

## Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association

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**Docosahexaenoic acid (DHA), a 22:6 n-3 polyunsaturated fatty acid, is the longest and most highly unsaturated fatty acid found in most membranes and has been shown to inhibit cancer cell growth in part by modifying cell signaling. In the current study, alterations to epidermal growth factor receptor (EGFR) signaling upon DHA supplementation are examined in A549 lung adenocarcinoma, WiDr colon carcinoma and MDA-MB-231 breast carcinoma cell lines. Interestingly, EGFR phosphorylation, most notably at the tyrosine 1068 residue, is dramatically upregulated, and EGFR association with the Sos1 guanine nucleotide exchange factor is concomitantly increased upon DHA supplementation. However, guanosine triphosphate-bound Ras and phosphorylated extracellular signal-regulated kinase (Erk)1/2 are paradoxically downregulated in the same treatments. Previous reports have noted changes in membrane microdomains upon DHA supplementation, and our findings confirmed that EGFR, but not Ras, is excluded from caveolin-rich lipid raft fractions in DHA-treated cells, resulting in a decreased association of Ras with Sos1 and the subsequent downregulation of Erk signaling. Xenografts of the A549 cell line implanted in athymic mice fed a control high-fat diet or a diet high in DHA confirmed our *in vitro* data. These results demonstrate for the first time a functional consequence of decreased EGFR protein in lipid raft microdomains as a result of DHA treatment in three different cancer models. In addition, we report the ability of DHA to enhance the efficacy of EGFR inhibitors on anchorage-independent cell growth (soft agar), providing evidence for the potential development of enhanced combination therapies.**

### Introduction

Dietary omega-3 fatty acids are known to be beneficial in the treatment of several types of disease (1), including cancers of mammary, lung and colonic origin (2–5). Of particular interest is docosahexaenoic acid (DHA), a 22 carbon omega-3 fatty acid with six *cis* double bonds, which represents an extreme, being the longest and most highly unsaturated fatty acid occurring naturally in organisms (6). Because of this, much attention has been given to how DHA can alter membrane properties (7–12) and the organization and composition of membrane microdomains (6,13–16), specifically lipid rafts, areas rich in cholesterol and sphingolipids postulated to serve as signaling platforms by clustering proteins (17–19).

Among the proteins known to be localized in lipid rafts is the epidermal growth factor receptor (EGFR) (20). EGFR is a membrane-bound receptor, which is frequently mutated or functions anomalously in cancer (21), and a number of monoclonal antibodies and chemical inhibitors targeting it have been developed as anticancer

**Abbreviations:** DHA, docosahexaenoic acid; EGFR, epidermal growth factor receptor; Erk, extracellular signal-regulated kinase; GTP, guanosine triphosphate; LA, linoleic acid; pErk, phosphorylated Erk.

therapeutics (22,23). Recent evidence suggests that DHA is capable of decreasing EGFR localization in the lipid rafts of the MDA-MB-231 breast carcinoma cell line, thereby altering its signaling properties (24), with a number of subsequent reports suggesting similar alterations in the lipid raft recruitment of other receptor proteins (25–27).

Key signaling pathways initiated by EGFR through its Grb2/Sos1 associations at the tyrosine 1068 residue involve Ras proteins (28). Ras activation is subsequently involved in cell growth, proliferation, motility, survival and transformation (28,29). Mutations resulting in constitutive activation of Ras are found in 20–30% of all human tumors (30), and sustained signals from Ras often contribute to the distorted signaling seen in cancers (31). Ras isoforms can also associate with lipid rafts through modifications by fatty acids allowing them to tether (32), and interestingly, DHA has also been shown to effect Ras localization to the plasma membrane by altering its intracellular trafficking (33).

Taken together, these reports suggest that a major consequence of DHA incorporation into cellular membranes is the disruption of signaling events prevalent in many cancers. This study examines the effect of DHA supplementation on breast, lung and colon carcinomas *in vitro*, as well as an *in vivo* model of lung adenocarcinoma on EGFR protein localization, activity and downstream signaling. In all three cell types, DHA diminished EGFR levels in lipid rafts, although increasing its phosphorylation, specifically at the tyrosine 1068 residue. Ras isoforms were found to be unaltered in lipid rafts with DHA treatment, but paradoxically, Ras activation and Ras/Sos1 association were significantly decreased. Finally, an EGFR inhibitor was used in conjunction with DHA supplementation demonstrating the potential for effective combination therapy in cancers demonstrating abnormal EGFR signaling.

### Materials and methods

#### Antibodies and reagents

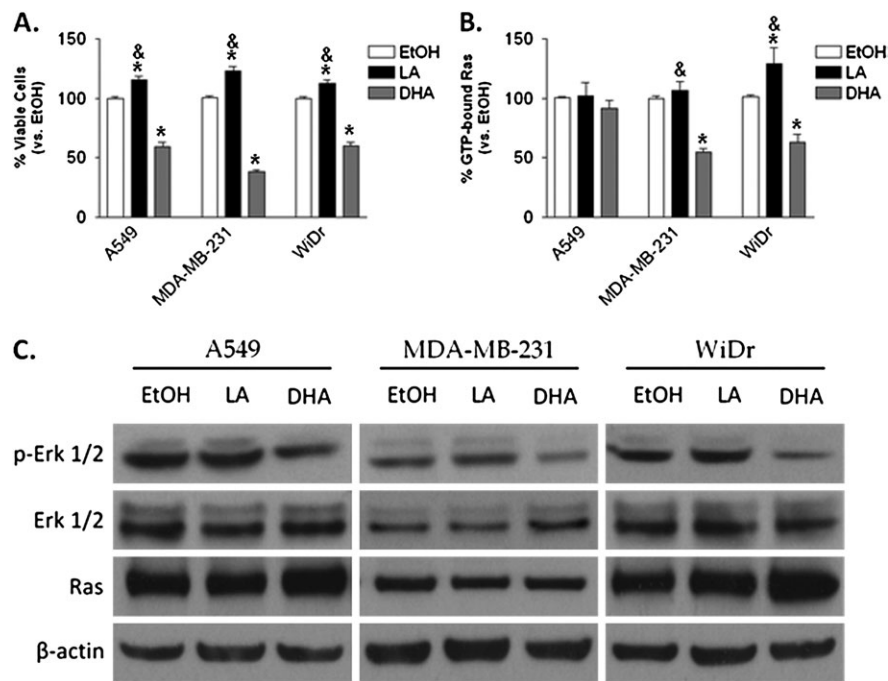
Fatty acid methyl esters of linoleic acid (LA) and DHA (Sigma, St Louis, MO) were dissolved in ethanol (EtOH), flushed with nitrogen gas and stored at –20°C for no >60 days. LA treatments were used in conjunction with DHA because LA has been shown to promote cancer cell growth and to verify the DHA effects are not due to some general effect from the addition of fatty acids. Specific antibodies to  $\beta$ -actin, EGFR pY1068 (Abcam, Cambridge, MA), phosphorylated Erk (pErk)1/2, extracellular signal-regulated kinase (Erk)1/2 and Sos1 (Santa Cruz Biotechnology, Santa Cruz, CA), pan Ras, clone 10 and phosphotyrosine/4G10 (Millipore, Temecula, CA) and EGFR (Cell Signaling Technology, Boston, MA) were purchased for the described studies. For EGFR and Ras immunoprecipitations, EGFR clone LA22 (Millipore) and pan Ras (Santa Cruz Biotechnology) were used. EGFR inhibitor PD153035 was purchased from Calbiochem (Gibbstown, NJ).

#### Cell culture

All cell lines were purchased from ATCC (Manassas, VA). WiDr cells were maintained in modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% bovine growth serum (Hyclone, Logan, UT). A549 and MDA-MB-231 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Hyclone). Cells were grown as monolayers at 37°C in a humidified environment with 5% CO<sub>2</sub>. Twenty-four hours after plating, A549 and MDA-MB-231 cultures were supplemented with 100  $\mu$ M fatty acid methyl esters, whereas WiDr cells received a 125  $\mu$ M concentration, and equal volumes of EtOH added as control treatments 48 h before experimental use. For cell viability assays, cells were trypsinized and counted using trypan blue staining and a hemacytometer. Unstained cells were counted as viable.

#### Immunoblotting and immunoprecipitation

Cells were washed with ice-cold phosphate-buffered saline and lysed using guanosine triphosphate (GTP)-lysis buffer [50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.5), 15 mM NaCl, 6 mM sodium deoxycholate,



**Fig. 1.** DHA inhibits cell growth and proliferation and reduces the levels of GTP-bound Ras and pErk1/2. (A) Cells were harvested with trypsin and counted using a hemacytometer and trypan blue staining. (B) Cells were lysed and the Ras GTPase Activation ELISA Kit was performed. Each data point represents the mean  $\pm$  SEM from three independent experiments. (C) Cells were lysed and protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with antibodies against pErk1/2, Erk1/2, Ras and  $\beta$ -actin. Each immunoblot is representative of three independent experiments. Statistical significance ( $P \leq 0.05$ ) between treatments was determined using analysis of variance and is indicated as followed: \* (versus EtOH); & (versus DHA). Redundant symbols were not shown.

1% nonyl phenoxyethylpolyethoxyethanol-40, 10% glycerol, 10 mM  $MgCl_2$  and 1 mM ethylenediaminetetraacetic acid] containing freshly added protease and phosphatase inhibitors. Tumors were homogenized in the same buffer. Samples were centrifuged at 16 000g for 10 min at 4°C. Supernatants were analyzed for protein concentration using Bio-Rad's DC assay (Hercules, CA). Samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad) and probed with specific antibodies. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and visualized with enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK). For immunoprecipitations, defined amounts of protein were incubated overnight with specific antibodies before the addition of protein G-agarose followed by washing. Densitometry was done using the Un-Scan-It® software using total protein levels to assess activity (i.e. Erk was used to compare levels of pErk).

#### Ras activation assay

Total GTP-bound Ras was assayed using a Ras GTPase Activation ELISA Kit (Millipore). Briefly, samples were assayed for protein content to standardize input amounts, before being analyzed by enzyme-linked immunosorbent assay using a Raf1 Ras-binding domain fusion protein that only binds active GTP-bound Ras as a substrate and then read on a luminometer.

#### Lipid raft isolations

Isolations were carried out using a modification method described previously (24). Briefly, cells were washed in ice-cold phosphate-buffered saline and scraped in ice-cold OptiPrep™ Base Buffer [20 mM Tris-HCl (pH 7.8), 250 mM sucrose, 6 mM ethylenediaminetetraacetic acid, 1 mM  $CaCl_2$  and 2.5 mM  $MgCl_2$ ] containing freshly added protease and phosphatase inhibitors. Tumors were homogenized in the same manner and buffer. Preparations were centrifuged at 1000g for 2 min at 4°C and the supernatants were assayed for protein content to standardize assay amounts. The membrane pellet was passed through a Sub-Q 26½ gauge needle in OptiPrep™ Base Buffer 20 times and centrifuged at 1000g for 10 min at 4°C. This step was repeated a second time. Samples preparations were mixed with 60% OptiPrep™ solution to form a 45% OptiPrep™ layer, which was overlaid with 35% OptiPrep™ followed by 5% OptiPrep™. Preparations were centrifuged at 268 000g for 2 h at 4°C in a ultracentrifuge (Beckman Coulter, Temecula, CA) using a SW55Ti swinging bucket rotor. To determine the location of the lipid rafts in the gradients, six fractions were collected sequentially from top to bottom and the aliquots were

assessed for a lipid raft-specific marker. Rafts were identified to be contained within fraction 3, which was added to an additional volume of 5% OptiPrep™ and centrifuged at 268 000g for 30 min at 4°C to pellet and concentrate the rafts. The pellet rafts were then resuspended in GTP-lysis buffer.

#### Anchorage-independent growth assay

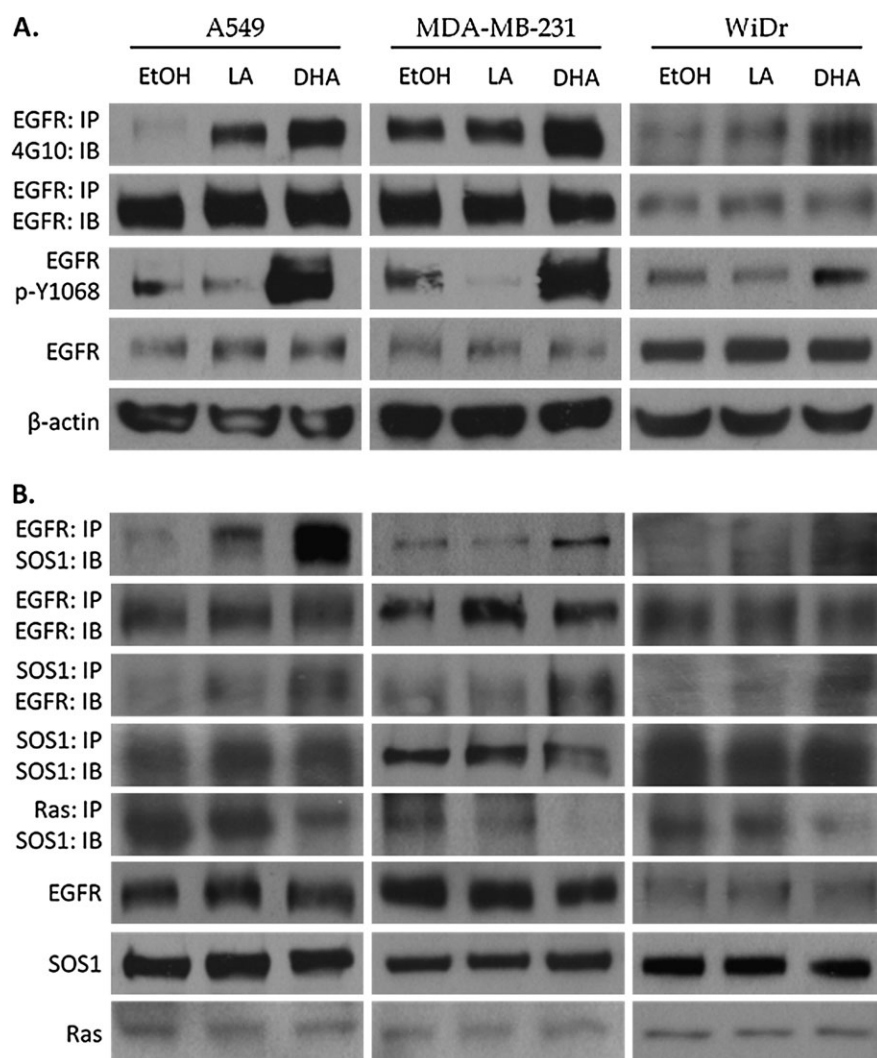
A 0.4% solution of noble agar (Sigma) was combined with the appropriate media containing specific treatments. The cells were seeded over a 0.8% noble agar base layer in triplicate. Cells were rehydrated every 3 days with the appropriate medium. After 3–5 weeks, cells were stained with crystal violet (Sigma) and colony numbers were determined.

#### Animal study

The feeding study was performed as described previously (3). Briefly, adult male NCr homozygous (nu/nu) athymic nude mice were maintained and bred under aseptic conditions with constant temperature and humidity. Animals were implanted with A549 xenografts in the right flank and randomly assigned to experimental treatment groups, which were fed diets composed of the American Institute of Nutrition-93M casein-based diet containing defined amounts of essential fatty acids starting the day of implantation (supplementary Figure 1 is available at *Carcinogenesis* Online). The experimental diets consisted of (i) a high-fat omega-6, 24% corn oil and (ii) a high-fat omega-3, 8% corn oil and 16% Dhasco™ oil (Martek, Columbia, MD) diet. Food intake was monitored daily and isocalorically controlled. Tumor growth was monitored biweekly by caliper measurement, with a volume of 3.38 cm<sup>3</sup> or signs of ulceration defined as the experimental end point. The study was ended 23 days after implantation because xenografts in the 24% corn oil group reached volumes defined as an end point for the study.

#### Fatty acid analysis

Lipid raft isolations of *in vitro* samples and pooled A549 xenografts were homogenized in 2.0 ml of GTP-lysis buffer containing 0.02% butylated hydroxytoluene [50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.5), 15 mM NaCl, 6 mM sodium deoxycholate, 1% nonyl phenoxyethylpolyethoxyethanol-40, 10% glycerol, 10 mM  $MgCl_2$ , 1 mM ethylenediaminetetraacetic acid and 0.02% butylated hydroxytoluene] containing freshly added protease and phosphatase inhibitors. Samples were analyzed for protein concentration using Bio-Rad's DC assay. Fatty acids were extracted from



**Fig. 2.** DHA increases total EGFR phosphorylation and pY1068 and alters Sos1 association with EGFR and Ras. **(A)** EGFR was immunoprecipitated from total cellular protein and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with phosphotyrosine/4G10 and EGFR. In addition, total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with antibodies against EGFR pY1068, EGFR and β-actin. Each immunoblot is representative of three independent experiments. **(B)** EGFR, Sos1 or Ras were immunoprecipitated from total cellular protein, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with antibodies against Sos1 and EGFR. We were unable to immunoblot for Ras in the Ras immunoprecipitation due to interference of the light chain of the antibody used for immunoprecipitation. In addition, total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with antibodies against EGFR, Sos1 and Ras. Each immunoblot is representative of three independent experiments.

whole-cell homogenates using a 2:1 chloroform:methanol mixture. The samples were vortexed and centrifuged at 1000g for 5 min. The chloroform layer was extracted and a 19:0 internal standard was added and dried under nitrogen. Boron trifluoride (14%, BF<sub>3</sub>/methanol) was added to dried sample and incubated at 110°C for 15 min. Petroleum ether containing 0.02% butylated hydroxytoluene was added to the samples. The petroleum ether fatty acid-containing fraction was dried in anhydrous Na<sub>2</sub>SO<sub>4</sub>/NaHCO<sub>3</sub> (2:1, wt, wt). The samples were flushed with nitrogen and stored at –20°C until use. An Agilent Technologies 6890N Chromatograph, which contained an SP2340 capillary column (Supelco, St Louis, MO), was used. A temperature program of a total time of 20 min with a starting temperature of 120°C was formulated. The injector was set at 245°C and flame ionization detector at 250°C. The program was modified so that rate 1 was set at 5°C/min to 160°C and rate 2 was set at 10°C/min to 240°C and held at 240°C for 4 min. Values for each fatty acid are reported as percentage of total fatty acids based on nanomoles calculated versus the 19:0 standard.

#### Statistical analysis

With the exception of the animal study, all experimental results were independently repeated at least three times. All quantitative data are presented with

SEM and statistical analyses were performed using analysis of variance with the Tukey method for pairwise comparison on SAS® software, except in the case of xenograft measurements where the Mann–Whitney test was used, with values of at least  $P \leq 0.05$  being considered significant.

## Results

### *DHA decreases cell growth and proliferation in A549, MDA-MB-231 and WiDr through decreases in GTP-bound Ras and pErk*

Numerous studies have demonstrated that DHA is capable of inhibiting proliferation through various mechanisms in *in vitro* cultures of cancer cells (1). Treatments yielded significant decreases versus control groups ( $P \leq 0.05$ ) between 40 and 60% in cell growth with DHA supplementation in A549 lung adenocarcinoma cells ( $40.9 \pm 4.0\%$ ), MDA-MB-231 breast cancer cells ( $62.7 \pm 2.2\%$ ) and WiDr colon cancer cells ( $39.9 \pm 3.2\%$ ), whereas equivalent concentrations of LA showed significant increases in cell numbers (Figure 1A).

Ras is activated in its GTP-bound state and initiates a number of proliferation signals. Previous findings have demonstrated that DHA decreases Ras activation in colonocytes (34). Current findings demonstrated that both MDA-MB-231 ( $45.1 \pm 3.9\%$ ) and WiDr cells ( $38.3 \pm 9.0\%$ ) display significant decreases versus EtOH control treatments ( $P \leq 0.01$ ) in GTP-bound Ras upon DHA treatment (Figure 1B), despite having no alterations in total Ras protein (Figure 1C). The A549 cell line has a known activating mutation of K-ras (35) and did not show any difference in Ras activity with DHA supplementation (Figure 1B).

Erk is a downstream component of Ras signaling and is involved in the propagation of proliferation pathways (28). Indeed, when changes in GTP-bound Ras were noted, declines in pErk levels in MDA-MB-231 ( $18.40 \pm 8.45\%$  down compared with EtOH control,  $P \leq 0.05$ ) and WiDr ( $30.74 \pm 21.84\%$  down compared with EtOH control) in cells treated with DHA were also seen (Figure 1C). A549 also showed diminished, although statistically insignificant decreases, in pErk levels ( $8.38 \pm 6.38\%$  down compared with EtOH control). These results indicate that DHA is probably decreasing cell proliferation in part, by downregulating Ras/Erk signaling.

#### DHA alters EGFR phosphorylation and associations

Ras/Erk signaling is often initiated by EGFR phosphorylation (21). A previous report noted that *in vitro* supplementation with a combination of DHA and another omega-3 fatty acid, eicosapentaenoic acid, resulted in increased total EGFR phosphorylation (24). The present study verified these findings as all three cell models showed increased EGFR phosphorylation upon DHA treatment compared with control groups (Figure 2A). LA, an omega-6 fatty acid, also displayed increases in EGFR phosphorylation in the three cell lines, though the noted alterations were not as large as what was noted with DHA treatments.

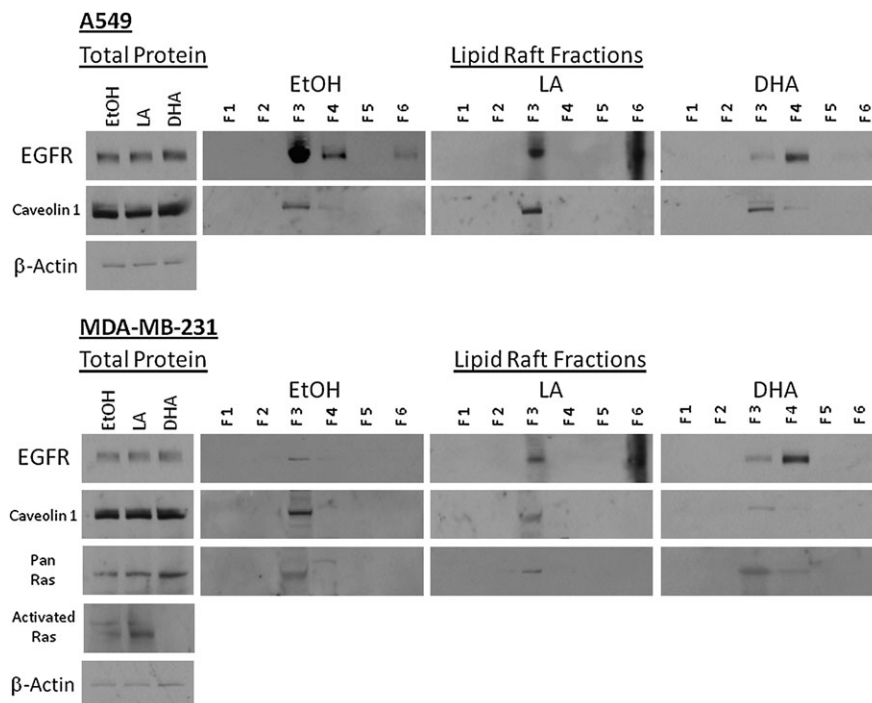
Although upregulation of EGFR phosphorylation does not correspond to the observed decreases in GTP-bound Ras and pErk, EGFR has a number of tyrosine residues that are phosphorylated in response to different ligands and dimerization partners (36). Phosphorylation of

EGFR at tyrosine 1068 (Y1068) is associated with Ras/Erk signaling, and although LA-treated cells showed decreases in EGFR phosphorylation at Y1068 versus control treatments, DHA paradoxically showed dramatic upregulations in phosphorylation at this site (Figure 2A).

After phosphorylation at Y1068, EGFR recruits the adaptor protein, Grb2, which facilitates binding to the guanine nucleotide exchange factor, Sos1, in the transduction of its signal to Ras/Erk (36). The observed changes in EGFR Y1068 phosphorylation upon DHA treatment were associated with increased EGFR/Sos1 association with DHA treatment in each of the three cell lines (Figure 2B). Again, this result was unexpected given decreased GTP-bound Ras and pErk. However, when Ras association with Sos1 was examined against control treatments, dramatic decreases were noted upon DHA supplementation in all cases, whereas no alterations in total EGFR, Ras or Sos1 protein expression were seen (Figure 2B). Thus, decreased activated Ras and Erk, despite upregulation of EGFR Y1068 phosphorylation in DHA treatments, appear to be due to the inability of Ras to complex with Sos1.

#### DHA disrupts the association of EGFR, but not Ras, from lipid raft microdomains

Lipid raft microdomains are thought to serve as platforms that aggregate specific proteins, including EGFR, to facilitate cell signaling (32). One recent report suggested that DHA treatment results in the exclusion of EGFR from these microdomains (24). Indeed, when lipid raft preparations of cell cultures supplemented with DHA were assessed, EGFR protein is no longer found predominately in the same major fraction as the lipid raft marker protein caveolin-1 (Figure 3). Moreover, despite the altered localization of EGFR, Ras protein appeared to be much less affected by DHA treatment, with most of the protein remaining in the same fraction as caveolin-1 (Figure 3). Lipid raft fractions were also distinguished by examining Gox protein (data not shown). In light of these findings, it seems probable that EGFR/Ras/Erk signaling is being disrupted in DHA treatments by the exclusion of EGFR protein from lipid raft microdomains.



**Fig. 3.** DHA disrupts EGFR from lipid rafts, whereas Ras association is unaffected. After fatty acid incubations, cells were mechanically lysed, standardized for equal protein content and lipid rafts were isolated via gradient centrifugation. Lipid raft fractions were solubilized in GTP-lysis buffer after isolation and equal amounts from each treatment were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with antibodies against EGFR, caveolin-1 and Ras. Total protein before lipid raft fractionation was also separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. Each immunoblot is representative of at least three independent experiments.

Fatty acid analyses confirmed that DHA was being incorporated at very high levels both *in vitro* and *in vivo* upon DHA supplementation compared with control treatments/diets (Table I). In both cases, the largest fold increase in the percentage of DHA occurred in fraction 3 (>8-fold increase *in vitro* and >10-fold increase *in vivo*), where EGFR and lipid raft markers are most highly localized in control treatments.

#### DHA modifies EGFR localization and phosphorylation of A549 cells *in vivo*

Although the *in vitro* studies on three independent cell models provided convincing evidence for the observations made, not all *in vitro* findings are relevant to *in vivo* models. To determine if the *in vitro* results were applicable to *in vivo* studies, athymic mice were im-

planted with xenografts from the A549 lung adenocarcinoma cell line and fed a high-fat diet of omega-6 fatty acids or a high-fat diet consisting mainly of the omega-3 fatty acid DHA.

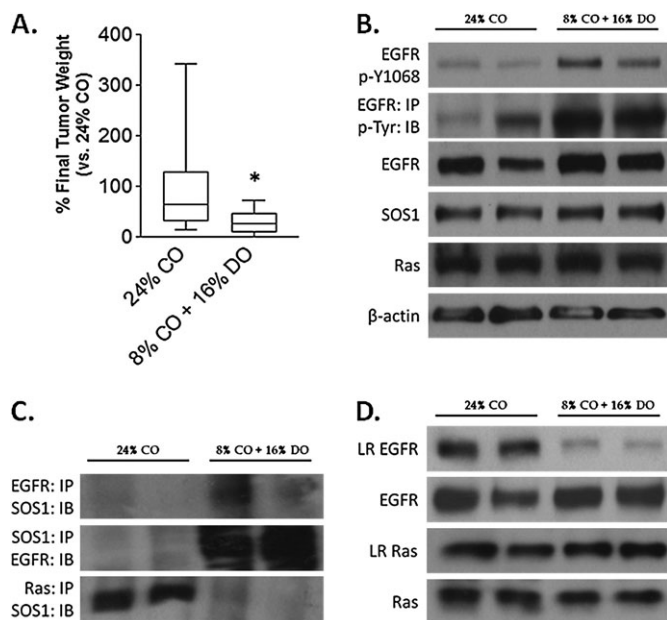
Mice fed the diet rich in DHA had significantly decreased final tumor weights ( $P \leq 0.05$ ) compared with animals fed the high-fat omega-6 diet ( $71.6 \pm 29.24\%$ ) (Figure 4A). Furthermore, xenograft homogenates confirmed upregulated EGFR phosphorylation, including Y1068, and increased association of EGFR and Sos1 with a corresponding decrease in Ras/Sos1 complexes in animals fed the diet high in DHA (Figure 4B and C). Lipid raft preparations of the *in vivo* samples from animals fed the high-DHA diet also showed decreases in EGFR protein in the lipid raft-containing fraction 3, with no concomitant alteration in Ras protein (Figure 4D).

**Table I.** Relative percentages of DHA increase in lipid raft fractions upon DHA supplementation

Fatty acids	A549 <i>in vitro</i> lipid fractions—control (EtOH) treatment						
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Total
16:1—Palmitic n-7	a	3.31 ± 0.26	0.94 ± 0.02	4.99 ± 0.23	a	3.64 ± 0.24	4.81 ± 0.09
18:0—Stearic	26.69 ± 4.67	29.44 ± 2.30	19.04 ± 0.50	34.04 ± 1.53	9.19 ± 0.26	3.53 ± 0.23	7.87 ± 0.15
18:1—Oleic n-9	57.21 ± 10.01	55.32 ± 4.33	36.00 ± 0.95	57.02 ± 2.57	31.23 ± 0.90	82.43 ± 5.33	50.16 ± 0.97
18:2—Linoleic n-6	a	a	37.97 ± 1.01	a	56.49 ± 1.62	a	32.65 ± 0.63
18:3—Linolenic n-3	a	a	0.86 ± 0.74	a	a	a	a
20:4—Arachidonic n-6	a	3.67 ± 0.29	3.91 ± 0.10	a	a	a	2.27 ± 0.04
20:5—EPA n-3	a	a	a	a	a	a	a
22:6—DHA n-3	16.10 ± 14.68	8.25 ± 7.18	2.14 ± 2.59	3.95 ± 4.33	3.09 ± 2.78	10.40 ± 5.79	2.24 ± 1.89
Total FA/fraction	7.05 ± 5.51	12.69 ± 9.31	26.79 ± 3.52	22.98 ± 19.85	22.40 ± 14.10	8.09 ± 6.29	
Fatty acids	A549 <i>in vitro</i> lipid fractions—DHA treatment						
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Total
16:1—Palmitic n-7	13.28 ± 3.31	13.07 ± 3.52	2.13 ± 0.54	0.12 ± 0.03	24.56 ± 6.85	13.80 ± 23.72	1.70 ± 0.81
18:0—Stearic	15.32 ± 3.82	14.88 ± 4.01	25.56 ± 6.42	20.23 ± 5.52	20.47 ± 5.71	32.46 ± 16.96	17.16 ± 8.16
18:1—Oleic n-9	14.52 ± 3.62	15.15 ± 4.08	20.61 ± 5.18	17.35 ± 4.73	29.54 ± 8.24	22.46 ± 17.83	19.63 ± 9.33
18:2—Linoleic n-6	35.36 ± 8.82	37.53 ± 10.12	27.25 ± 6.85	44.06 ± 12.02	0.12 ± 0.03	4.34 ± 4.97	24.71 ± 11.74
18:3—Linolenic n-3	a	a	a	a	a	a	a
20:4—Arachidonic n-6	0.88 ± 0.22	1.08 ± 0.29	2.88 ± 0.72	0.21 ± 0.06	2.99 ± 0.83	1.35 ± 2.14	1.86 ± 0.89
20:5—EPA n-3	1.60 ± 0.40	1.75 ± 0.47	4.12 ± 1.04	2.82 ± 0.77	4.09 ± 1.14	1.62 ± 1.86	2.61 ± 1.24
22:6—DHA n-3	19.05 ± 20.19	16.54 ± 22.50	17.45 ± 20.75	15.22 ± 23.13	18.24 ± 22.80	23.96 ± 38.20	32.33 ± 32.17
Total FA/fraction	21.03 ± 26.80	24.28 ± 20.62	27.38 ± 32.17	19.72 ± 15.48	4.06 ± 5.16	3.53 ± 4.37	
Fatty acids	A549 <i>in vitro</i> lipid fractions—control diet (24% corn oil)						
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Total
16:1—Palmitic n-7	2.25 ± 0.23	2.16 ± 0.16	1.35 ± 1.91	a	a	a	2.89 ± 0.23
18:0—Stearic	22.33 ± 0.10	26.24 ± 1.09	24.06 ± 2.85	25.71 ± 2.21	14.35 ± 7.03	16.54 ± 1.85	24.94 ± 0.70
18:1—Oleic n-9	28.65 ± 2.14	27.01 ± 1.76	30.94 ± 9.36	31.02 ± 12.38	21.93 ± 12.24	22.64 ± 5.62	23.73 ± 1.00
18:2—Linoleic n-6	31.04 ± 0.19	28.20 ± 0.15	28.23 ± 0.48	30.59 ± 3.34	21.67 ± 17.37	30.26 ± 0.31	28.25 ± 0.90
18:3—Linolenic n-3	0.35 ± 0.49	0.38 ± 0.54	0.25 ± 0.35	a	a	a	0.49 ± 0.04
20:4—Arachidonic n-6	10.42 ± 1.43	10.90 ± 0.69	12.83 ± 1.41	6.34 ± 8.97	34.73 ± 26.29	12.08 ± 1.10	13.74 ± 0.24
20:5—EPA n-3	a	a	a	a	a	a	a
22:6—DHA n-3	4.96 ± 1.34	5.13 ± 0.57	2.34 ± 3.32	6.34 ± 8.97	7.32 ± 10.36	18.48 ± 8.26	5.96 ± 1.16
Total FA/fraction	21.80 ± 2.64	22.03 ± 2.91	32.39 ± 43.02	13.22 ± 15.63	3.96 ± 0.88	6.61 ± 0.36	
Fatty acids	A549 <i>in vitro</i> lipid fractions—DHA diet (8% corn oil + 16% DHASCO)						
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Total
16:1—Palmitic n-7	1.32 ± 1.86	2.11 ± 0.26	1.62 ± 2.29	a	a	a	3.60 ± 0.62
18:0—Stearic	19.51 ± 3.11	23.58 ± 1.52	23.60 ± 0.33	15.38 ± 5.28	12.76 ± 5.28	15.05 ± 0.60	9.55 ± 10.74
18:1—Oleic n-9	25.22 ± 1.60	26.04 ± 0.72	24.50 ± 0.67	19.24 ± 5.24	21.49 ± 2.10	23.69 ± 0.02	30.52 ± 0.52
18:2—Linoleic n-6	17.18 ± 0.13	17.23 ± 0.60	19.09 ± 0.52	8.38 ± 11.85	14.82 ± 6.12	17.00 ± 1.10	22.87 ± 2.06
18:3—Linolenic n-3	0.35 ± 0.49	a	a	a	a	a	0.51 ± 0.10
20:4—Arachidonic n-6	0.54 ± 0.76	1.29 ± 0.09	2.17 ± 0.73	a	a	a	1.89 ± 1.01
20:5—EPA n-3	1.13 ± 1.60	2.71 ± 0.22	4.17 ± 0.13	a	a	a	3.80 ± 0.95
22:6—DHA n-3	34.76 ± 9.56	27.04 ± 0.16	24.85 ± 2.29	57.00 ± 22.37	50.94 ± 13.51	44.26 ± 1.72	27.25 ± 5.50
Total FA/fraction	24.83 ± 3.10	26.33 ± 7.06	32.00 ± 8.58	7.93 ± 3.98	3.88 ± 0.94	5.04 ± 2.05	

*In vitro* A549 cell treatments, as well as A549 xenograft samples from lipid raft isolations and matched total lysates were analyzed by gas chromatography for fatty acid content. All values are represented as the percentage of each fatty acid relative to total fatty acid content ± standard deviation. DHASCO, DHA-rich single cell oil; EPA, eicosapentaenoic acid; FA, fatty acid.

<sup>a</sup> Indicates no measurable value could be detected in the sample for the listed fatty acid.



**Fig. 4.** DHA inhibits A549 tumor growth *in vivo* by altering EGFR signaling. Adult male NCr (nu/nu) athymic nude mice were xenografted with the lung tumor line A549 and fed the indicated experimental diets. (A) Final tumor weights were estimated using the formula (tumor weight in milligram =  $A \times B \times C/2$ ) where A, B and C represent the three perpendicular diameters of the tumor in millimeters. Each data point represents the mean  $\pm$  SEM from 14 animals at day 24. (B) EGFR was immunoprecipitated from total cellular protein and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with phosphotyrosine/4G10 and EGFR. In addition, total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with antibodies against EGFR pY1068, EGFR, Sos1, Ras and  $\beta$ -actin. Each immunoblot is representative of three independent experiments. (C) EGFR, Sos1 and Ras were immunoprecipitated from total cellular protein from two sets of three different pooled tumors from each experimental diet, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with phosphotyrosine/4G10, EGFR and Sos1. Total proteins from the same sets were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with antibodies against EGFR pY1068, EGFR, Ras and  $\beta$ -actin. (D) Two sets of three pooled tumors from each experimental diet were homogenized and standardized to total protein and lipid rafts were isolated via gradient centrifugation. Lipid raft fractions were solubilized in GTP-lysis buffer after isolation and equal amounts from each treatment were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with antibodies against EGFR and Ras. Total protein before lipid rafts isolation was also separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. The membranes were immunoblotted with antibodies against EGFR and Ras. Statistical significance ( $P \leq 0.05$ ) between treatments was determined using the Mann–Whitney test and is indicated as followed: \* (versus 24% corn oil diet).

#### DHA supplementation enhances the efficacy of EGFR inhibitors *in vitro*

Inhibitors of EGFR have been widely developed for the treatment of cancer but have demonstrated only limited effectiveness in clinical trials (36–38). PD153035 (Calbiochem, San Diego, CA), although not employed clinically, is a common chemical inhibitor of EGFR used for *in vitro* studies. Treatment of A549 cells with PD153035 is capable of reversing changes in EGFR phosphorylation upon DHA supplementation (Figure 5A). Although the inhibitor ( $14.52 \pm 4.02\%$  at  $2 \mu\text{M}$  and  $28.53 \pm 4.02\%$  at  $5 \mu\text{M}$  concentrations) and DHA ( $55.1 \pm$

$3.79\%$ ) alone were capable of significantly decreasing cell proliferation versus control treatments ( $P \leq 0.01$ ) in a dose-dependent manner (Figure 5B), a combination of the two further inhibited the growth of A549 cells ( $69.6 \pm 3.75\%$  at  $2 \mu\text{M}$  and  $83.37 \pm 3.44\%$  at  $5 \mu\text{M}$  concentrations overall inhibition and by  $32.9 \pm 4.15\%$  at  $2 \mu\text{M}$  and  $63.4 \pm 3.77\%$  at  $5 \mu\text{M}$  versus DHA alone) (Figure 5B).

Interestingly, when examining colony formation and anchorage-independent growth in soft agar, our laboratory has found concentrations of DHA necessary to inhibit cell growth by 50% (IC<sub>50</sub>) are less than one-fourth the amount noted in standard tissue cultures (Kikawa KD, unpublished results). DHA ( $51.49 \pm 3.99\%$ ) and PD153035 ( $20.95 \pm 4.39\%$ ) both significantly inhibited the growth of A549 cells in soft agar ( $P \leq 0.01$ ) and noted further reductions when used in combination ( $72.31 \pm 3.65\%$  overall inhibition and by  $47.0 \pm 2.40\%$  versus DHA treatment) (Figure 5C). These data provide strong preliminary data that DHA supplementation could nutritionally enhance *in vivo* treatments using drugs targeting EGFR.

#### Discussion

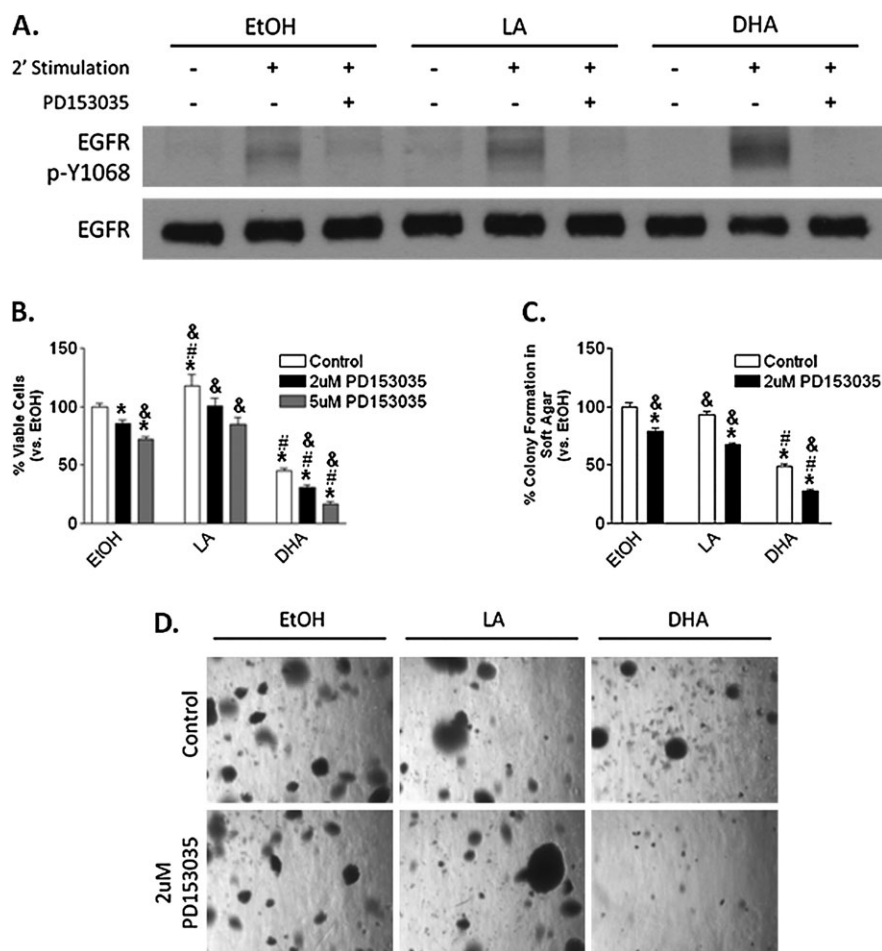
This novel study establishes a functional consequence of DHA's alterations to membrane microdomains by demonstrating EGFR exclusion from lipid rafts results in subsequent decreases in activated Ras and Erk downstream and decreased cell proliferation. Moreover, it affirms that the mechanism may be widely applicable, by confirming the findings in three different *in vitro* cancer cell models of different tissue origins (lung, breast and colon) and one *in vivo* model for lung cancer. Finally, the therapeutic potential of combining DHA supplementation with the plethora of EGFR inhibitors currently being developed for cancer treatment is validated and warrants further review.

Consistent results in numerous independent reports conclude DHA is capable of inhibiting cancer cell growth (1,5,39–48), and although the mechanisms behind its actions are many, it is clear that DHA-induced alterations in membrane microdomains resulting in the disruption of proliferation pathways are involved. Given the number of cancers displaying dysregulation of EGFR, DHA supplementation provides a unique approach to treatment and prevention because disrupting membrane localization would probably interfere with Ras/Erk signaling regardless of EGFR mutational status or the overexpression of other EGFR family members associated with aggressive transformation (36,38).

Additionally, it seems probably that EGFR is not the only protein being differentially localized due to DHA's effect on membrane microdomains. Various changes in signaling due to altered lipid raft associations, including alterations in Toll-like receptor 4 (25), the chemokine receptor CXCR4 (49) and even the lipid raft marker caveolin 1 (50) have been reported upon treatment with DHA, and a number of other pathways are disrupted by similar mechanisms. Although this study did note slight changes in caveolin-1 fraction localization between LA- and DHA-treated cells *in vitro* (Figure 3), the DHA treatments did not appear to be significantly altered compared with the EtOH-treated control group, indicating that perhaps high amounts LA and/or the saturated fatty acid, stearic acid, used in the earlier reports (49,50) magnified the alteration in caveolin-1 localization.

Given the large increases in DHA noted in the primary lipid raft fractions in the fatty acid analyses performed (Table 1), it is clear that DHA is dramatically altering lipid microdomain composition to generate these signaling changes. Further study to determine whether DHA-induced changes on lipid raft domains can enhance therapeutics targeting lipid raft-associated proteins is warranted.

EGFR inhibitors developed for use clinically have shown only limited effectiveness in specific subsets of patients, who are predominantly of Japanese or East Asian descent (51). Although this has been postulated to be due to the prevalence of somatic mutations in exons 18–21 (52–55), these populations also tend to consume diets higher in DHA than other groups, which given the findings of this report, might also enhance efficacy of EGFR-targeted therapies. Additional studies looking at an *in vivo* model combining DHA and EGFR inhibitors could yield promising new avenues of research.



**Fig. 5.** DHA decreases transformation by altering EGFR signaling. (A) A549 was treated for 24 h with either 100  $\mu$ M DHA, LA or control volume of EtOH in 10% serum media. Media was replaced with 0.01% serum and cells were treated with the same indicated fatty acid concentrations for 48 h. Prior to cell lysis, the cells were treated with 5  $\mu$ M PD153035 or control volume of dimethyl sulfoxide for 30 min and were then stimulated for 2 min with 10% serum. Total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were immunoblotted with antibodies against EGFR pY1068 and EGFR. Each immunoblot is representative of three independent experiments. (B) A549 was treated for 48 h with either 100  $\mu$ M DHA, LA or control volume of EtOH and either 2  $\mu$ M or 5  $\mu$ M PD153035 or control volume of dimethyl sulfoxide. Cells were harvested with trypsin and counted using a hemacytometer and trypan blue staining to determine cell viability. (C and D) A549 was suspended in 0.4% noble agar mixed with the appropriate medium and either a control volume of EtOH, 25  $\mu$ M LA or 25  $\mu$ M DHA. In addition to the fatty acids, EGFR inhibitor PD153035 was added at 2  $\mu$ M concentration or a control volume of dimethyl sulfoxide. The cells were seeded over a 0.8% noble agar base layer. After 3–5 weeks, cells were stained with crystal violet and colonies were counted. Each experiment represents the mean  $\pm$  SEM from three independent experiments. Statistical significance ( $P \leq 0.05$ ) between treatments was determined using analysis of variance and is indicated as followed: \* (versus EtOH); # (versus EtOH + 2  $\mu$ M PD153035); & (versus DHA). Redundant symbols were not shown.

Nutritional intervention in the treatment of cancer provides a promising approach to enhancing more conventional therapeutics, or potentially lowering effective doses, and does not further reduce a patient's quality of life. Thus, more research into the potential of using DHA supplementation in combination with drugs like EGFR inhibitors is of critical importance.

#### Supplementary material

Supplementary Figure 1 can be found at <http://carcin.oxfordjournals.org/>

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