC differentiation leads to lineage-specific lipidomic remodeling, resulting in unique membrane phenotypes for osteoblasts and adipocytes, which may reflect the specific membrane demands of these disparate cell types. The distinct membrane features of each cell type instructed strategies for tuning lineage specification in MSCs by rational remodeling of the PM. Specifically, supplementing cells with the osteoblast-characteristic die-

tary component ω -3 DHA induced lipidomic and biophysical changes that mimic aspects of the osteoblastic membrane phenotype. These lipid-induced changes to the PM phenotype altered signaling at the PM and potentiated osteogenic differentiation in MSCs. We identified Akt phosphorylation as a convergent node in DHA-mediated promotion of osteogenesis and showed that enhanced Akt activation, potentially associated with more stable membrane microdomains, was essential for the effect of DHA on osteogenesis; however, we note that other microdomain-dependent PM pathways are likely involved in this process. These findings suggest a potential mechanism for the many profound physiological effects of dietary lipids, including the previously reported links between DHA and bone health (16–20). Namely, dietary fats may influence cell signaling and physiology by incorporating into lipids and remodeling membrane biochemical, biophysical, and therefore functional phenotypes.

MATERIALS AND METHODS

Materials

MK2206, zaragozic acid, and myriocin were obtained from Cayman Chemical. Oil Red O and Alizarin Red stainings were bought from Sigma-Aldrich. The Alkaline Phosphatase Assay Kit was purchased from Abcam. Antibodies used were as follows: caveolin (polyclonal; Santa Cruz Biotechnology), Na^+ , K^+ adenosine triphosphatase (polyclonal; Cell Signaling), calnexin (polyclonal; Abcam), GM130 (polyclonal; Cell Signaling), LAMP1 (polyclonal; Abcam), RUNX2 (polyclonal; Cell Signaling), GOLGA7 (monoclonal; Abcam), Sec61a (Abcam), hexokinase II (polyclonal; Cell Signaling), phospho-Akt (pS473; polyclonal; Cell Signaling), phospho-Akt (pS473; monoclonal; Cell Signaling), and Akt (polyclonal; Cell Signaling). The plasmids for Lyn-AktAR and Kras-AktAR were provided by J. Zhang (University of California, San Diego). The plasmid for GFP-PH-Akt was a gift from T. Balla [National Institutes of Health (NIH)].

Cell culture

BHK cells were maintained in 10% fetal bovine serum (FBS) in Eagle's minimum essential medium containing 1% penicillin-streptomycin. Human adult bone marrow MSCs were obtained as a gift from M. Andreeff (MD Anderson). They were isolated by bone marrow aspirates from the iliac crest of normal, healthy volunteers after informed consent. Mononuclear cells were collected by gradient centrifugation and seeded at a density of 1×10^5 cells/cm² in growth medium containing minimum essential medium α , 20% heat-inactivated FBS, and 1% L-glutamine. Nonadherent cells were removed after 2 days. Medium was changed every 3 to 4 days thereafter. When the cells reached 70 to 80% confluence, adherent cells were trypsinized and expanded for 3 to 5 weeks. MSCs were checked for positivity of CD105, CD73, and CD90 and the lack of expression of CD45 and CD34 (65). The purity of MSC preparation was >99%. Cells were maintained in growth medium containing Dulbecco's modified Eagle's medium with 1% L-glutamine, 10% FBS, and 1% penicillin-streptomycin and analyzed up to passage 6. For all results shown, three or more different human donors were compared, unless otherwise stated.

For osteogenic or adipogenic differentiation, cells were plated at approximately 5×10^3 cells/cm², and differentiation medium was added the following day, for up to 20 days. Osteogenic medium consisted of MSC growth medium supplemented with 50 μM ascorbate 2-phosphate, 10 μM β -glycerol phosphate, and 100 μM dexamethasone. Adipogenic medium consisted of MSC growth medium supplemented with 1 μM

dexamethasone, 500 μ M isobutylmethylxanthine, 100 μ M indomethacin, and insulin (1 μ g/ml).

For all experiments with fatty acid (DHA, EPA, or OA) supplementation, cells were incubated with 20 μ M fatty acid for the indicated time periods. This concentration was chosen to approximate conditions observed *in vivo* under DHA-enriched diets in mammals: plasma free fatty acid concentrations ranged from 300 to 750 μ M (24, 66), and up to 10 mol % of plasma fatty acids were ω -3 DHA in rats fed a high-fish oil diet (24). Further, diets rich in ω -3 PUFAs led to significant incorporation of these fats into cell membrane lipids (23, 24, 65), similar to the levels we observed under our culture feeding conditions (Fig. 2A). For these reasons, we believe that our culture conditions reasonably approximate physiological dietary membrane perturbations. No notable cell toxicity was observed for any of the culture conditions or inhibitor treatments. Fatty acids (Sigma-Aldrich) were received as pure liquid oils in hermetically sealed ampules. Fatty acid stock solutions (2 mM) were prepared by stirring the fatty acid with 1 mM bovine serum albumin (BSA) (in water), sterile-filtering, and purging with nitrogen before aliquoting and storage at -80°C . These steps were taken to guard against oxidation of the PUFAs during storage.

T_{misc} measurements

GPMVs were isolated and imaged as described (26), with T_{misc} values calculated as previously (30, 32, 67). Briefly, cells were washed with phosphate-buffered saline (PBS) and stained with FAST DiO (5 μ g/ml) (Invitrogen), a green fluorescent lipid dye that strongly partitions to the disordered phase, for 10 min on ice. Then, the cells were washed twice with GPMV buffer [10 mM Hepes, 150 mM NaCl, and 2 mM CaCl_2 (pH 7.4)] and incubated with 25 mM paraformaldehyde (PFA) and 2 mM dithiothreitol for 1 hour at 37°C . GPMVs were decanted and placed in a temperature-regulated imaging chamber, as described (26). GPMVs were imaged from 4° to 28°C , counting phase-separated and uniform vesicles at each temperature. For each temperature, 25 to 50 vesicles were counted, and the percentage of phase-separated vesicles was calculated, plotted versus temperature, and fitted to a sigmoidal curve to determine the temperature at which 50% of the vesicles were phase-separated (T_{misc}).

C-laurdan spectroscopy

Membrane order was determined via C-laurdan spectroscopy, as described (26). Briefly, cells were washed with PBS and stained with C-laurdan (20 μ g/ml) (gift from B. R. Cho) for 10 min on ice. After this, the cells were washed twice with GPMV buffer [10 mM Hepes, 150 mM NaCl, and 2 mM CaCl_2 (pH 7.4)] and incubated with 15 mM *N*-ethylmaleimide (NEM) for 1 hour at 37°C to isolate GPMVs. GPMVs were decanted away from the cells and then concentrated by centrifugation (20,000g for 1 hour at 4°C). GPMVs were resuspended in 100 μ l of GPMV buffer, and the emission spectrum was measured at 23°C from 400 to 550 nm with excitation at 385 nm. The GP was calculated according to the following equation

$$\text{GP} = \frac{\sum_{420}^{460} I_x - \sum_{470}^{510} I_x}{\sum_{420}^{460} I_x + \sum_{470}^{510} I_x} \quad (1)$$

C-laurdan microscopy

Cells were stained with C-laurdan, and GPMVs were prepared as above. Instead of centrifugation, GPMVs were allowed to sediment, then

collected from the bottom of microcentrifuge tubes, and imaged via confocal microscopy at $60\times$ magnification with excitation by a 405-nm laser on a Nikon A1R confocal microscope with a spectral imaging detector. The emission was collected in two channels: 433 to 463 nm corresponding to the ordered channel and 473 to 503 nm corresponding to the disordered channel. The GP images in Fig. 3E were generated by subtracting the background, thresholding the image to reduce noise (keeping only pixels that are greater than 3 SDs of the background value), and then calculating the GP at each pixel using the equation above from the two-dimensional fluorescence intensity matrices. For each image, the average GP of the coexisting phases (disordered and ordered) was calculated by averaging the pixels within a region of interest representing a clearly distinct ordered or disordered domain. A minimum of 10 vesicles per experiment was quantified, and three independent experiments were performed.

Membrane isolation for lipidomics

All lipidomic data sets are included in the Supplementary Materials. For lipidomic analysis, GPMVs were prepared at either 14 or 21 days of differentiation or 4 days of treatment with DHA. Cells were washed with GPMV buffer and incubated with 15 mM NEM in GPMV buffer for 1 hour at 37°C . GPMVs were decanted, and the suspension was centrifuged at 100g for 5 min to remove cell debris. The supernatant was then centrifuged at 20,000g for 1 hour at 4°C , and then the GPMV-containing pellet was resuspended in 150 mM ammonium bicarbonate.

To prepare CM fractions, cells were washed with PBS and scraped in hypotonic buffer [10 mM tris (pH 7.4)]. The cells were then homogenized with a 27-gauge needle, and the nuclei were pelleted by centrifugation at 300g for 5 min. The supernatant was pelleted by centrifugation at 100,000g for 1 hour at 4°C . The membrane pellet was then resuspended in 150 mM ammonium bicarbonate.

Lipidomics by ESI-MS/MS

Lipidomics was performed at Lipotype GmbH, as described previously (1, 2, 68, 69). Lipidomes were prepared from at least three individual human donors for all experiments using the following procedures.

Nomenclature

The following lipid names and abbreviations are used: Cer, ceramide; Chol, cholesterol; DAG, diacylglycerol; HexCer, glucosyl/galactosyl ceramide; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; and their respective lysospecies lyso-PA (LPA), lyso-PC (LPC), lyso-PE (LPE), lyso-PI (LPI), and lyso-PS (LPS); and their ether derivatives PC O-, PE O-, LPC O-, and LPE O-; SE, sterol ester; SM, sphingomyelin; SLs, sphingolipids; and TAG, triacylglycerol. Lipid species were annotated according to their molecular composition as follows: [lipid class]-[sum of carbon atoms in the fatty acids]:[sum of double bonds in the fatty acids]:[sum of hydroxyl groups in the long-chain base and the fatty acid moiety] (for example, SM-32:2;1). Where available, individual fatty acid composition following the same rules is given in brackets (for example, 18:1;0-24:2;0).

Lipid extraction for MS lipidomics

MS-based lipid analysis was performed by Lipotype GmbH (Dresden, Germany), as described (2). Lipids were extracted using a two-step chloroform/methanol procedure (68). Samples were spiked with internal lipid standard mixture containing cardiolipin (CL), 16:1/15:0/15:0/15:0; Cer, 18:1/2/17:0; DAG, 17:0/17:0; HexCer, 18:1/2/12:0; LPA, 17:0; LPC, 12:0; LPE, 17:1; LPG, 17:1; LPI, 17:1; LPS, 17:1; PA, 17:0/17:0; PC, 17:0/17:0; PE, 17:0/17:0; PG, 17:0/17:0; PI, 16:0/16:0; PS, 17:0/17:0;

cholesterol ester (CE), 20:0; SM, 18:1;2/12:0;0; TAG, 17:0/17:0/17:0; and Chol. After extraction, the organic phase was transferred to an infusion plate and dried in a speed vacuum concentrator. First-step dry extract was resuspended in 7.5 mM ammonium acetate in chloroform/methanol/propanol (1:2:4, v/v/v), and second-step dry extract was resuspended in 33% ethanol solution of methylamine/chloroform/methanol (0.003:5:1, v/v/v). All liquid handling steps were performed using Hamilton Robotics STARlet robotic platform with the Anti-Droplet Control feature for organic solvent pipetting.

MS data acquisition

Samples were analyzed by direct infusion on a Q Exactive mass spectrometer (Thermo Scientific) equipped with a TriVersa NanoMate ion source (Advion Biosciences). Samples were analyzed in both positive and negative ion modes with a resolution of 280,000 at $m/z = 200$ for MS and 17,500 for MS/MS experiments in a single acquisition. MS/MS was triggered by an inclusion list encompassing corresponding MS mass ranges scanned in 1-Da increments (69). Both MS and MS/MS data were combined to monitor CE, DAG, and TAG ions as ammonium adducts; PC and PC O⁻ as acetate adducts; and CL, PA, PE, PE O⁻, PG, PI, and PS as deprotonated anions. Only MS was used to monitor LPA, LPE, LPE O⁻, LPI, and LPS as deprotonated anions; Cer, HexCer, SM, LPC, and LPC O⁻ as acetate adducts; and Chol as an ammonium adduct of an acetylated derivative (70).

Data analysis and postprocessing

Data were analyzed with in-house developed lipid identification software based on LipidXplorer (71). Data postprocessing and normalization were performed using an in-house developed data management system. Only lipid identifications with a signal-to-noise ratio of >5 and a signal intensity fivefold higher than in corresponding blank samples were considered for further data analysis.

Lipidomic data processing

The lipidomic analysis yielded a list of >600 individual lipid species and their picomolar abundances. These were processed by first transforming into mol % of all lipids detected. Next, the TAG and SEs were removed from the analysis, and the remaining data were analyzed as mol % of membrane lipids. From here, the data sets were broken down further into class composition. In some cases, the distribution and structural characteristics (for example, the number of carbons or unsaturations in the acyl chains) of the individual species were analyzed. To determine the degree of GPL unsaturation and length, the data were first recalculated as mol % of GPLs (PA, PC, PC O⁻, PE, PE O⁻, PG, PI, and PS). For the total carbon length or unsaturation, the number of carbons or unsaturations from the individual acyl chains of each individual species was combined values (for example, PC 18:1-18:1 contains 36 carbons and 2 unsaturations). Lipids that contained ω -3 PUFAs were defined as those in which a 22:6 and 20:5 fatty acid was detected. These are not the only possible ω -3 PUFAs in mammalian cells, but they are by far the most abundant. Further, they were the only ones that could be definitively identified as ω -3 rather than ω -6 using our lipidomic setup. Thus, the measurements of ω -3-containing lipids may be very slight underestimates. To describe membrane remodeling after DHA supplementation, ω -3 fatty acyl chain-containing lipids (that is, containing a 22:6 or 20:5 acyl chain) were removed from the analysis, and the data were recalculated as mol % of non-DHA/EPA-containing lipids.

PCA was performed using shareware software (Multibase Excel Add-in; www.numericaldynamics.com). All membrane lipids were included in the analysis, with the features included being the major structural features (lipid length and unsaturation) combined with structural

category (sphingolipid, GPLs, glycolipid, and sterol). Running the PCA with more specific lipid categories (for example, including headgroup information such as “PC with two unsaturations”) led to essentially similar separation of the three cell lineages and identification of their defining features. The following abbreviations of structural features were used: GL, glycerolipid; GPL, glycerophospholipid; SL, sphingolipid; C, number of carbons per lipid; DB, number of double bonds/unsaturations per lipid.

Osteogenic morphology quantification

Osteogenic nodules (areas of insoluble calcified matrix deposition) are obviously detectable by bright-field microscopy as dark spots on a bright background. Because osteogenic deposition is a direct consequence of osteogenesis, the quantification of these nodules was used as a proxy for osteogenic differentiation. Bright-field images were taken at indicated times after osteogenic medium ($\pm$ fatty acids) was added to the cells. The bright-field images were automatically processed by thresholding the dark spots and calculating the area covered by thresholded pixels for each image. Thresholded areas from a matrix of nine systematically assigned locations per treatment per experiment were summed.

Adipogenic morphology quantification

The bright-field images were automatically processed by contrast thresholding, which clearly identified accumulated lipid droplets (fig. S8A). The area covered by pixels above the intensity threshold was calculated for each image and used as a correlate for the extent of adipogenic differentiation. Thresholded areas from a matrix of five to nine systematically assigned locations per treatment per experiment were averaged.

Alizarin Red S staining

Cells were washed briefly with PBS, fixed with absolute ethanol for 30 min at room temperature, and allowed to dry completely. Alizarin Red stain (2%, w/w; in distilled water, pH 4.1; Sigma-Aldrich) was added to the wells and incubated for 15 min. The wells were then carefully washed three times with distilled water and allowed to dry before imaging. To quantify Alizarin Red staining, five random images per well per experiment were taken at 10 $\times$ magnification and analyzed by an automatic image processing protocol. The images were thresholded using a color hue/saturation/intensity threshold, and the area of the image covered by the thresholded pixel (that is, positive Alizarin Red staining) intensity was measured.

ALP activity

ALP activity was measured following kit instructions (Abcam ALP Fluorometric kit).

Oil Red O staining

Oil Red O (185 mg) was dissolved in 50-ml isopropanol, stirred overnight at 4°C, and filtered through Whatman paper. Immediately before use, Oil Red O was diluted in water (3:2) and filtered again. Cells were washed with PBS and fixed in 4%PFA on ice for 1 hour. After washing with PBS, the cells were washed briefly with 60% isopropanol and incubated with the diluted Oil Red O solution for 5 min at room temperature. The wells were washed with PBS before imaging.

Western blot

Undifferentiated MSCs were incubated with or without 20 μ M DHA for 3 days. Cells washed with ice-cold PBS were scraped into Laemmli lysis

buffer [50 mM tris-HCl (pH 8.0), 2% SDS, and 5 mM EDTA (pH 8.0)] supplemented with protease inhibitor cocktail. Protein concentration was determined using a bicinchoninic acid assay (Pierce), and equal amounts of protein were mixed with reducing Laemmli sample buffer and loaded onto SDS–polyacrylamide gel electrophoresis gels. Gels were transferred to polyvinylidene difluoride membranes, which were blocked in either 5% BSA in tris-buffered saline + 0.1% Tween 20 (TBST) or 5% milk in TBST. Membranes were incubated with primary antibodies overnight on a rocker at 4°C, washed with TBST, and incubated with either Alexa Fluor or horseradish peroxidase–tagged secondary antibodies for 1 hour before visualization. Membranes were imaged using a Bio-Rad ChemiDoc imager. The intensity of the bands was quantified, normalized to actin or calnexin, and plotted as a relative mean $\pm$ SD of $n \geq 3$ experiments. One-sample *t* test analysis was performed to test for significance.

Real-time qPCR

Total RNA was isolated via TRIzol (Sigma-Aldrich) following the manufacturer's protocol. Reverse transcription PCR was performed on these samples using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems according to the manufacturer's protocol. To quantify mRNA expression, SYBR Fast Master Mix (2 $\times$) Universal Dye (#KK4602) from Kapa Biosystems was used in the Eppendorf RealPlex2 Mastercycler. Each primer set for each sample was run in triplicate with 1 ng of complementary DNA (cDNA) per well. The primer sets used are as follows: *GAPDH*, 5'-CTATAAATTGAGCCCG-CAGCC-3' (forward) and 5'-ACGACCAAATCCGTTGACTCC-3' (reverse); *ALP*, 5'-CTGGGCTCCAGGGATAAAGC-3' (forward) and 5'-GTGGAGACACCCATCCCATC-3' (reverse); *BSP2*, 5'-CAATCTGTGC-CACTCACTGC-3' (forward) and 5'-GGCCTGTACTTAAAGACCC-CA-3' (reverse); *CEBPa*, 5'-CCTTGTGCCTTGGAAATGCAAC-3' (forward) and 5'-CTGCTCCCTCCTTCTCTCA-3' (reverse); *LPL*, 5'-CCGCCGACCAAGAAGAGAT-3' (forward) and 5'-TAGCCACG-GACTCTGCTACT-3' (reverse).

Expression changes were calculated using the $\Delta\Delta C_T$ method. The data were standardized using a previously published protocol (72), in which the average fold change was log-transformed and mean-centered, and confidence intervals were determined to evaluate statistical significance.

Microarray analysis

Each sample (100 ng) was hybridized on the Illumina HumanHT-12 v4 Expression BeadChip and scanned at the University of Texas Health Science Center Quantitative Genomics and Microarray Core Lab. IlluminaExpressionFileCreator module in GenePattern was used to preprocess the raw data without background subtraction, and then the data were quantile-normalized (73). For the scatterplot in Fig. 4A, osteogenic- and DHA-induced gene expression was calculated by subtracting the signal in untreated, undifferentiated MSCs from the treated sample (that is, osteogenic or U+DHA). For the Venn diagram in Fig. 4B, the genes varying by $\geq 30\%$ in either condition are plotted. The 816 genes that varied in both osteogenic- and DHA-induced gene expression were first filtered to select only those that varied in the same direction in both DHA and osteogenic (that is, either both up or both down; 757 of 816) and then analyzed using DAVID (74) to determine the most statistically enriched gene ontologies associated with these overlapping genes.

Pathway analysis was performed using the GSEA module available at GenePattern (75, 76) using the C2 curated gene sets in the MSigDB

database version 5.0. To ensure that genes represented by multiple probes on the array are not overweighted in the score, only probes containing the maximum score were used. Network analysis was done with the EnrichmentMap (77) plug-in in Cytoscape. The network consists of the gene sets with significance $P < 0.01$ and false discovery rate < 0.25 . Edges connect pairs of nodes with overlap coefficient > 0.5 .

Changes to PPAR target genes [as annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (78)] were calculated as the difference in expression compared to untreated undifferentiated controls. CEBPa target genes were identified from Lefterova *et al.* (79) as those up-regulated in adipogenesis and contained CEBPa-binding sites within 50 kb. Expression changes to these genes were determined as with PPAR target genes above.

Reverse phase protein array

Cells were lysed after 3 days of treatment (DHA or osteogenic supplementation). Cellular proteins were denatured by 1% SDS (with β -mercaptoethanol) and diluted in five twofold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serially diluted lysates were arrayed on nitrocellulose-coated slides (Grace Bio-Labs) by Aushon 2470 Arrayer. A total of 5808 array spots were arranged on each slide including the spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. Only antibodies with a Pearson correlation coefficient between RPPA and Western blotting of greater than 0.7 were used in the RPPA study. Antibodies with a single or dominant band on Western blotting were further assessed by direct comparison to RPPA using cell lines with differential protein expression or modulated with ligands/inhibitors or small interfering RNA for phosphoproteins or structural proteins, respectively. The signal obtained was amplified using a Dako Cytomation–catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using customized software to generate spot intensity.

Each dilution curve was fitted with a logistic model (“Supercurve Fitting” developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center; <http://bioinformatics.mdanderson.org/OOMPA>). This fits a single curve using all the samples (that is, dilution series) on a slide with the signal intensity as the response variable and the dilution steps as the independent variable. The fitted curve was plotted with the signal intensities—both observed and fitted—on the *y* axis and the $\log_2$ concentration of proteins on the *x* axis for diagnostic purposes. The protein concentrations of each set of slides were then normalized by median polish, which was corrected across samples by the linear expression values using the median expression levels of all antibody experiments to calculate a loading correction factor for each sample.

The heatmap was generated in R of all proteins that were significantly different ($P < 0.05$) in osteogenic cells as compared to untreated cells. The columns represent independent repeats of individual donors presented as $\log_2$ of the changes in expression levels between the supplemented or untreated samples. The covariance of the individual post-translational modifications was calculated by first calculating the modification level relative to total protein expression (for example, AktpS473/Akt). Then, the differences from untreated, undifferentiated cells were calculated for each modification. These differences were multiplied [(U+DHA – Undiff) $\times$ (Osteo – Undiff)] to yield a covariance value for each posttranslational modification.

Immuno-EM spatial mapping

Immuno-EM was conducted as previously described (37, 80, 81). Because of technical limitations in transfecting primary hMSCs, BHK cells were used to image and spatially map Akt localization at the PM. Briefly, intact cell PM sheets of BHK cells expressing the GFP-tagged protein of interest were attached to EM grids, washed with PBS, fixed with 4% PFA and 0.1% glutaraldehyde, labeled with 4.5-nm gold particles coupled to anti-GFP antibody, and embedded in uranyl acetate. Gold particle distribution on the PM sheets was imaged using a JEOL JEM-1400 TEM at $\times 100,000$ magnification, and (x,y) coordinates of each gold particle within a selected $1\text{-}\mu\text{m}^2$ area were determined using ImageJ. The clustering of gold particles was analyzed using variations of Ripley's K -function

$$K(r) = An^{-2} \sum_{i \neq j} w_{ij} 1(\|x_i - x_j\|) \quad (2)$$

$$L(r) - r = \sqrt{\frac{K(r)}{\pi}} - r \quad (3)$$

where $K(r)$ is the univariate K -function for n gold particles in the area A , r is the radius, $\|\cdot\|$ is the Euclidean distance, $1(\cdot)$ is the indicator function that has a value of 1 if $\|x_i - x_j\| \leq r$ and 0 otherwise, and w_{ij}^{-1} is the proportion of the circumference of a circle with center at x_i and a radius $\|x_i - x_j\|$ located within A . $L(r) - r$ is a parameter that described this clustering and was standardized on the 99% confidence interval estimated from Monte Carlo simulations. An $L(r) - r$ value greater than the confidence interval indicates significant clustering, and the maximum value of the function ($L_{\max}$) estimates the extent of clustering. A minimum of 15 PM sheets were imaged and analyzed for each experiment. Differences between replicated point patterns were analyzed by constructing bootstrap tests, as described previously (81), and statistical significance was evaluated against 1000 bootstrap samples. We note that the effects of the rip-off procedure on inositol abundance or phosphorylation state have not been investigated and that these effects may affect the absolute, although not relative, values derived from this technique.

AktAR quantification

BHK cells were treated with $20\text{ }\mu\text{M}$ DHA for 3 days. The cells were then transfected with Lyn-AktAR and AktAR-Kras (61) using Lipofectamine 3000 following the manufacturer's protocol. Six hours after transfection, the medium was changed to include DHA or 25 mM myriocin and 25 mM Zaragozic acid and to remove the Lipofectamine reagent. After 24 hours, cells were imaged at $20\times$ magnification with excitation by a 405-nm laser on a Nikon A1 confocal microscope. The emission was collected in two channels: 465 to 500 nm corresponding to CFP and 515 to 550 nm corresponding to YFP. Regions of interest were drawn around individual cells, and the ratio of YFP to CFP was calculated per cell. At least 25 cells per construct per condition were imaged and averaged. Four independent experiments were performed.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/3/11/eaao1193/DC1>

fig. S1. hMSCs differentiate into adipogenic and osteogenic lineages.

fig. S2. GPMVs prepared from hMSCs.

fig. S3. Validation of GPMVs as PM-enriched preparations.

fig. S4. Lipidomic differentiation of CMs.

fig. S5. DHA-induced remodeling of the MSC lipidome.

fig. S6. Distribution of lipids containing ω -6 PUFAs.

fig. S7. Supplementation with DHA increases the order difference between ordered and disordered domains in GPMVs.

fig. S8. DHA supplementation does not enhance adipogenic differentiation of hMSCs.

fig. S9. DCA treatment phenocopies membrane effects of DHA and enhances osteogenic differentiation.

fig. S10. Correlation between DHA- and osteogenic-induced effects on protein expression.

fig. S11. DCA treatment enhances Akt signaling.

fig. S12. Uncropped representative Western blots.

fig. S13. Effects of DHA and MZ on membrane organization and Akt activity in BHKs.

fig. S14. Row identifiers for Fig. 4D.

table S1. Variables and principal component correlation coefficients (loadings) for PCA of CM and PM lipids isolated from adipogenic, osteogenic, and undifferentiated cells (fig. S3).

table S2. Variables and principal component correlation coefficients (loadings) for PCA of PM lipids isolated from adipogenic, osteogenic, and undifferentiated cells (Fig. 11).

table S3. Variables and principal component correlation coefficients (loadings) for PCA of CM lipids isolated from adipogenic, osteogenic, and undifferentiated cells treated with DHA (Fig. 2L).

table S4. PPAR target genes are induced by adipogenesis but not induced or enhanced by DHA supplementation.

Excel file

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Acknowledgments: All confocal microscopy was performed at the Center for Advanced Microscopy, Department of Integrative Biology and Pharmacology McGovern Medical School at the University of Texas Health Science Center at Houston. We acknowledge the anonymous reviewers for their careful and constructive comments, which have significantly improved the manuscript. All confocal microscopy was performed at the Center for Advanced Microscopy, Department of Integrative Biology and Pharmacology McGovern Medical School at the University of Texas Health Science Center at Houston. **Funding:** I.L., K.R.L., and J.H.L. are funded by the Cancer Prevention Research Institute of Texas (CPRIT; R1215), NIH/National Institute of General Medical Sciences (grant no. 1R01GM114282), and the Volkswagen Foundation (grant no. 93091). J.F.H. is partially supported by CPRIT (RP170233). Y.Z. is supported by NIH (P30 DK056338). **Author contributions:** I.L. and K.R.L. designed the study, performed experiments, and wrote the paper. M.A.S. and C.K. performed lipidomic experiments and assisted in data analysis. A.D.S., J.H.L., and Y.Z. performed experiments. J.T.C. assisted in the interpretation of transcriptomic and proteomic data. J.F.H. designed EM experiments. **Competing interests:** The authors have read the journal's policy and have the following conflicts: M.A.S. and C.K. have paid employment at Lipotype GmbH. This does not alter the authors' adherence to all policies on sharing data and materials. The other authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 15 June 2017

Accepted 16 October 2017

Published 8 November 2017

10.1126/sciadv.aao1193

Citation: K. R. Levental, M. A. Surma, A. D. Skinkle, J. H. Lorent, Y. Zhou, C. Klose, J. T. Chang, J. F. Hancock, I. Levental, ω -3 polyunsaturated fatty acids direct differentiation of the membrane phenotype in mesenchymal stem cells to potentiate osteogenesis. *Sci. Adv.* **3**, eaao1193 (2017).