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Small Amounts of Isotope-reinforced Polyunsaturated Fatty Acids Suppress Lipid Autoxidation

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Abstract

Polyunsaturated fatty acids (PUFAs) undergo autoxidation and generate reactive carbonyl compounds that are toxic to cells and associated with apoptotic cell death, age-related neurodegenerative diseases, and atherosclerosis. PUFA autoxidation is initiated by the abstraction of bis-allylic hydrogen atoms. Replacement of the bis-allylic hydrogen atoms with deuterium atoms (termed site-specific isotope-reinforcement) arrests PUFA autoxidation due to the isotope effect. Kinetic competition experiments show that the kinetic isotope effect for the propagation rate constant of Lin autoxidation compared to that of 11,11-D_2-Lin is 12.8 ± 0.6. We investigate the effects of different isotope-reinforced PUFAs and natural PUFAs on the viability of coenzyme Q-deficient *Saccharomyces cerevisiae coq* mutants and wild-type yeast subjected to copper stress. Cells treated with a C11-BODIPY fluorescent probe to monitor lipid oxidation products show that lipid peroxidation precedes the loss of viability due to H-PUFA toxicity. We show that replacement of just one bis-allylic hydrogen atom with deuterium is sufficient to arrest lipid autoxidation. In contrast, PUFAs reinforced with two deuterium atoms at mono-allylic sites remain susceptible to autoxidation. Surprisingly, yeast treated with a mixture of approximately 20%:80% isotope-reinforced D-PUFA: natural H-PUFA are protected from lipid autoxidation-mediated cell killing. The findings reported here show that inclusion of only a small fraction of PUFAs deuterated at the bis-allylic sites is sufficient to profoundly inhibit the chain reaction of non-deuterated PUFAs in yeast.

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Keywords

C11-BODIPY; chain reaction; coenzyme Q; kinetic isotope effect; lipid autoxidation; oxidative stress; polyunsaturated fatty acid; ubiquinone

Introduction

PUFAs[1] are essential nutrients and are avidly taken up by cells [1]. As components of phospholipids, PUFAs are key building blocks of membrane bilayers; they facilitate the assembly and stability of protein complexes, and play major roles in cellular metabolism [2]. PUFAs can be metabolized into several major classes of hormones by oxidative enzymes, including cyclooxygenases, lipoxygenases, and epoxygenases, generating prostaglandins, hydroxyl-fatty acids, leukotrienes and other mediators collectively known as eicosanoids [3, 4].

PUFAs comprise the most vulnerable components of cells and are highly susceptible to non-enzymatic oxidation by reactive oxygen species (ROS) [5, 6]. ROS initiate the free radical chain reaction of PUFA autoxidation, resulting in changes in membrane permeability and fluidity due to accumulation of lipid peroxides [7] and *cis* to *trans* isomerisation [8]. The lipid peroxides resulting from PUFAs autoxidation may play a role in DNA damage [9] and carcinogenesis [10]. Because of their ability to generate oxyradicals, lipid peroxides may initiate degenerative processes and promote disorders, including inflammation [11] and cancer [12]. A separate class of non-enzymatic lipid peroxidation products comprises arachidonic acid-derived isoprostanes, which play a role in cellular signalling [13]; and PUFA-derived resolvins and protectins, which act as lipid mediators to resolve inflammation [14]. Oxidative damage to PUFAs also leads to a smorgasbord of reactive carbonyl electrophiles including products such as *trans*-4-hydroxy-2-nonenal, *trans,trans*-2,4-decadienal, malondialdehyde, crotonaldehyde, 4-hydroxyhexenal, acrolein, and others [15–17]. These carbonyl electrophiles cause harm by reacting with cellular components such as proteins and nucleic acids [16, 18, 19]. The majority of cellular electrophiles are generated from PUFAs by a peroxidation chain reaction that is readily triggered by ROS, but propagates without their further input. Thus, the formation of lipid-derived electrophiles such as trans-4-hydroxy-2-nonenal is relatively insensitive to the level of initiating ROS, but depends mainly on the availability of PUFAs and O_2. This is consistent with observations that life span is inversely correlated to membrane peroxidizability [20].

We have demonstrated that PUFAs harboring deuterium atoms at the bis-allylic sites are much more resistant to autoxidation reactions [21]. This is due to the isotope effect, whereby abstraction of the bis-allylic H atom, the rate-limiting step of PUFA autoxidation, is substantially slowed down by the presence of the D atoms at the bis-allylic site. Isotope-reinforced PUFAs were shown to protect coenzyme Q-deficient (*coq*) mutants of *Saccharomyces cerevisiae* and heat-stressed wild-type yeast against the toxic effects of lipid autoxidation products [21]. Isotope-reinforced PUFAs are not diluted by endogenous PUFAs in yeast, because yeast synthesize only saturated and monounsaturated fatty acids and do not

[1] *Abbreviations:* αLnn, α-linolenic acid (C18:3, *n*–3); ARA, arachidonic acid (C20:4, *n*–6); BHT, butylated hydroxytoluene; D, deuterium; D_2-Lin, 11,11-D_2-linoleic acid or ethyl ester; D_4-αLnn, 11,11,14,14-D_4-α-linolenic acid; EPA, eicosapentaenoic acid (C22:5, *n*–3); EtOAc, ethylacetate; GC-MS, gas chromatography-mass spectrometry; HODE, hydroxyoctadecadienoic acid or hydroxyoctadecadienoate; IE, isotope effect; KIE, kinetic isotope effect; KODE, keto-octadecadienoic acid or ketooctadecadienoate; Lin, linoleic acid or ethyl ester (C18:2, *n*–6); MeOAMVN, 2,2'-azobis(4-methoxy-2-dimethylvaleronitrile); Ole, oleic acid (C18:1, *n*–9); PPh_3, triphenylphosphine; PUFA, polyunsaturated fatty acids; Q, coenzyme Q or ubiquinone; QH_2, coenzyme QH_2 or ubiquinol; ROS, reactive oxygen species; YPD, rich growth medium with dextrose.

require PUFAs as essential nutrients [22]. Thus, PUFAs content can be readily manipulated, and isotope-reinforced PUFAs can provide the sole source of PUFAs in the yeast cell.

However, PUFAs are essential components of animal cells, and the total replacement of essential PUFAs in animals with isotope-reinforced PUFAs is a daunting prospect. We report herein the kinetic isotope effect of autoxidation of 11,11-D_2-Lin in solution. Inclusion of only a small fraction of PUFAs deuterated at the bis-allylic sites is sufficient to profoundly inhibit the chain reaction in non-deuterated PUFAs in yeast. The exogenously added D-PUFAs slow detrimental lipid autoxidation within live yeast cells, and are effective even when present at low ratios in cell lipids. The results suggest that it may be practical to ameliorate ROS-initiated PUFA damage with the isotope-reinforcement approach.

Experimental Procedures

Fatty acids

The fatty acids used in this study are shown in Fig. 1. Ole, Lin, and αLnn (99% pure) were from Sigma-Aldrich. The synthesis of 11,11-D_2-Lin and 11,11,14,14-D_4-αLnn was described previously [21]. The synthesis of 8,8-D_2-Lin, 11,11-D,H-Lin, and 11-^{13}C-Lin is described in Supplementary Material.

Radical clock and co-oxidation experiments

Determination of rate constants for peroxidation of Lin and D_2-Lin were performed as previously described [23, 24]. PUFAs were purified by flash column chromatography (10% EtOAc in hexanes to 20% EtOAc in hexanes) and dried overnight on vacuum. A stock solution of 0.1 M 2,2'-azobis(4-methoxy-2,4-dimethyl)valeronitrile (MeOAMVN) in benzene was used to initiate all reactions. Standards used in analysis were 4-methoxybenzyl alcohol (HPLC-UV) and D_4-13-*trans,cis*-HODE (HPLC-MS). In all clocking or competition experiments, reagents were added in the order of: 1) benzene, 2) Lin/11,11-D_2-Lin (ethyl esters or free acids), 3) MeOAMVN. Reaction vials were vortexed for 5 seconds, and then heated at 37 °C for one hour. Each reaction was quenched by addition of 25 μL of both 0.5 M BHT (to quench radicals) and 0.5 M PPh_3 (to reduce hydroperoxide to alcohol). All experiments were carried out in triplicates.

In clocking experiments, Lin or 11,11-D_2-Lin ethyl esters were used and the oxidation products, HODEs, were analyzed by normal phase HPLC-UV (250 × 4.6 mm silica column; 5 μm; elution solvent: 0.5% 2-propanol in hexanes; monitoring wavelength: 234 nm). The residual amount of 11-D_1-Lin and D_0-Lin in the 11,11-D_2-Lin starting material was determined to be 2.9 and 0.8 mole %, respectively, from ^{1}H NMR analysis and these values were used to correct the data from 11,11-D_2-Lin assuming that 11-D_1-Lin is half as reactive as D_0-Lin.

In competition experiments, the total amount of Lin free acids (Lin + 11,11-D2-Lin) in each experiment was held constant at 0.64 M. After the reaction was quenched by addition of BHT and PPh_3 (*vide supra*), 4-methoxybenzyl alcohol was added as an internal standard for HPLC-UV analysis and each reaction was divided into two parts. One part was analyzed by HPLC-UV for quantification of total HODEs formed in each reaction (250 × 4.6 mm silica column; 5 μm; elution solvent: 1.4% 2-propanol and 0.1% acetic acid in hexanes; monitoring wavelength: 234 nm). The other part was analyzed by HPLC-MS using D_4-13-*trans,cis*-HODE as the internal standard (150 × 4.6 mm silica column; 3 μm; elution solvent: 1.4% 2-propanol and 0.1% acetic acid in hexanes) with selective reaction monitoring [24, 25]. Among these HPLC-MS analyses, the reactions that have large D_2-Lin:Lin ratio (>5:1) were used to calculate the KIE by comparing the total D_0-HODEs vs. total D_1-HODEs. Note that D_1-HODEs formed from 11-D_1-Lin and isotopic contribution from D_0-HODE were

taken into consideration when calculating the actual amount of D_1-HODEs that were formed from 11,11-D_2-Lin. Specifically, the percentage of 11-D_1-Lin in the 11,11-D_2-Lin starting material, 2.9 mole % (*vide supra*), was used to correct the D_1-HODEs formed from D_1-Lin assuming that 11-D_1-Lin is half as reactive as D_0-Lin in solution. The isotopic contribution to the D_1-HODEs from D_0-HODEs was determined by running the same HPLC-MS analysis on the autoxidation of pure D_0-Lin.

KODEs (corresponding ketones from HODEs) were also analysed [25] in reactions with D_2-Lin: Lin ratios varying from 0 to 9. After HPLC-UV analysis was complete for each co-oxidation, solvent was removed from each vial. The resulting oil was dissolved in $CDCl_3$ for ^{1}H-NMR analysis to determine the true ratio of D_2-Lin: Lin in each sample (excluding vials which were exclusively Lin) by comparing the integration of the vinyl protons and the bis-allylic protons.

Yeast strains and growth media

Yeast strains used in this study included wild-type, W303-1B (Mat α *ade2-1 his3-1,15 leu2–3,112 trp1-1 ura3-1*) [26], W303ΔCOR1 (Mat α *ade2-1 his3-11, 15 leu2–3, 112 ura3-1 trp-1 can1-110 cor1::HIS3*) [26], CC303.1 (Mat α *ade2-1 his3-1,15 leu 2–3, 112 trp1-1 ura3-1 coq3::LEU2*) [27], and W303Δcoq9 (Mat α *ade2-1 his3-1,15 leu2–3,112 trp1-1 ura3-1 coq9::URA3*) [28]. Yeast growth media were prepared as described [29] and included YPD (1% yeast extract, 2% yeast peptone, 2% dextrose). Solid plate medium contained 2% Bacto agar. Components for growth media were obtained from Difco, Fisher, and Sigma.

Fatty acid sensitivity assays

Fatty acid sensitivity assays were performed as described [21]. Yeast were inoculated overnight in 5 ml YPD with aeration (250 rpm at 30 °C). Yeast cells were diluted to 0.2 OD/ml in YPD media and were grown to mid-log phase (0.2–1.0 OD_{600}). Yeast cells were harvested at mid-log phase and collected by centrifugation (5 min at 1,000 × *g*) followed by two washes with sterile water. Yeast cells were diluted to 0.2 OD/ml in 0.10 M phosphate buffer (0.2% dextrose, pH 6.2). Aliquots (5 ml) were placed in overnight tubes and treated with the designated fatty acid and incubated at 30 °C with aeration (250 rpm). Aliquots were removed at the designated times and viability was assessed by spotting 2 μl of 1:5 serial dilutions (starting at 0.20 OD/ml) onto YPD plate medium. Pictures were taken after two days of growth at 30 °C.

Yeast cell lipid peroxidation assay

Yeast cells were subjected to treatment with fatty acids as described above and the assay of lipid peroxidation was performed as described [30], with the following modifications. Aliquots (10 ml) were removed from incubator (250 rpm at 30 °C) at the designated time and cells were washed twice with sterile water to remove excess fatty acids. Cells (2.0 OD_{600}) were resuspended in 1 ml 0.10 M phosphate buffer (0.2% dextrose, pH 6.2) and treated with 4 μl of an ethanolic stock of 2 mM C11-BODIPY(581/591) (Molecular Probes) for 30 min at room temperature with shaking on a flat surface. Cells were collected by centrifugation at 10,000 × *g* for 30 sec, washed and resuspended in 1 ml 0.10 M phosphate buffer (0.2% dextrose, pH 6.2). For assays performed in wild-type cells, lipid peroxidation was induced with 50 μM $CuSO_4$ at room temperature. Aliquots (100 μl) were placed into a black, flat-bottomed 96-well plate in quadruplicates and the OD_{595} was measured. Fluorescence was measured with a 485 nm excitation and a 520 nm emission filter in a Perkin Elmer, 1420 Multi label Counter and data was obtained with the Wallac workstation. Cells were visualized by fluorescent microscopy using excitation at 490 nm with a 520 nm emission filter. An aliquot of resuspended cells (9 μl) were placed on microscope slides (Fisher Scientific, 3” × 1” × 1mm) containing 1 μL of an ethanolic stock of 0.25 mg/ml

DAPI (Molecular Probes). Microscope covers were pre-incubated with 30 μL of poly-L-lysine solution (Sigma-Aldrich) and were placed on microscope slides.

Fatty acid uptake and GC-MS detection of fatty acids

Fatty acid uptake and saponification, lipid extraction and alkaline methanolysis were performed as described [21]. GC-MS analyses of fatty acid methyl esters were performed as described [21], with the following modifications. Samples were subjected to analyses by GC-MS (Agilent 6890–6975) with a DB-wax column (0.25 mm inner diameter × 30 m length × 0.25-μm film thickness) (Agilent catalog number 122–7032). The temperature program was set to 100 °C for 1 min, 20 °C/min to 200 °C for 10 min, 15 °C/min to 250 °C, final temperature for 5 min and 1 μl was injected, with a split ratio of 1:10.

RESULTS

Synthesis of site specifically deuterated PUFAs

Isotope-reinforced Lin and αLnn containing two or four D at the bis-allylic sites (11,11-D_2-Lin and 11,11,14,14-D_4-αLnn; Figure 1) were synthesized as described previously [21]. Modifications of the published protocols [31, 32] were used to synthesize 8,8-D_2-Lin, 11,11-D,H-Lin, and 11-^{13}C-Lin (Fig. 1) as described in Supplemental Materials. The structures and purity of deuterated PUFAs were confirmed by ^{1}H and ^{13}C NMR (Supplemental Materials).

Kinetic measurements reveal a large KIE for the chain reaction of Lin autoxidation

Autoxidation of Lin is known to occur by a chain reaction process described by the following equations [6]:

$$\textbf{Initiation}: \mathrm{In{-}In} \rightarrow \mathbf{2}\ \mathrm{In}^{\bullet} \quad (1)$$

$$\mathrm{In}^{\bullet} + \mathrm{LH} \xrightarrow{k_{\mathrm{iLH}}} \mathrm{In{-}H{+}L}^{\bullet} \quad (2)$$

$$\textbf{Propagation}: \mathrm{L}^{\bullet} + \mathrm{O_2} \xrightarrow{k_{\mathrm{perox}}} \mathrm{LOO}^{\bullet} \quad (3)$$

$$\mathrm{LOO}^{\bullet} + \mathrm{LH} \xrightarrow{k_{\mathrm{p}}} \mathrm{LOOH{+}L}^{\bullet} \quad (4)$$

$$\textbf{Termination}: \mathbf{k_t}\ \mathbf{2}\ \mathrm{LOO}^{\bullet} \rightarrow [\mathrm{L{-}OO{-}OO{-}L}] \quad (5)$$

$$[\mathrm{L{-}OO{-}OO{-}L}] \rightarrow \mathrm{Non{-}radical\ products{+}O_2} \quad (6)$$

We utilized MeOAMVN to initiate formation of two carbon-centered radicals (equations 1 and 2) that add oxygen at diffusion-controlled rates to generate two peroxyl radicals (equation 3). The rate of propagation of the chain reactions is determined by k_p (equation 4). To determine the effect of the isotope reinforcement on the propagation rate constants of Lin autoxidation as compared to that of 11,11-D_2-Lin, we performed parallel clocking experiments utilizing a linoleate peroxyl radical clock [6, 23, 24], as well as competition experiments by co-oxidizing 11,11-D_2-Lin and Lin in solution.

In clocking experiments, the concentrations of Lin or 11,11-D_2-Lin ethyl esters were varied from 0.14 to 2.1 M. The ratios of *trans,cis*-HODEs to *trans,trans*-HODEs were plotted against the concentrations of Lin or 11,11-D_2-Lin to obtain the slope that was used to calculate the KIE [23] (Fig. 2). The slopes obtained for the 11,11-D_2-Lin clocking experiments were corrected based on the content of 11-D_1-Lin and D_0-Lin in the starting material assuming that 11-D_1-Lin is half as reactive as D_0-Lin in free radical chain oxidation. As the propagation rate constant is proportional to the slope of clocking plots [23] shown in Fig. 2, the KIE (i.e., ratio of the slopes for Lin and 11,11-D_2-Lin) was thus found to be 9.3 ± 1.1 by the application of this method.

To further elucidate the kinetics and KIE of 11,11-D_2-Lin autoxidation, competition experiments (co-oxidation of Lin and 11,11-D_2-Lin) were carried out. A typical chromatogram of HODE free acids is shown in Fig. 3A. The order of elution is as follows: 13-t,c-HODE (t = 9.5 min.), 13-t,t-HODE (t = 14 min.), 9-t,c-HODE (t = 16.5 min), 9-t,t-HODE (t = 18.5 min.), 4-methoxybenzyl alcohol (t =20.5 min.). The same mobile phase is used for HPLC-MS analyses, and the same order of elution is observed as shown in Fig. 3B, in which both D_0-HODEs and D_1-HODEs were analyzed.

By keeping the total concentration of (11,11-D_2-Lin + Lin) constant and varying the ratio of 11,11-D_2-Lin: Lin, a linear relationship was observed in co-oxidation experiments between the total amounts of HODEs formed and the percentage of D_2-Lin (no turning point was observed), suggesting D_2-Lin did not act as an antioxidant in the co-oxidation reactions and only acted as a less reactive co-oxidant in the autoxidations carried out in solution (Fig 3C).

The KIE was also measured by a direct competition method. When Lin and D_2-Lin are co-oxidized, the ratios of the total HODEs formed from Lin (HODEs) and those from D_2-Lin (D-HODEs) reflects the pseudo-first-order rate constant of the Lin and D_2-Lin ([HODEs]/[D -HODEs] = k_{Lin}[D_0-Lin]/$k_{D2\text{-}Lin}$[D_2-Lin]). Ratios of $k_{Lin}/k_{D2\text{-}Lin}$ can thus be calculated. Because D-HODEs formed from H,D-Lin (present in D_2-Lin as determined by NMR) and isotopic peaks of HODEs will contribute to the D-HODEs signal, these contributions were subtracted from the total D-HODEs signals (see Materials and Methods). It is also important to note that the percentage of oxidation after one hour was measured to be approximately 2 % for pure Lin. Based on this it can be assumed that the percentage of oxidation in other samples with higher concentrations of D_2-Lin will be lower than that for Lin (< 2%). The small oxidation extent suggests that the concentrations of Lin and D_2-Lin did not change significantly during oxidation, a requirement for calculating the KIE. The average KIE value calculated from HPLC-MS data of co-oxidation experiments for samples with D_2-Lin: Lin ratios greater than 5:1 was found to be 12.8 ± 0.6 (Table 1).

KODEs can be formed from the radical termination reaction. In the series of co-oxidation reactions carried out here with D_2-Lin: Lin ratios varying from 0 to 9, KODEs were formed at very low levels (0.5–0.8% of the levels of HODEs), suggesting that, based on these products, the termination step was not affected significantly by the presence of D_2-Lin in solution oxidation.

Isotope reinforcement at the bis-allylic position of PUFAs is required to protect Q-less yeast against lipid autoxidation

Previous studies have shown that Q-less yeast *coq* mutants are exquisitely sensitive to PUFA treatment [27] and can be rescued by isotope-reinforced PUFAs [21]. Here we show that site-specific reinforcement at the bis-allylic position is essential for this protection, since treatment with mono-allylic 8,8-D_2-Lin failed to protect Q-less yeast (Fig. 4A). The sensitivity of the yeast *coq3* null mutant to PUFA treatment is not due to the inability to

respire since the respiratory deficient *cor1* null mutant lacking complex III does not show sensitivity to PUFA treatment.

A lipophilic dye, C11-BODIPY(581/591) was used to monitor lipid peroxidation in the *coq3* null mutant yeast cells treated with PUFAs. This C11-BODIPY(581/591) probe is a fluorescent fatty acid analogue used as an indicator of lipid peroxidation and antioxidant efficacy in membrane systems and living cells [33]. Upon oxidation of C11-BODIPY(581/591) there is a fluorescent shift from red to green [33]. Yeast cells were treated with PUFAs for 2 h, and then treated with C11-BODIPY(581/591) for 30 min. Following 2 h of PUFA treatment, the yeast *coq3* mutant cells are still viable as determined by the number of colony forming units present (Fig. 4B). Lipid peroxidation in the yeast *coq3* null mutant cells was monitored with a multi-well fluorescent microplate reader and by fluorescent microscopy. The yeast *coq3* null mutants treated with Lin or mono-allylic 8,8-D_2-Lin have much higher levels of lipid peroxidation as compared to either untreated cells or cells treated with bis-allylic 11,11-D_2-Lin (Fig. 4C and 4D). As shown in Figure 4, lipid peroxidation resulting from treatment with Lin or 8,8-D_2-Lin is evident after 2 h as detected with the C11-BODIPY probe and precedes cell death.

Q-less yeast are protected by mono-deuterated isotope-reinforced PUFA

We wished to investigate further the dramatic protection afforded by the site-specific deuteration of the bis-allylic H atoms of PUFAs. We reasoned that if the resistance to autoxidation was attributed primarily to the rate-limiting abstraction of a H atom from the bis-allylic site, then a “half-deuterated” Lin (11,11-D,H-Lin) would be expected to lose most of its protective effect. Surprisingly, treatment with the mono-deuterated Lin afforded the same degree of protection to the Q-less *coq3* yeast mutant as the 11,11-D_2-Lin (Fig. 5A). A shorter treatment with Lin (3 hours) resulted in a 50% loss of cell viability in the yeast *coq3* mutant (Fig. 5B). In contrast, no loss in cell viability was detectable after 3 hours of treatment with either mono-deuterated Lin, or 11,11-D_2-Lin. Yeast *coq3* mutant cells treated with either mono-deuterated Lin or the 11,11-D_2-Lin were resistant to lipid peroxidation, as monitored with the C11-BODIPY(581/591) probe (Fig. 5C).

Q-less yeast are protected by small amounts of isotope-reinforced PUFA

Our previous study showed that isotope-reinforcement of only one of the two bis-allylic sites present in linolenic acid (11,11-D_2-αLnn or 14,14-D_2-αLnn) provided a similar degree of protection against PUFA sensitivity as compared to the fully reinforced 11,11,14,14-D_4-αLnn [21]. These results, together with the protection afforded by the mono-deuterated 11,11-D,H-Lin (Fig. 5), suggested that mixtures containing isotope-reinforced PUFA together with natural PUFA may mimic the robust protection afforded by partial isotope reinforcement. To test the effect of PUFA mixtures in the yeast cell model, we subjected yeast to PUFA treatments containing only a small proportion of isotope-reinforced PUFAs. As shown in Fig. 6A, PUFA mixtures containing as little as 20% of isotope-reinforced PUFAs afforded dramatic protection, as shown by the viability of the *coq3* Q-less yeast following serial dilution and plating onto rich growth medium. Although treatment for 30 min with αLnn did not affect *coq3* mutant cell viability (Fig. 6B), this brief treatment resulted in lipid peroxidation products as assayed with the C11-BODIPY(581/591) probe (Fig. 6C). In contrast, inclusion of only 20% 11,11,14,14-D_4-αLnn inhibited the formation of lipid peroxidation products from the 80% αLnn present in the mixture (Fig. 6C).

A small fraction of either type of reinforced PUFA afforded “cross-over” protection as shown by the efficacy of 11,11-D_2-Lin to protect against αLnn treatment, or 11,11,14,14-D_4-αLnn to protect against Lin treatment (Fig. 7). Yeast cells do not appear to discriminate among the PUFAs provided, since the content of PUFAs taken up by yeast cells roughly

reflects the ratios added (Table 2). The results of these studies suggest that the dramatic protection afforded by small amounts of isotope-reinforced PUFAs are likely to derive from the multi-step process of lipid autoxidation, which serves to amplify even small kinetic isotope effects. Alternatively, it is possible that the D-PUFA• radical may have properties different from the standard H- PUFA• radical.

The yeast coq3 null mutant is sensitive to a fatty acid mixture containing monounsaturated and polyunsaturated fatty acids

To determine whether the protection afforded by partial amounts of isotope-reinforced PUFA may simply reflect the decreased amount of vulnerable (natural) PUFA present in the mixing experiments, yeast *coq3* mutants were treated with a 50:50 mixture of Ole: αLnn. Plate dilution assays revealed that after four hours of treatment there was a profound loss of yeast *coq3* null mutant viability (Fig. 8A). Similarly, yeast *coq9* mutants were sensitive when treated with a 50:50 mixture of Ole: Lin, but were protected upon treatment with 50:50 Lin: 11,11-D_2-Lin (Fig. 8B). These results suggest that the isotope-reinforced PUFAs inhibit the process of lipid autoxidation and do not merely act to dilute the H-PUFAs susceptible to lipid autoxidation.

To investigate the protection afforded by the mono-deuterated 11,11-D,H-Lin, we reasoned that secondary isotope effects may govern the abstraction of the bis-allylic H atom adjacent to the D at position 11. Such small effects, when amplified over many chain reaction steps might be sufficient to slow the generation of toxic autoxidation products and preserve cell viability. The value of the primary KIE for abstraction of the bis-allylic H from 11-^{13}C-Lin is roughly comparable to the secondary KIE for the H abstraction off the 11-CHD methylene group [34, 35]. However, we observed that yeast *coq9* mutants were nearly as sensitive to treatment with 11-^{13}C-Lin as they were to treatment with Lin (Fig. 8B). This result argues against the importance of small effects on the abstraction stage of the process as accounting for the protection afforded by the 11,11-D,H-Lin to inhibit the chain reaction. Instead, the presence of the D-Lin• radical may be important. Accordingly, we tested whether the 11,11-D,H-PUFA could cross-protect when mixed with Lin. As shown in Fig. 8B, although yeast *coq9* mutants were resistant to treatment with mono-deuterated 11,11-D,H-Lin, the mutants remained sensitive when treated with a 50:50 mix of mono-deuterated 11,11-D,H-Lin:Lin. Taken together, these observations hint that both the abstraction step, and the presence of D in the radical system, are important for cross protection.

Copper-treated wild-type yeast treated with isotope-reinforced PUFAs have low levels of lipid peroxidation

We employed a model of copper-induced PUFA autoxidation previously developed by Avery et al., [36]. Wild-type yeast cells do not exhibit high levels of lipid peroxidation when treated with PUFAs. However, wild-type yeast cells treated with natural PUFAs and copper have increased levels of lipid peroxidation as compared to no addition of copper (Fig. 9A and 9B). We found that wild-type cells treated with either Lin or mono-allylic 8,8-D_2-Lin failed to protect against lipid peroxidation induced by copper stress. In contrast, copper-stressed wild-type yeast cells treated with 11,11-D_2-Lin have lower levels of lipid peroxidation similar to yeast not treated with PUFAs (Fig. 9). In this copper-stress yeast model, the mono-deuterated 11,11-D,H-Lin offers protection similar to the 11-11-D_2-Lin (Fig. 10).

Small amounts of isotope-reinforced PUFA protect yeast cells from long chain PUFAs stress

We tested the effect of small amounts of 11,11-D_2-Lin on the stress imposed by a biologically important class of long chain PUFAs, including arachidonic acid (ARA, 20:4,

n–6), and eicosapentaenoic acid (EPA, 20:5, n–3). As shown in Fig. 11, Q-less yeast were sensitive to treatment with either ARA or EPA long chain PUFAs, and inclusion of just 20% of 11,11-D_2-Lin provided dramatic protection.

DISCUSSION

Membrane lipids are highly vulnerable to oxidative damage due to the facile abstraction of bis-allylic H atoms from PUFAs [37]. Here we assess the effects of site-specific isotope-reinforcement of PUFAs, where the vulnerable bis-allylic H atoms are replaced with deuterium. We exploit the yeast *S. cerevisiae* to monitor lipid autoxidation within cells and to assess the toxicity of lipid autoxidation products. We also utilize *in vitro* assays of non-enzymatic PUFA autoxidation reactions to determine the kinetic D/H isotope effect. Both the yeast cell and *in vitro* assays suggest that substitution of bis-allylic H atoms with D atoms profoundly slows PUFA autoxidation and the generation of toxic products.

In this study we determine the KIE for PUFA autoxidation in solution. It is well established that deuteration substantially slows down the rate of enzymatic oxidation of 11,11-D_2-Lin, with KIE values in the range of 80–100 [38, 39]. We found that there is a substantial KIE (12.8 ± 0.6) for the 11,11-D_2-Lin autoxidation as compared to a non-deuterated Lin control from competition kinetic measurements (Fig. 3 and Table 1). The KIE obtained using parallel radical clock measurements of Lin and D_2-Lin ethyl ester, 9.3 ± 1.1 (see results section and Fig. 2) is somewhat less than the values obtained from the competition method. However, the KIE obtained from the competition experiments is more reliable because of the error that occurs in the clocking experiments of 11,11-D_2-Lin due to the low slope of the plot of *trans*,*cis*-/*trans*,*trans*-HODEs for this deuterated compound.

The HODE measurement mainly reflects the propagation step of the chain reaction (k_p, equation 4). The KIE of 12.8 falls in the upper range of previously reported values for primary deuterium KIE of free radical peroxidation, which normally are less than 10 [40–43]. The reaction was initiated by controlled decomposition of an azo initiator (MeOAMVN, $k_d = 3.2 \times 10^{-5}\ s^{-1}$, $\tau_{1/2} = 6$ h) [44]. In addition, the termination step was not affected significantly by the presence of 11,11-D_2-Lin in co-oxidation in solution based on the fact that the levels of KODEs, known peroxyl radical termination products [40], did not change significantly with the levels of 11,11-D_2-Lin. Thus, all three stages of the free radical peroxidation (initiation, propagation, and termination) were accounted for in our analysis of Lin autoxidation in solution.

The measured KIE value for 11,11-D_2-Lin autoxidation by analyzing the oxidation products (HODEs), fell short of the biological effect of the yeast viability rescue observed with D_2-Lin and partially deuterated αLnn, which were found to be two to three orders of magnitude less toxic to the yeast mutants than the non-deuterated forms [21].

Also, the linear dependence of the total amount of HODEs formed on the fraction of D-PUFA in H-PUFA (Fig. 3C) did not correlate with our previous yeast results on the total inhibition of autoxidation achieved with partial deuteration at only one of the two bis-allylic sites in both 11,11-D_2-αLnn and 14,14-D_2-αLnn [21], which essentially amounted to an inhibition of autoxidation at bis-allylic sites by a 50% fraction of deuterated bis-allylic sites, i.e. a non-linear dependence.

The initiation reaction in *in vitro* studies employs azo compounds, which form a nitrogen molecule and two autoxidation initiating radicals. This may be quite different from the natural processes typically initiated by one radical moiety, making a caging effect more likely. The arrangement of PUFAs in membranes is also quite different from their distribution in solution.

We therefore turned to a yeast model to further investigate the effects of isotope-reinforced deuterated PUFAs. *S. cerevisiae* provides a sensitive model for monitoring the toxic products of lipid autoxidation. Yeast cells normally do not produce or require PUFAs, but are able to take them up and incorporate them into membrane phospholipids [45–47]. Although wild-type yeast cells are quite tolerant of PUFAs, *coq* mutant yeast lacking Q, an endogenously produced antioxidant lipid, are exquisitely sensitive to PUFA treatment [27, 48]. The site-specific replacement of the 11,11-bis-allylic H atoms of Lin with D rescues the hypersensitivity of the yeast Q-less mutants to PUFA treatment (Fig. 3) [21]. In contrast, replacement of the 8,8-mono-allylic H atoms with D provides no protection to Q-less yeast (Fig. 3). In these studies, lipid autoxidation was monitored with a membrane-intercalated indicator lipid, C11-BODIPY(581/591) [33]. Oxidation stimulates a fluorescence shift of this indicator lipid, and the time-dependent increase in fluorescence reflects the degree of lipid autoxidation [33]. Although the fluorescence does not yield quantitative information about lipid oxidation, it does give a sensitive read-out of radical processes that oxidize lipids in membranes [49]. We detected increased fluorescence and hence autoxidation products prior to loss of yeast cell viability. The results indicate that treatment of *coq* mutant yeast with either Lin or 8,8-D_2-Lin produce marked levels of lipid peroxidation as compared to either untreated cells, or cells treated with 11,11-D_2-Lin. We also used the yeast copper-dependent oxidation model developed by Avery et al., [36] to test the effects of isotope-reinforcement on copper-stressed wild-type yeast cells. We showed that copper-stressed wild-type yeast treated with isotope-reinforced PUFAs have lower levels of lipid autoxidation products as compared to treatment with standard PUFAs (Fig. 9). These results show that the protection afforded by bis-allylic isotope-reinforcement results from an enhanced resistance to autoxidation, and is not an artifact of the organic synthetic method, nor a property exerted by the presence of D atoms located at non-susceptible sites, and instead depends on the site-specific deuteration at the bis-allylic site of Lin.

We also used the yeast model to explore whether mixtures containing small proportions of isotope-reinforced PUFAs were protective. We showed that as little as 15 mol percent 11,11-D_2-Lin in Lin, or 20 mol percent 11,11,14,14-D_4-αLnn in αLnn, was sufficient to rescue the *coq* mutant hypersensitivity (Fig. 6). This protective effect is also observed when 11,11-D_2-Lin is mixed with αLnn, or when 11,11,14,14-D_4-αLnn is mixed with Lin (Fig. 7). A somewhat larger fraction of between 20% and 50% of D_2-Lin is required to inhibit the autoxidation of αLnn, while (less than) 20% D4-αLnn inhibits Lin peroxidation. Yeast cell PUFA uptake reflects the ratios of exogenously supplied PUFAs (Table 2), thus these effects are unlikely to be due to differential uptake. However, direct comparison of the optimal ratios of D_2-Lin: αLnn and D_4-αLnn: Lin that afford protection is difficult due to the different relative toxicities of Lin and αLnn. Inclusion of isotope-reinforced PUFAs along with vulnerable PUFAs slowed autoxidation as assayed in the Q-less yeast *coq* mutant (Fig. 6). In contrast, inclusion of Ole, a monounsaturated fatty acid resistant to oxidation, neither rescued cell viability nor slowed autoxidation (Fig. 8). Thus the rescue afforded by isotope-reinforced PUFAs cannot be explained by a simple dilution of the toxicant. These studies indicate that the protection exerted by small amounts of isotope-reinforced PUFAs is due to inhibition of autoxidation, and illuminate our previous findings with partially reinforced PUFAs. In previous studies we noted that treatment of yeast *coq* mutants with either 11,11-D_2-αLnn or 14,14-D_2-αLnn, (reinforced at only one of the two bis-allylic positions), were as nontoxic as 11,11,14,14-D_4-αLnn [21]. Treating yeast with mixtures of standard and reinforced PUFAs may mimic treatment with the partially reinforced 11,11-D_2-αLnn or 14,14-D_2-αLnn. Indeed, inclusion of the isotope-reinforced 11,11-D_2-Lin (20 mol%) protected *coq* mutant yeast from long chain PUFAs stress mediated by either ARA or EPA.

In yeast cells, the observed chain reaction inhibition effects essentially amount to D-PUFAs playing an antioxidant role of terminating/inhibiting the chain process. PUFA autoxidation

is initiated by the hydrogen (or deuterium, for D-PUFA) abstraction from the bis-allylic sites (Eq. 2 and Eq. 4) [6]. For 11,11-D_2-Lin, the resulting conjugated radical will still contain one deuterium atom. Conceivably, the chain reaction inhibition effects observed (large KIE and 'preservative/antioxidant') may be influenced by both the initial step (deuterium abstraction) as well as by the presence of the remaining D in the resulting radical. To analyze these two steps separately, we tested the effectiveness of mono-deuterated 11,11-D,H-Lin. Although we anticipated that the mono-deuterated 11-H atom would be nearly as vulnerable as the H atoms on standard Lin, treatment of the *coq* Q-less yeast with the mono-deuterated 11,11-D,H-Lin afforded the same degree of protection as the 11,11-D_2-Lin (Fig. 5). Our observation that the mono-deuterated 11,11-D,H-Lin is as non-toxic as the 11,11-D_2-Lin suggests that the presence of a deuterium atom in the conjugated radical system may be important to the mechanism of protection. It is possible that the D-PUFA$^{\bullet}$ radical may have altered reactivity with respect to peroxyl radical formation and/or termination reactions in a biological environment. For example, the D-PUFA$^{\bullet}$ radical generated from either 11,11-D,H-Lin or 11,11-D_2-Lin (similar to the pentadien-1,4-yl-3D$^{\bullet}$) might be less reactive than the equivalent standard PUFA$^{\bullet}$ radical (similar to the pentadien-1,4-yl-3H$^{\bullet}$) [40, 50].

We also considered the possibility that a small secondary KIE may impact the abstraction of the bis-allylic 11-H atom adjacent to the D at position 11 in the mono-deuterated Lin. Typical values of secondary KIE are 1.2 to 1.4 [51], and a small KIE are amplified over multiple steps would be expected to significantly inhibit the progress of the chain reaction of lipid autoxidation. However, the lack of protection afforded by 11-^{13}C-Lin, argues against this idea. This is because the bis-allylic H atom at position 11 is predicted to have a primary ^{13}C KIE (with values up to 1.25 [35]) comparable to the secondary KIE for the bis-allylic 11-H atom adjacent to the D at position 11 in the mono-deuterated Lin. Thus it appears that the presence of D in the radical system is important for this inhibition effect.

The profound protection afforded by the presence of a small fraction of isotope-reinforced PUFAs amidst vulnerable PUFAs is shown in a simple scheme in Fig. 12. Once a PUFA radical is produced it can attack other PUFAs in the lipid membrane (Fig. 12A). The probability of this reaction is higher in a bilayer of hydrogenated PUFAs than in a bilayer containing deuterated PUFAs because hydrogen abstraction is much easier than deuterium abstraction. The degree to which membranes are enriched in PUFAs correlates with the degree of electrophilic stress [20]. Conversely, membranes harboring just 20% D-PUFAs would be expected to impede the progress of the chain reaction (Fig. 12B). Deuterated PUFAs may provide a new approach to keeping electrophilic stress in check. Given that electrophilic stress is implicated in aging and neurodegenerative diseases [20], there are numerous applications where isotope-reinforced PUFAs may show efficacy [52, 53]. However, a major concern regarding the use of D-PUFAs as therapeutic agents has been that crucial enzymatic reactions involving PUFAs (e.g. lipoxygenase), are likely to be compromised due to large KIEs [38, 39]. The results presented here suggest that the inhibition of detrimental autoxidation can be achieved by employing relatively small amounts of D-PUFAs, so that most of the PUFAs will be in non-deuterated form, compatible with the enzymatic transformations. The described effect also makes it more feasible to build up necessary therapeutic levels of D-PUFAs *in vivo*.

The limitation of the current study is our incomplete understanding of the mechanism of the reported non-additivity (antioxidant) effect of 11,11-D_2-Lin in yeast (which is contrary to the effect observed in solution autoxidation), and the influence of deuterium atom(s) in the conjugated system (known to be able to transmit the secondary KIE across unsaturated sections due to hyperconjugation effects [54]) on the outcome of the chain reaction. Both the initial hydrogen/deuterium abstraction from the bis-allylic site and the presence of deuterium in the conjugated system seem to play a role, but their relative impacts are unknown,

although the KIE of the H atom abstraction by the cumylperoxyl radical is around 5 at low temperature [43], and it is as large as 13 for abstraction by the linoleate-peroxyl radical at 37 °C as measured in this report. At present, we are synthesizing an additional set of variously di- and mono-deuterated and ^{13}C-labelled Lin and αLnn to address these issues. Further insight will be obtained from *in vitro* kinetic studies and radical clock experiments with the isotope-reinforced PUFAs [6].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Replacement of bis-allylic H atoms with deuterium (D) arrests autoxidation of PUFA.
- Linoleic acid with just one bis-allylic D replacement also resists autoxidation.
- Linoleic acid with D at other sites (e.g. mono-allylic) remains susceptible.
- 20 mol % of deuterated PUFA profoundly inhibits the autoxidation of other PUFA
- It may be feasible to build up necessary therapeutic levels of D-PUFA in vivo.

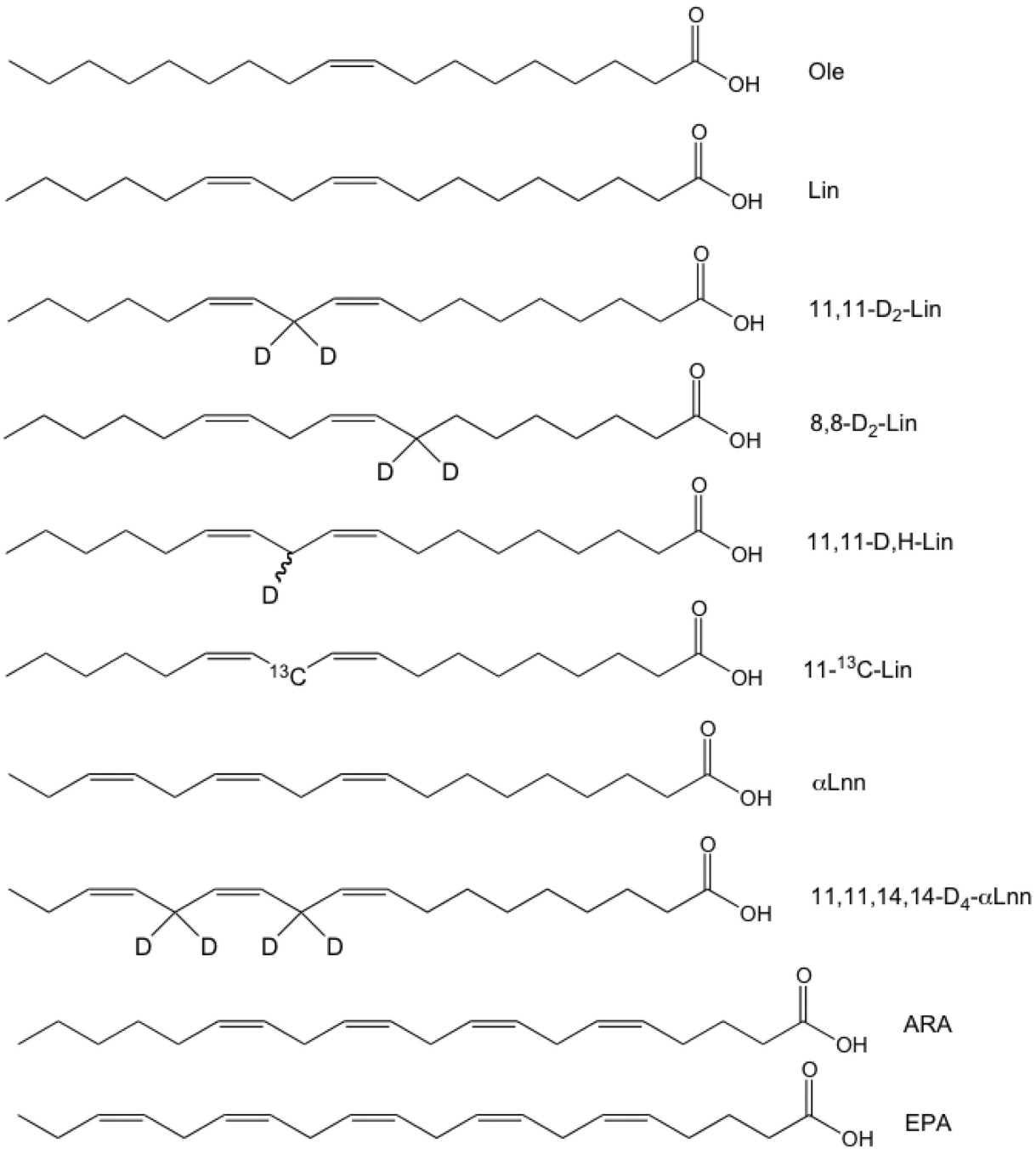

Figure 1. Structures of fatty acids used in this study

Ole, oleic acid (18:1, *cis*-9-octadecenoic acid); Lin, linoleic acid (18:2, *cis,cis*-9,12-octadecenoic acid); 11,11-D_2-Lin (11,11-D_2-18:2; 11,11-D_2-*cis,cis*-9,12-octadecenoic acid); 8,8-D_2-Lin (8,8-D_2-18:2; 8,8-D_2-*cis,cis*-9,12-octadecenoic acid); 11,11-D,H-Lin (11,11-D,H-18:2; 11,11-D,H-*cis,cis*-9,12-octadecenoic acid); 11-^{13}C-Lin (11-^{13}C-18:2; 11-^{13}C-*cis,cis*-9,12-octadecenoic acid); αLnn, linolenic acid (18:3, *cis,cis,cis*-9,12,15-octadecenoic acid); 11,11,14,14-D_4-αLnn (D_4-18:3; 11,11,14,14-D_4-*cis,cis,cis*-9,12,15-octadecenoic acid).

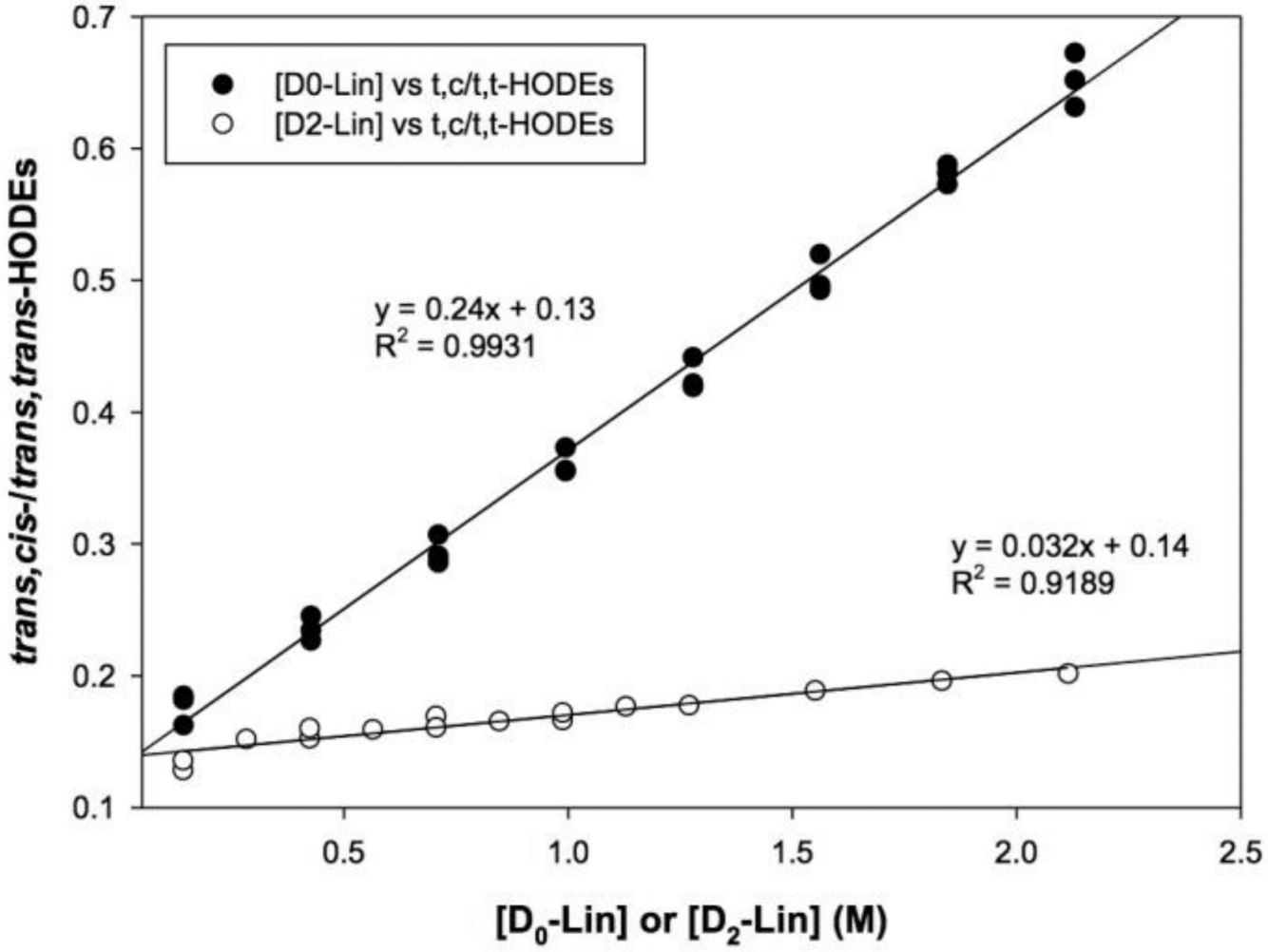

Figure 2. Clocking experiments on Lin and 11,11-D_2-Lin ethyl esters

Ratios of *trans,cis-*/*trans,trans-* HODEs versus concentration of ethyl linoleate (D_0 or D_2) were plotted. Oxidations were carried out at 37 °C in benzene for 1 h and initiated with MeOAMVN. HODE ethyl esters were analyzed by HPLC-UV (250 × 4.6 mm silica column; 5 μm; elution solvent: 0.5% 2-propanol in hexanes; monitoring wavelength: 234 nm).

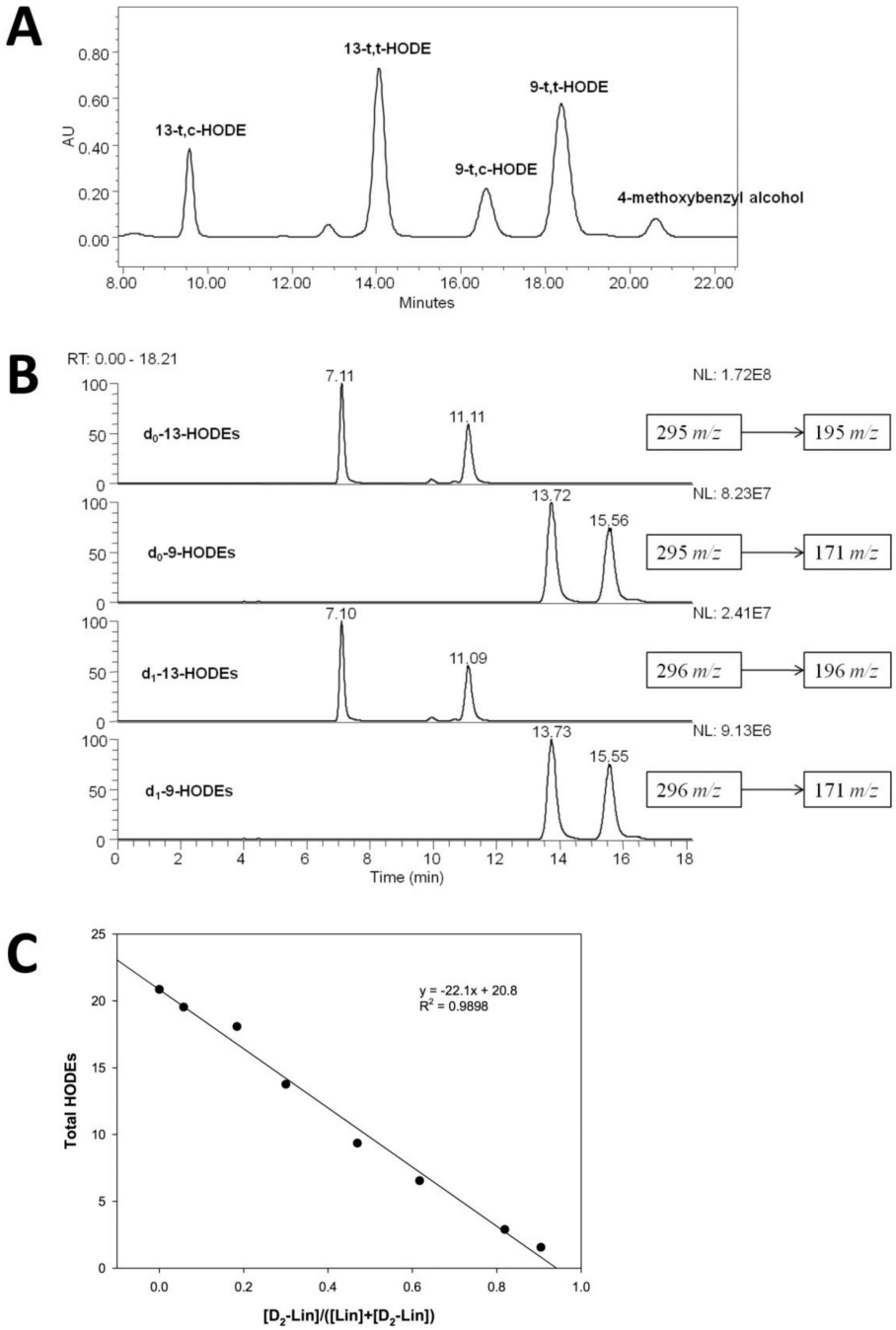

Figure 3. *In vitro* kinetic studies of PUFA autoxidation

(*A*) Typical chromatogram of HPLC-UV (250 × 4.6 mm silica column; 5 μm; elution solvent: 1.4% isopropanol, 0.1% acetic acid in hexanes; 1.0 mL/min; monitoring wavelength: 234 nm; analysis of D_0-Lin: D_2-Lin co-oxidation products. (*B*) Typical chromatogram from HPLC-MS analysis (150 × 4.6 mm silica column; 3 μm; elution solvent: 1.4% isopropanol, 0.1% acetic acid in hexanes; 1.0 mL/min) of D_0-Lin: D_2-Lin co-oxidation. The first two panels are monitoring for D_0-fragmentation ions. The last two panels are monitoring for D_1-fragmentation ions (which have a *m/z* value corresponding to a mono-deutero ion). (*C*) Total HODEs vs. percentage of 11,11-D_2-Lin in co-oxidation experiments. Co-oxidation mixtures of D_0-Lin and D_2-Lin were analyzed by HPLC-UV as

shown in panel A. Total amount of HODEs were quantified relative to the level of the internal standard, 4-methoxybenzyl alcohol, present in the sample.

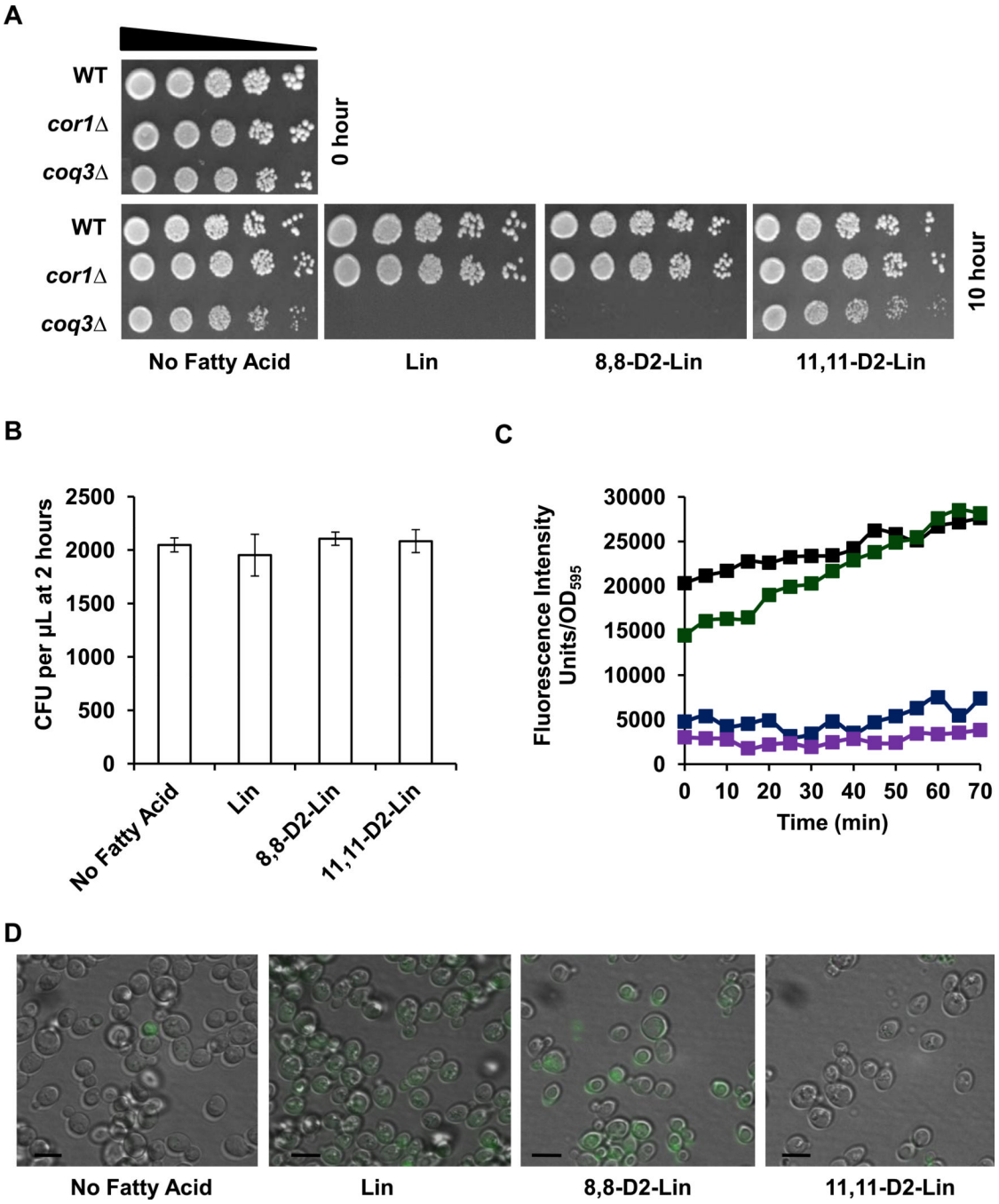

Figure 4. Isotope reinforcement at the bis-allylic position of polyunsaturated fatty acids is required for protection against lipid autoxidation

(*A*) Wild-type, yeast Q-less *coq3,* or respiratory deficient *cor1* null mutants were harvested during mid-log phase growth (0.2–1.0 OD_{600}). Yeast cells were washed twice with sterile water and diluted to 0.2 OD_{600} in phosphate buffer. Yeast cells were treated with 200 µM of the designated fatty acid. Serial dilutions (1:5) starting at 0.2 OD/ml were spotted on YPD solid plate medium. A zero-time untreated control is shown on the top left. Pictures were taken after 2 days of growth at 30 °C. (*B*) Yeast *coq3* null mutant cells were treated with the designated fatty acid for 2 hours and three 100 µl aliquots were removed and spread onto YPD plate medium after dilution. The chart shows the number of colony forming units

(CFU) per µl after 2 hours of PUFA treatment. There was no significant difference between CFU of different treatments. (*C*) After 2 hours of PUFA treatment *coq3* null mutant cells were treated with 8 µM C11-BODIPY 581/591 for 30 min at room temperature. Four 100 µl aliquots were plated in a 96-well plate and the fluorescence was measured by fluorimetry with a 485 nm excitation and a 520 nm emission filter using a Perkin Elmer, 1420 Multilabel counter, Victor3 in 5 min increments following 30 min of C11-BODIPY 581/591 treatment. Fatty acid treatments include: no treatment, *blue*; Lin, *black*; 8,8-D_2-Lin, *green*; 11,11-D_2-Lin, *purple*. (*D*) Lipid peroxidation in cells treated with the designated fatty acid was examined as described in (*C*) except cells were visualized by fluorescent microscopy using excitation at 490 nm with a 520 nm emission filter within 45 min after C11BODIPY 581/591 treatment. Lipid peroxidation was visualized by an Olympus IX70 fluorescence microscope with 100X oil objective and a 490 nm excitation and a 520 nm emission filter (FITC). (Scale bar= 6.6 µm).

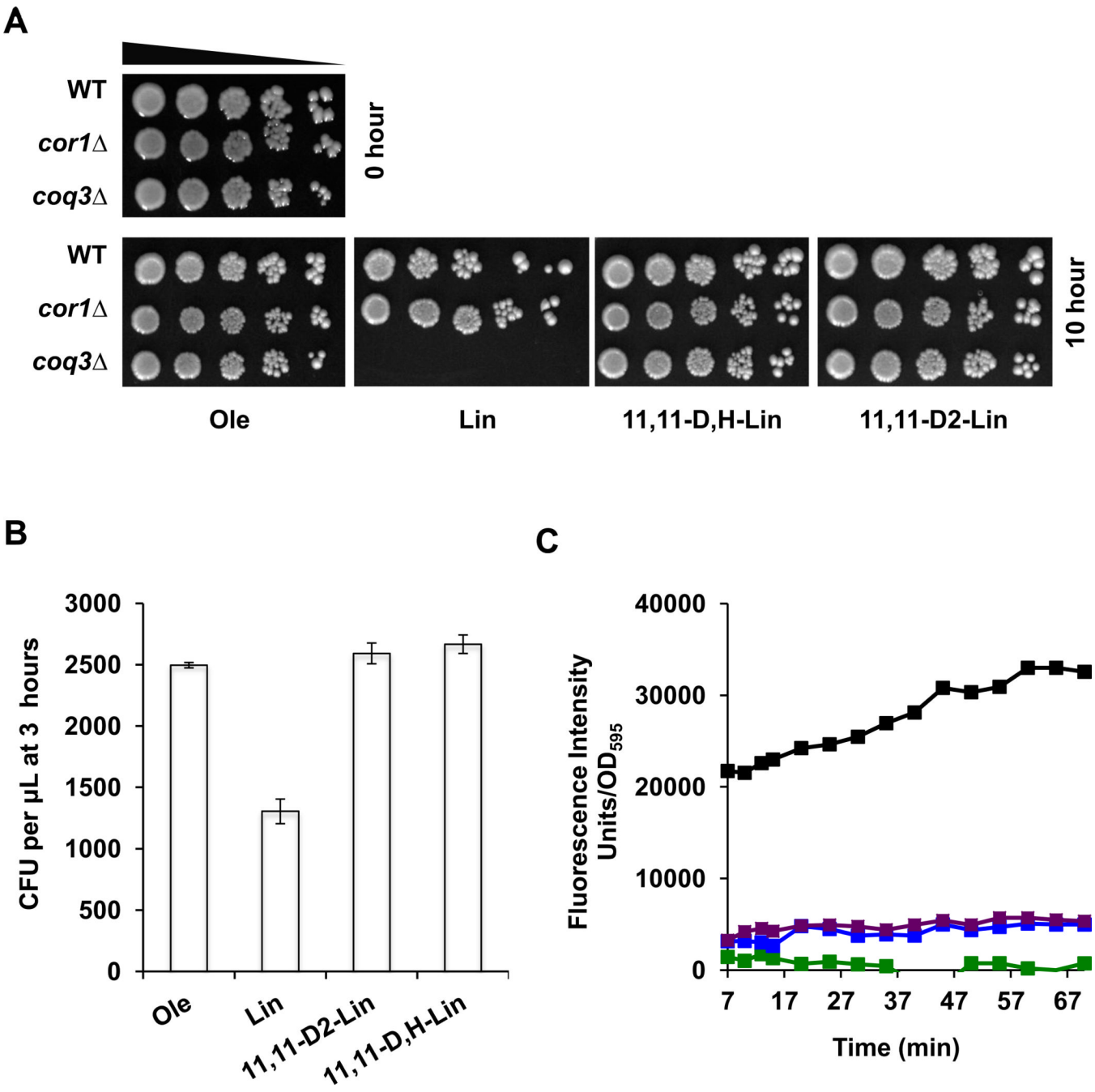

Figure 5. Yeast *coq3* null mutants treated with mono-deuterated 11,11-D,H-Lin are resistant to PUFA-induced sensitivity

(*A*) Fatty acid sensitivity assays were performed as described in Figure 4, except yeast cells were treated with 200 µM of the designated fatty acid for 10 h. A zero-time untreated control is shown in the top left. Pictures were taken after 2 days of growth at 30 °C. (*B*) *Coq3* null mutant cells were treated with the designated fatty acid for 3 hours and three 100 µl aliquots were removed and spread onto YPD plates after dilution. The chart shows the number of colony forming units (CFU) per µl after 3 hours of PUFA treatment. (*C*) After 3 hours of PUFA treatment *coq3* null mutant cells were treated with 8 µM C11-Bodipy 581/591 for 30 min at room temperature. Four 100 µl aliquots were plated in a 96-well plate and the

fluorescence was measured as described in Fig 4C. Fatty acid treatments include: Ole, *green*; Lin, *black*; 11,11-D,H-Lin, *purple*; 11,11-D_2-Lin, *blue*.

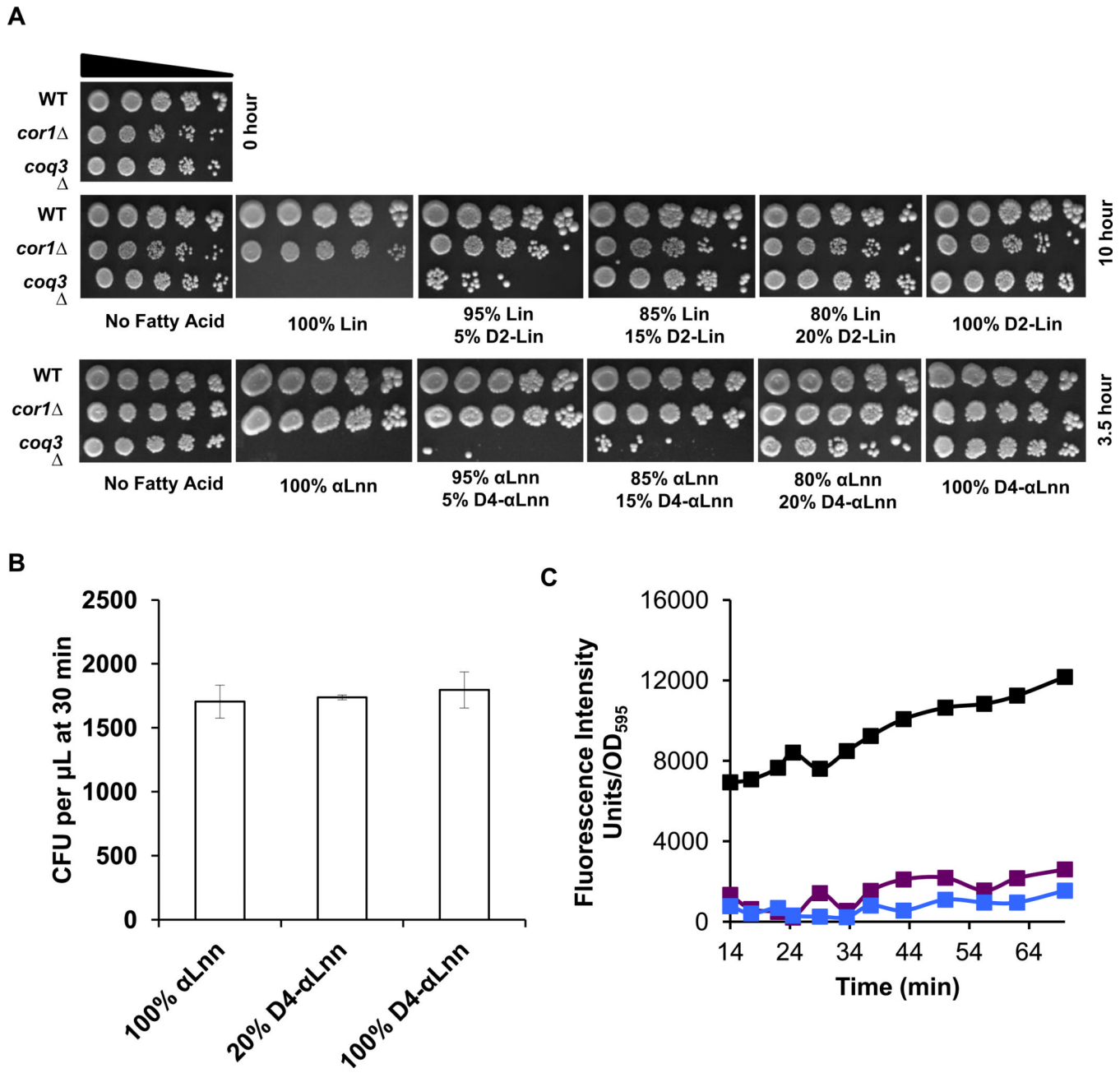

Figure 6. Only a small fraction of isotope-reinforced PUFAs is required for rescue

(*A*) Fatty acid sensitivity assays were performed as described in Figure 4 except that yeast were treated with 200 µM of the designated PUFA mixture (natural PUFA : isotope-reinforced PUFA) for either 3.5 or 10 hours. A zero-time untreated control is shown on the top left. Pictures were taken after 2 days of growth at 30 °C. (*B*) Yeast *coq3* null mutant cells were treated with the designated fatty acid mixture for 30 min and three 100 µl aliquots were removed and spread onto YPD plates after dilution. The chart shows the number of colony forming units (CFU) per µl after 30 min of PUFA treatment. There was no significant difference between CFU of different treatments. (*C*) Yeast *coq3* null mutant cells were treated with 8 µM C11-Bodipy 581/591 for 30 min at room temperature. Four 100 µl aliquots were plated in a 96-well plate and the fluorescence was measured as described in

Figure 4C. Fatty acid treatments include: 100% αLnn, *black*; 20% D_4-αLnn, *purple*; 100% D_4-αLnn, *light blue*.

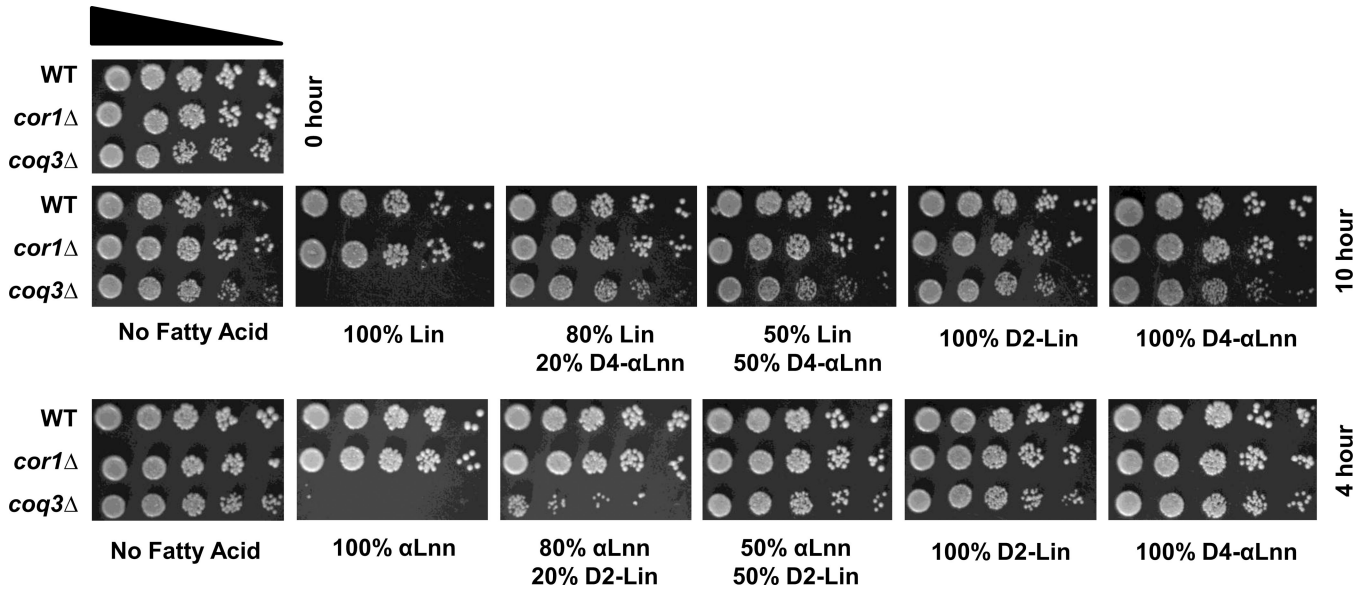

Figure 7. A small fraction of either D_2-11,11-Lin or D_4-11,11,14,14-αLnn is sufficient to rescue sensitivity of *coq3* mutant yeast cells to either PUFA treatment

Fatty acid sensitivity assays were performed as described in Figure 4 except that yeast were treated with 200 μM of the designated PUFA mixture (natural PUFA : isotope-reinforced PUFA) for either 4 or 10 hours. A zero-time untreated control is shown on the top left. Pictures were after taken 2 days of growth at 30 °C.

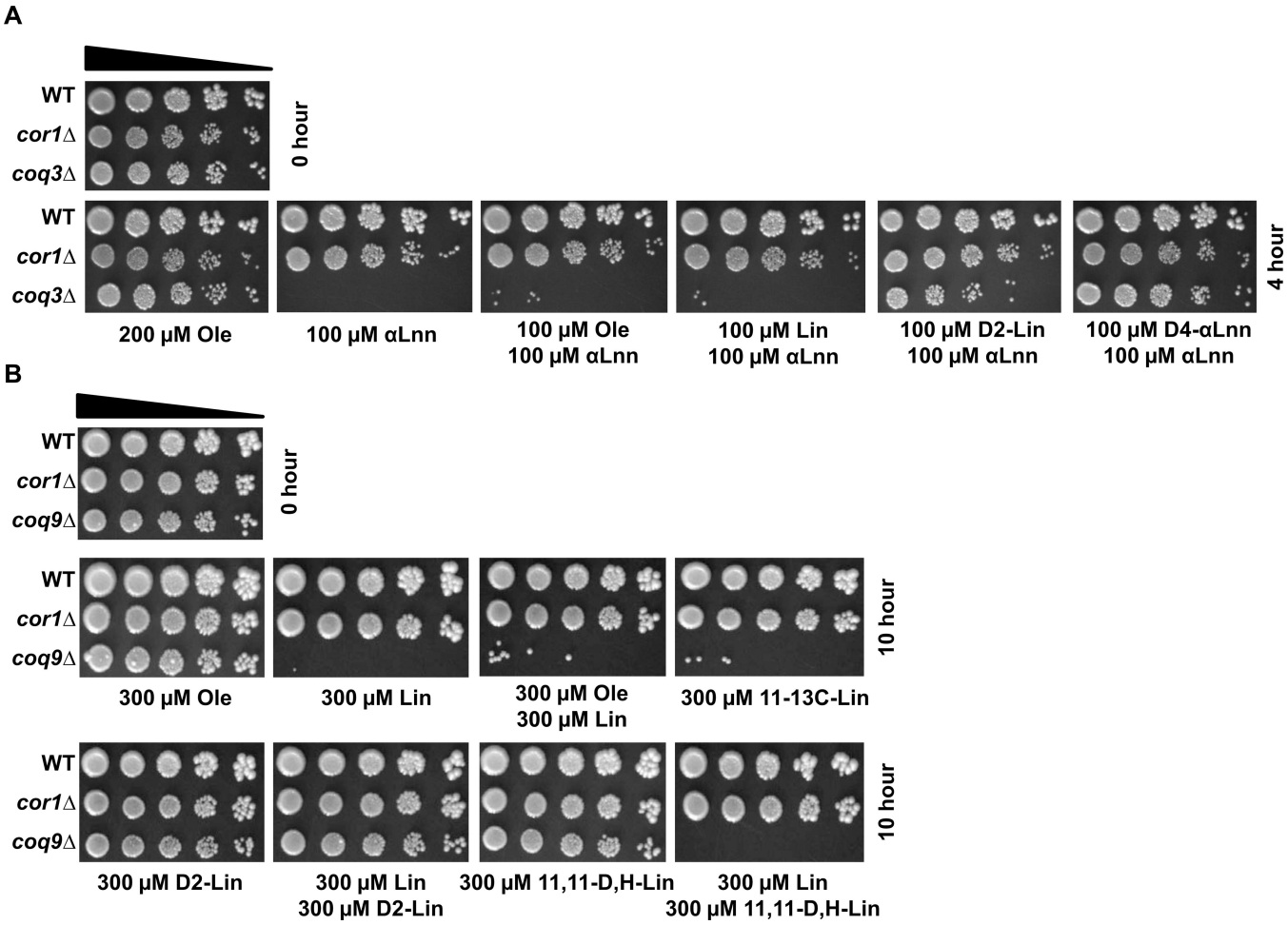

Figure 8. Yeast *coq3* and *coq9* null mutant cells are sensitive to a fatty acid mixture containing monounsaturated (Ole) and αLnn or Lin

(A) Fatty acid sensitivity assays were performed as described in Figure 4 except that yeast were treated with 200 μM Ole or 100 μM of αLnn in the presence of 100 μM of the designated fatty acid for 4 hours. A zero-time untreated control is shown on the top left. Pictures were taken after 2 days of growth at 30 °C. (*B*) Fatty acid sensitivity assays were performed as described in Figure 4 except that yeast were treated with 300 μM Ole or 300 μM of Lin in the presence of 300 μM of the designated fatty acid for 10 hours. A zero-time untreated control is shown on the top left. Pictures were taken after 2 days of growth at 30 °C.

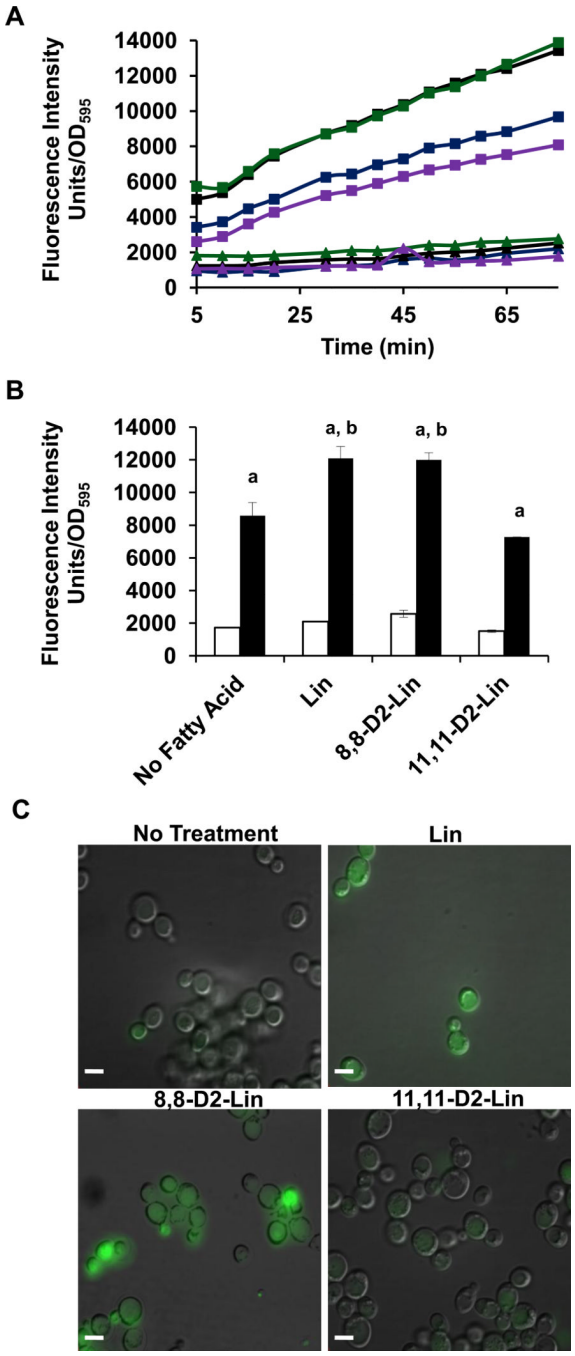

Figure 9. Isotope-reinforcement of Lin at the bis-allylic position protects copper-stressed wild-type cells from lipid peroxidation

(*A*) Wild-type yeast cells were treated as described in Figure 4 except yeast were treated with 200 μM of the designated fatty acid for 2 hours, washed with sterile water, and were either not treated (*triangles*) or treated with 50 μM $CuSO_4$ (*squares*) at room temperature. After 60 min of copper treatment cells were treated with 8 μM C11-Bodipy 581/591 for 30 min at room temperature. Four 100 μl aliquots were plated in a 96-well plate and the fluorescence was measured as described in Figure 4C. Fatty acid treatments include: no treatment, *blue*; Lin, *black*; 8,8-D_2-Lin, *green*; or 11,11-D_2-Lin, *purple*. (*B*) The chart shows the fluorescence intensity per OD_{595} at the 60 min time point shown in (A) and corresponds

to no copper (*white bars*) or copper treatment (*black bars*). Wild-type yeast cells treated with copper in the absence or presence of PUFA have significantly higher levels of lipid peroxidation as compared to yeast not treated with copper; *a*, $p<1.0E{-}4$. Wild-type yeast cells treated with copper in the presence of Lin or 8,8-D_2-Lin have significantly higher levels of lipid peroxidation as compared to yeast treated with copper in the absence of PUFA; *b*, $p<1.0E{-}4$. Differences in fluorescence signals were assessed with one-way ANOVA test followed by a Tukey comparison test (Stat View 5.0.1, SAS, CA). (*C*) Lipid peroxidation of the designated fatty acid was examined as described in Figure 4D except cells were visualized by fluorescent microscopy after 60 min of 50 μM $CuSO_4$ treatment. (Scale bar = 5 μm).

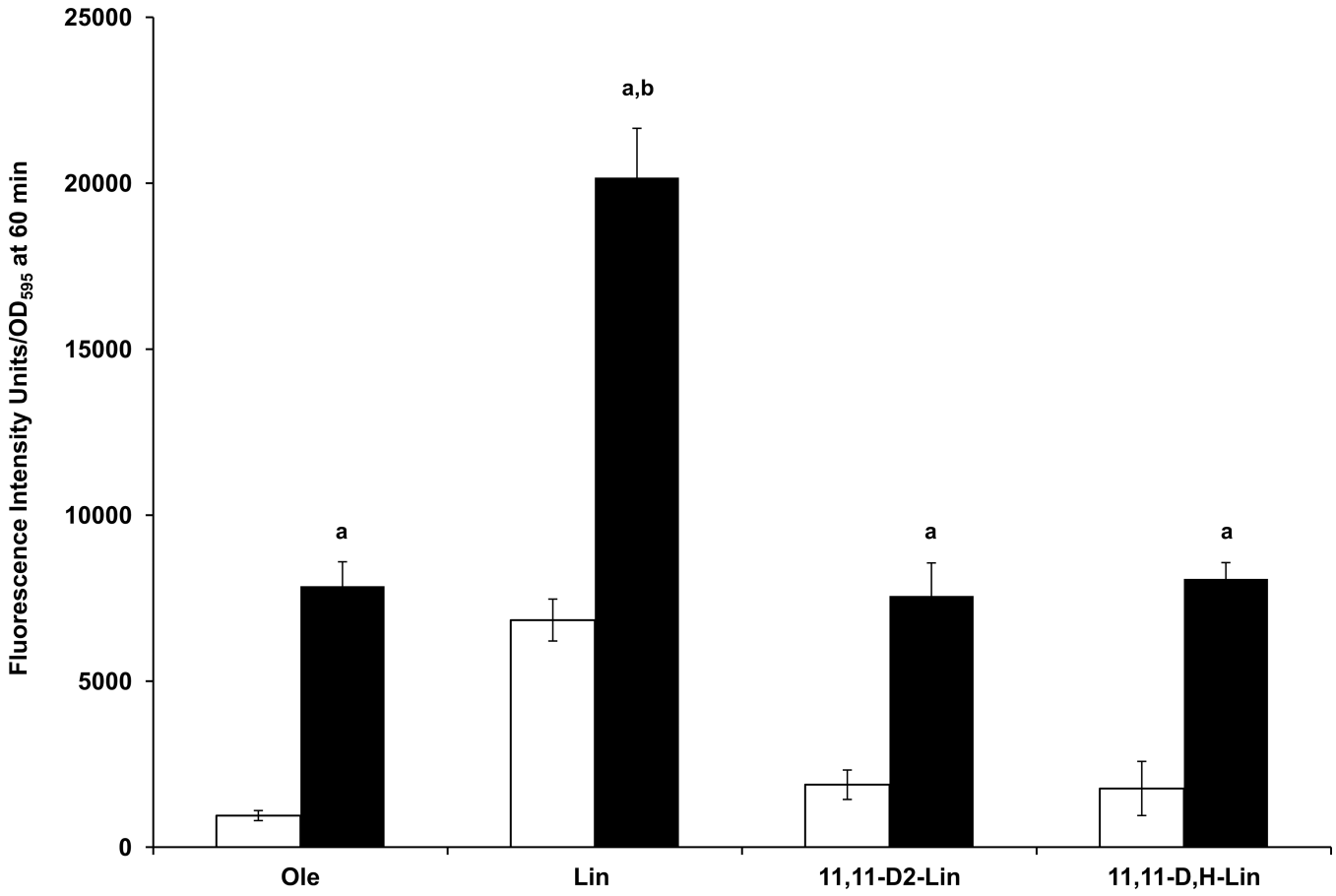

Figure 10. Both mono- and di-deuterated Lin at the bis-allylic position protect copper-stressed wild-type cells from lipid peroxidation

Wild-type cells were treated as described in Figure 4 except yeast were treated with the designated fatty acid for 3 hours, washed with sterile water, and treated with 8 μM C11-BODIPY 581/591 for 30 min at room temperature. Following BODIPY 581/591 treatment wild-type yeast were either not treated (*white bars*) or treated with 50 μM $CuSO_4$ (*black bars*) at room temperature. The chart shows the fluorescence intensity per OD_{595} after 60 min of either no copper (*white bars*) or plus copper (*black bars*) treatment. Wild-type yeast treated with copper in the absence or presence of PUFAs have significantly higher levels of lipid peroxidation as compared to yeast not treated with copper; a, $p<1.0E–4$. Wild-type yeast treated with copper in the presence of Lin have significantly higher levels of lipid peroxidation as compared to yeast treated with copper in the presence of Ole; b, $p < 1.0E–4$. Differences in fluorescence signals were assessed with one-way ANOVA test followed by a Tukey comparison test (Stat View 5.0.1, SAS, CA).

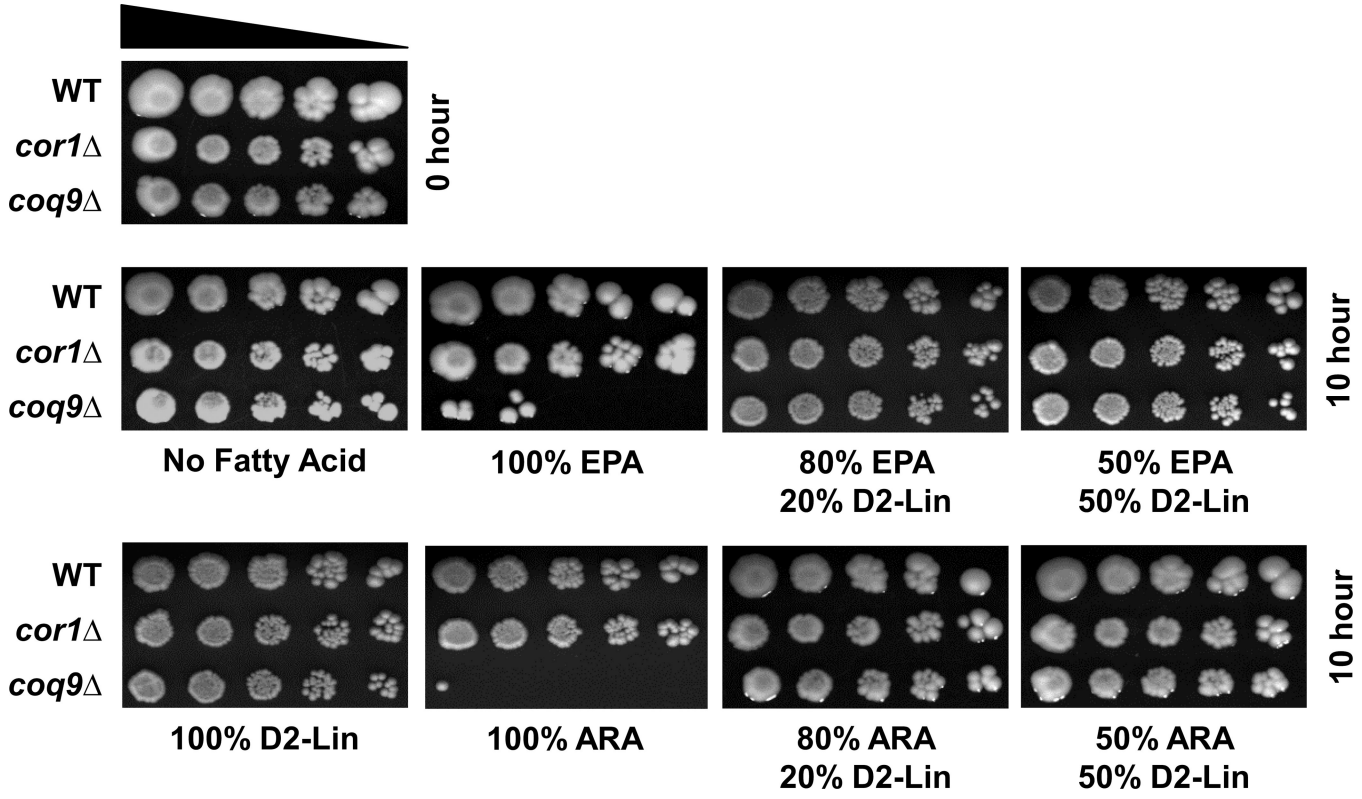

Figure 11. Small amounts of isotope-reinforced PUFAs protect yeast cells from long chain PUFA stress

Fatty acid sensitivity assays were performed as described in Figure 4 except that yeast were treated with 300 μM of the designated PUFA or PUFA mixture for 10 hours. A zero-time untreated control is shown on the top left. Picture were taken after 3 days of growth at 30 °C.

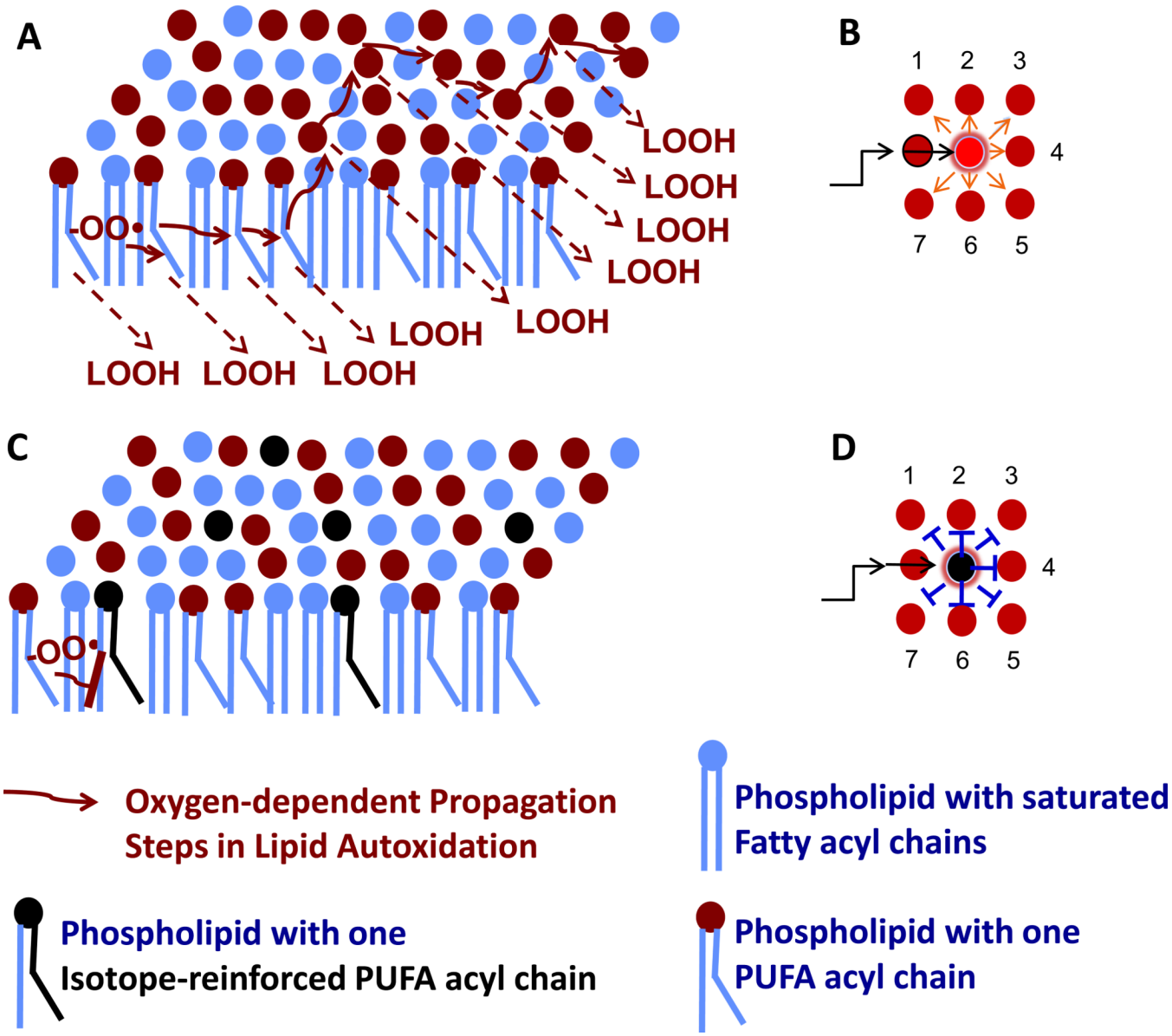

Figure 12. Isotope-reinforced PUFAs limit the chain reaction of lipid autoxidation when present at only 20%

A theoretical chain reaction is depicted where a single initiation event producing a lipid peroxyl radical (denoted by –OO•) starts a chain reaction of lipid autoxidation that in the presence of O_2, may continue indefinitely (*A*) (red arrows), and produce many molecules of lipid peroxides; susceptible phospholipid molecules containing a PUFA acyl chain are designated by a kinked blue line and a red dot. (*B*) Propagation of PUFA autoxidation can progress by interaction with any neighboring PUFAs. (*C*) The presence of 20% isotope-reinforced PUFA (denoted by a black kinked line and a black dot) inhibit (or slow) chain propagation. (*D*) Propagation is inhibited for PUFAs neighboring the D-PUFA.

Table 1

KIE of 11,11-D_2-Lin oxidation obtained from co-oxidation of 11,11-D_2-Lin and D_0-Lin in solution.

Run	$D_0 : D_2$	D_0-HODEs/D_1-HODEs	KIE
1	1 : 5.1	2.4	12.4
2	1 : 6.5	2.0	13.1
3	1 : 9.5	1.3	12.8

Table 2

Relative fatty acid uptake of PUFA-fed wild-type yeast

	Relative Ratios of PUFA Uptake[a]		
Ratios of PUFAs Added	**αLnn**	**Lin**	**11,11-D_2-Lin**
αLnn:Lin	57 ± 2.3%	43 ± 2.3%	0%
αLnn:11,11- D_2-Lin	55 ± 1.5%	0%	45 ± 1.8%
Lin:11,11- D_2-Lin	0%	44 ± 2.7%	56 ± 2.7%
αLnn:Lin:11,11- D_2-Lin	40 ± 3.1%	28 ± 0.7%	32 ± 2.4%

[a]Relative ratios of PUFA uptake in wild-type cells after 4 hours of incubation with exogenously added PUFAs. Results are representative of two independent experiments.