

Preprints are preliminary reports that have not undergone peer review. They should not be considered conclusive, used to inform clinical practice, or referenced by the media as validated information.

7-Dehydrocholesterol is an endogenous suppressor of ferroptosis

Jose Pedro Friedmann Angeli (pedro.angeli@virchow.uni-wuerzburg.de)

University of Würzburg https://orcid.org/0000-0001-7706-1379

Florencio Porto Freitas Rudolf Virchow Center

Palina Nepachalovich

University of Leipzig

Lohans Puentes

University of Köln

Omkar Zilka

University of Ottawa

Alex Inague

University of Würzburg

Svenja Lorenz

Helmholtz Zentrum München

Viktoria Kunz

Universitätsklinikum Würzburg (UKW)

Helene Nehring

University of Würzburg

Thamara Nishida Xavier da Silva

University of Würzburg

Zhiyi Chen

University of Würzburg

Sebastian Doll

Helmholtz Zentrum München Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH)

Werner Schmitz

University of Würzburg https://orcid.org/0000-0003-0485-7303

Peter Imming

University of Halle

Sayuri Miyamoto

Universidade de Sao Paulo

Judith Klein-Seetharaman

Arizona State Universtiy

Lokender Kumar

Colorado School of Mines

Thiago Cardoso Genaro-Mattos

University of Nebraska

Karoly Mirnics

University of Nebraska Medical Center https://orcid.org/0000-0002-5521-0254

Svenja Meierjohann

University of Würzburg

Matthias Kroiss

Ludwig Maximillian University

Isabel Weigand

Ludwig Maximillian University

Kurt Bommert

Universitätsklinikum Würzburg (UKW)

Ralf Bargou

University Hospital Wuerzburg https://orcid.org/0000-0002-1221-7421

Ana Garcia-Saez

University of Cologne https://orcid.org/0000-0002-3894-5945

Derek Pratt

Department of Chemistry and Biomolecular Sciences, University of Ottawa https://orcid.org/0000-0002-7305-745X

Maria Fedorova

University of Leipzig

Ann Wehmann

University of Denver

Aline Horling

University of Halle

Georg Bornkamm

University of Ulm

Marcus Conrad

Helmholtz Zentrum München https://orcid.org/0000-0003-1140-5612

Biological Sciences - Article

Keywords: cell death, 7-dehydrocholesterol reductase, pro-survival function

Posted Date: October 6th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-943221/v1

License: © (1) This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: Yes there is potential Competing Interest. Marcus Conrad holds patents for some of the compounds described herein, and is co-founder and shareholder of ROSCUE Therapeutics GmbH.

Version of Record: A version of this preprint was published at Nature on January 31st, 2024. See the published version at https://doi.org/10.1038/s41586-023-06878-9.

Abstract

Ferroptosis is a form of cell death that has received considerable attention not only as a means to eradicate defined tumour entities but also because it provides unforeseen insights into the metabolic adaptation exploited by tumours to counteract phospholipid oxidation. Here, we identify a pro-ferroptotic activity of 7-dehydrocholesterol reductase (DHCR7) and an unexpected pro-survival function of its substrate, 7-dehydrocholesterol (7-DHC). Although previous studies suggested that high levels of 7-DHC are cytotoxic to developing neurons and favour lipid peroxidation, we now demonstrate that 7-DHC accumulation confers a robust pro-survival function in cancer cells. 7-DHC, due to its far superior reactivity towards peroxyl radicals, is shown here to effectively shield (phospho)lipids from autoxidation and subsequent fragmentation. We further demonstrate in a subset of ferroptosis-sensitive Burkitt lymphomas - where DHCR7 mutations have been reported - that the accumulation of 7-DHC is sufficient to suppress the basal sensitivity of cells toward ferroptosis, thereby translating into an unexpected growth advantage. Conclusively, our findings provide compelling evidence of a yet-unrecognised anti-ferroptotic activity of 7-DHC as a cell-intrinsic mechanism that could be exploited by cancer cells to escape ferroptosis.

Main Text

Ferroptosis has attracted considerable attention in recent years¹, and a detailed characterisation of this pathway has uncovered it's implication in a series of pathological conditions ranging from tissue ischaemia/reperfusion injury to infection^{2,3}. Moreover, the modulation of ferroptosis is increasingly recognised as a potential avenue for developing therapeutics against various diseases⁴. At the molecular level, ferroptosis was initially characterised as the cell death process induced by cysteine starvation, usually caused by lack or insufficient activity of the cystine-glutamate antiporter (designated system x_c^{-})⁵. Low system system x_c⁻ activity directly impacts substrate availability for the key enzyme regulating ferroptosis, namely glutathione peroxidase 4 (GPX4), leading to lipid hydroperoxide accumualtion and cell death⁶. Early works have established the central role played by the enzymatic activity of GPX4 in suppressing the process of ferroptosis⁷⁻⁹. GPX4 is the sole enzyme responsible for reducing peroxidised phospholipids¹⁰ and can be inhibited by a series of alkylating small molecules, such as RSL3 and ML210⁸, leading to cell death in ferroptosis-sensitive cancer cell lines. The initial characterisation of this pathway demonstrated the critical role of esterified polyunsaturated fatty acid (PUFA) oxidation downstream of GPX4 inhibition as a driver of ferroptosis^{11,12}. Specifically, we and others have shown that the activity of the acyl-CoA-synthetase long-chain family 4 (ACSL4) is required for the enrichment of phospholipids with PUFAs¹¹. The enrichment of phospholipids with PUFAs results in a marked dependency on GPX4 activity^{11,13}. Accordingly, the inhibition of GPX4 in ferroptosis-prone cell lines leads to the characteristic oxidation fingerprint entailing the accumulation of peroxidised products of phosphatidylethanolamine (PE) containing arachidonic acid (AA) and adrenic acid (AdA)¹². It has been further demonstrated that the sole accumulation of peroxidised fatty acids is not sufficient to induce

ferroptosis, and a central role in the free radical-mediated propagation step defined¹⁴. This process has been shown to contribute to the formation of pore-like structures of ill-defined identity¹⁵ that drive the osmotic lysis of the cells¹⁶.

The present study uncovered and characterised an unexpected role for 7-dehydrocholesterol reductase (DHCR7) in the ferroptotic process. DHCR7 catalyses the final step in cholesterol biosynthesis, and its inhibition leads to the accumulation of 7-dehydrocholesterol (7-DHC). 7-DHC was initially reported to accumulate in preputial gland tumours by Kandutsh and Russel¹⁷ and whose function, at that time, was only assumed to function as a spare capacity for cholesterol synthesis. We show that the accumulation of 7-DHC translates into an increased tolerance towards (phospho)lipid peroxidation, thus providing a robust and unexpected resistance to ferroptosis. This mechanism could be potentially exploited by Burkitt 's lymphomas (BL) to overcome the metabolic stress characteristic of their lineage specific low system x_c^- activity¹⁸. Moreover, the detailed characterisation of the unique protective effect of 7-DHC on (phospho)lipid peroxidation allows us to provide evidence that ferroptotic cell death is a consequence of the accumulation of oxidatively truncated (phospho)lipid species rather than solely lipid hydroperoxide accumulation and that these species are integral players in ferroptosis execution.

DHCR7 is a pro-ferroptotic gene

Spurred by the still incomplete understanding of the ferroptotic process and the development of nextgeneration single guide RNAs (sgRNAs)¹⁹, we performed a genome-wide reverse genetic CRISPR screen using second-generation CRISPR libraries to identify genes that may confer robust protection against ferroptosis. To this end, the Pfa1 cell line⁶ was transduced with a CRISPR library covering 18,424 genes with a total representation of 90,230 sgRNAs followed by a stringent selection for fourteen days using 200 nM of the GPX4 inhibitor (1S,3R)-RSL3, from now on only RSL3 (Fig. 1a). Consistent with the results of our previous screen and those of other groups, *Acs/4* emerged as the highest-scoring hit^{11,13,20-22}. The second top-scoring gene with multiple sgRNAs enriched was *Dhcr7* (Fig. 1b). The identification of *Dhcr7* as a potential pro-ferroptotic gene was unexpected in light of a number of studies indicating that loss or inhibition of DHCR7 is associated with increased lipid peroxidation^{23,24}. Intrigued by this finding, we set out to explore the basis of this unanticipated discovery. Using the bona fide ferroptosis cell line model HT1080, we generated DHCR7-deficient cell lines using two independent sgRNAs. The successful loss of DHCR7 (Fig. 1c) was mirrored by the accumulation of its direct substrate 7-DHC. Notably, cholesterol depletion was not observed as most of it stems from the uptake of cholesterol present in the serum. Importantly, knockout of *DHCR7* did not affect the expression of known ferroptosis regulators (Fig. 1d). In support of this conclusion, we could confirm the screening results showing that *DHCR7*-deficient HT1080 cells present a marked resistance towards ferroptosis inducing compounds (Fig. 1e). Similar results were obtained with three independent clonal cell lines derived from Pfa1, HT1080 and MDA-MB-435 cells, supporting the general impact of this system in preventing cell death (Extended Data Fig. 1). We further show that DHCR7 loss does not modulate sensitivity to a panel of cytotoxic compounds, thus highlighting its specificity to the ferroptotic process (Extended Data Fig. 1d, f). We further corroborated

these findings with studies of a clonal cell line derived from the HT1080 *DHCR7* knockout (KO) pool to avoid confounding results from non-edited cells. Using this cell line, we could unequivocally show the proferroptotic activity of DHCR7 as genetic reconstitution of a sgRNA resistant *DHCR7* variant abolished 7-DHC levels and re-sensitised cells to ferroptosis without impacting on the cell's response to other cytotoxic agents (Fig. 1f, g and Extended Data Fig. 1e, f).

7-DHC is a bona fide anti-ferroptotic metabolite

In the penultimate step of the cholesterol biosynthesis pathway, lathosterol, through lathosterol oxidase (SC5D), is converted to 7-DHC, which, in turn, is reduced to cholesterol by DHCR7 in the final step of the cholesterol pathway (Fig. 2a). Several earlier studies have pointed to a toxic effect of 7-DHC accumulation via an increased susceptibility toward lipid peroxidation due to the very high inherent reactivity of 7-DHC²⁴. To shed light on these seemingly contradictory findings, we generated a DHCR7/SC5D double mutant cell line to address whether 7-DHC accumulation indeed mediates the protective effects induced by the loss of DHCR7. In agreement with a protective effect of 7-DHC, the loss of SC5D in the DHCR7 knockout cell line completely abolished the protective effect conferred by the single loss of DHCR7 (Fig. 2b). As expected, deletion of both genes led to a detectable accumulation of lathosterol and completely abolished the formation of 7-DHC (Fig. 2c). Subsequently, the serial reconstitution of DHCR7 and SC5D in a DHCR7/SC5D knockout background demonstrated that only the re-expression of SC5D resulted in an accumulation of 7-DHC (Extended Data Fig. 2a), and consequently a marked resistance to ferroptosis induced by GPX4 inhibition (Fig. 2d), but not to other cytotoxic agents (Extended Data Fig. 2b). Similarly, pharmacological inhibition of several upstream steps of the cholesterol biosynthetic process also resulted in the complete loss of the protective effect conferred by loss of DHCR7 (Extended Data Fig. 2c, d). Using the DHCR7 and the DHCR7/SC5D deficient cell lines in a series of sterol supplementation experiments, we further demonstrate that exogenous supplementation of 7-DHC protected all cell lines from ferroptosis (Fig. 2e). Additionally, lathosterol only increased ferroptosis resistance in cell lines able to accumulate 7-DHC. Interestingly, the high levels of cholesterol assayed here blunted the protective effect in all genotypes, likely due to feedback inhibition of upstream steps of the mevalonate pathway (Fig. 2e). To further corroborate the anti-ferroptotic role of 7-DHC in ferroptosis, we could show that the protective effect was also observed in the Pfa1/TAM system, a genetic model of Gpx4 deficiency⁶ (Extended Data Fig. 2e). In line with these observations, 7-DHC was the only sterol able to suppress BODIPY-C11 oxidation, a marker of lipid peroxidation (Fig. 2f). These results firmly establish a yet-unrecognised role of the endogenous metabolite, 7-DHC, in preventing (phospho)lipid oxidation and associated death by ferroptosis.

7-DHC protects phospholipids from autoxidation

7-DHC is reported to be among the most autoxidisable lipid components *in vivo*²⁴. To investigate the impact of this sterol in a well-defined (phospho)lipid autoxidation model, we prepared unilamellar liposomes of soy phosphatidylcholine (PC) loaded with 7-DHC (Fig. 3a). We used the recently developed FENIX assay²⁵, which employs the lipophilic radical initiator (E)-1,2-bis((2-methyldecan-2-yl)oxy)diazene

(DTUN) to specifically generate lipid peroxyl radicals. STY-BODIPY competes with phospholipids for propagating lipid peroxyl radicals, and the fluorescence of its oxidised product(s), STY-BODIPY_{ox}, can be monitored by fluorescence (Fig. 3a, b and c). Typical radical trapping antioxidants (RTA), such as tocopherol or its truncated form, 2, 2, 5, 7, 8-pentamethyl-6-hydroxychromane (PMC), inhibit autoxidation and thus retard STY-BODIPY oxidation until the RTA is consumed (Fig. 3A). Interestingly, 7-DHC-enriched liposomes resulted in a dose-dependent suppression of the rate of STY-BODIPY oxidation, albeit without the evident inflexion point characteristic of good RTAs, exemplified by PMC (Fig. 3b and c). Since the suppression of STY-BODIPY oxidation could arise from dilution of the pool of autoxidisable phospholipids upon supplementation of the liposomes with 7-DHC, similar experiments wherein nonoxidisable DPPC were incorporated in place of 7-DHC were performed, allowing us to demonstrate no difference from the native soy PC liposomes (Fig. 3b). Furthermore, since sterols alter membrane fluidity and could confer protection through dynamic parameters²⁶ that could impact lipid peroxidation²⁷, corresponding experiments were carried out on cholesterol-loaded liposomes (Fig. 3c). Yet again, there was no effect on the rate of STY-BODIPY oxidation - even beyond concentrations of 7-DHC used (Extended Data Fig. 3a) – suggesting that physical changes in the bilayer imparted by the sterol framework do not impact the oxidation rates in our model system, neither do impact their integrity (Extended Data Fig. 3b). Given the indirect nature of the assay, we also directly measured the impact of 7-DHC on soy PC peroxidation, i.e. PLPC-00H, DLPC-00H and DLPC-200H, by LC-MS/MS (Fig. 3e, f). While supplementation of the liposomes with DPPC (up to 32 mol%) had no effect on the rate of PLPC and DLPC oxidation, cholesterol (at 8 mol%) had a modest effect on the accumulation of PLPC-OOH, DLPC-OOH and DLPC-20OH. Entirely consistent with the FENIX results, 7-DHC supplementation led to a dosedependent suppression in the rate of PLPC and DLPC oxidation. To demonstrate that this suppression corresponded with the intervention of 7-DHC in the radical chain reaction, the consumption of 7-DHC was monitored spectrophotometrically via its characteristic absorbance (Fig. 3f, g and Extended Data Fig. 3c). This data suggests that the oxidation of 7-DHC *in vitro* is responsible for the inhibition of (phospho)lipid peroxidation a notion we could further validate in a model using iron/ascorbate as the source of oxidation (Extended Data Fig. 3d). Hence, if this hypothesis were correct, 7-DHC oxidation should lead to the accumulation of these products during the course of ferroptosis, and by doing so, it could spare phospholipids from accumulating oxidative damage. Fig. 3h illustrates the major detectable products formed upon 7-DHC oxidation, where the major quantifiable product is the oxysterol 3β,5αdihydroxycholest-7-en-6-one (DHCEO). To assess if 7-DHC oxidation products also accumulate upon triggering ferroptosis in cells, we treated the HT1080 DHCR7/SC5D double-knockout cell line expressing SC5D and an empty vector with the GPX4 inhibitor RSL3. While no major loss in the total content of 7-DHC was noticeable (Extended Data Fig. 3e), the exact quantification of the two major non-enzymatic oxidation products of 7-DHC, namely DHCEO and 4a-OH 7-DHC and, revealed a significant increase (Fig. 3i and Extended data 3e). To demonstrate that the 7-DHC products originate from the peroxyl radicalmediated oxidation of 7-DHC, we further incubated these cells with the RTA and ferroptosis inhibitor liproxstatin-1 (Lip1)⁹. In agreement with the free radical-mediated formation of DHCEO and 4a-OH 7-DHC, Lip1 fully inhibited their accumulation (Fig. 3i). Hence, these results strongly suggest that due to its

inherent reactivity, 7-DHC autoxidises preferentially, thereby suppressing the propagation of peroxyl radical-mediated (phosphor)lipid damage.

7-DHC suppresses the formation of truncated phospholipids and membrane rupture

Following these results, we reasoned that the presence of 7-DHC in phospholipid bilayers generates a strong pro-survival effect by increasing the resistance of membranes to oxidation-mediated permeabilisation. Therefore, a model system was employed that consists of 5(6)-carboxyfluorescein (CF) encapsulated in liposomes allowing for the detection of a fluorescent signal upon membrane permeabilisation (Extended Data Fig. 4a). Using the iron/ascorbate couple as a well-established oxidation model, we now show that liposomes containing 7-DHC are remarkably resistant to oxidation-mediated membrane permeabilisation (Extended Data Fig. 4b). To further support the relevance of this simplified system for ferroptosis, we could show that the process of vesicle rupture could be prevented entirely by the ferroptosis inhibitor Lip1 (Extended Data Fig. 4c), indicating that Lip1 acts similarly to prevent membrane permeabilisation in cells. Recent reports studying the relative contribution of different photosensitisation mechanisms to membrane permeabilisation suggested that truncated phospholipid species rather than phospholipid hydroperoxide are key in generating membrane pores and consequently mediating the loss of membrane integrity²⁸. Therefore, we reasoned that a similar mechanism could be at play during iron-induced permeabilisation and ferroptosis execution²⁹. As such, we next explored the feasibility of this mechanism using our cellular models treated with a GPX4 inhibitor. In-depth epilipidomics analysis indeed detected a substantial accumulation of PE and plasmalogen PE truncated products in cells undergoing ferroptosis (Fig. 4a). Notably, cell permeabilisation, monitored as PI-positive cells, was only detectable in conditions marked by an increase in these oxidised and truncated species (Fig. 4a). We also show that Lip1 fully inhibited the formation of these species, thus confirming their origin from the autocatalytic lipid peroxidation process (Fig. 4a). In accordance, cells accumulating 7-DHC behaved similarly to Lip1-treated cells and the specific oxidation product of 7-DHC, DHCEO, accumulated in these cells. This thus demonstrates that 7-DHC is preferentially oxidised in cells, thereby sparing phospholipids and preventing the formation of oxidised and truncated species (Fig. 4a). To establish the functional link between truncated lipids and ferroptosis execution, we assayed a panel of different truncated species regarding their capacity to destabilise membranes in model systems and in cells (assayed structures are depicted in Extended Data Fig. 4d). Accordingly, all tested truncated lipids were able to permeabilise liposomal membranes and to kill cells more efficiently than the parental lipid and the corresponding hydroperoxide (Extended Data Fig. 4e). In line with the proposed mechanism 7-DHC did not affect permeabilization mediated by truncated phospholipid species (Extended Data Fig. 4eh). We reasoned that the extent of the bilayer packing alterations induced by the truncated lipids, and thereby their potency to destabilise the membrane in order to generate pores and induce cell death, would depend on their acyl chain length, with shorter truncated chains being more cytotoxic. Although no apparent relationship with the length of the truncated tail was evident in the above experiments, it was clear that exogenous addition of the lipids could result in less efficient membrane incorporation of the shorter and more hydrophilic species. To circumvent this issue, a system in which the species are formed

in situ would be required. We took advantage of the cell's own fatty acid incorporation machinery to achieve this goal. ACSL4-deficient cells have a profound loss of PUFA content in membranes, resulting in a marked resistance to ferroptosis due to the lack of oxidisable substrates. Sensitivity to ferroptosis in this setting can be regained by feeding exogenous PUFAs¹¹. This feature should facilitate a better control of the substrates utilised for ferroptosis execution. Using this model, we compared side-by-side the sensitisation provided by a-linolenic acid (aLNN) and y-linolenic acid (yLNN). Both fatty acids have an identical structure in length and number of double bonds leading to a similar propensity to be oxidised, yet the position of the last double bond determines the structure of the resulting truncated product. Analysis of the lipidomic changes of ACSL4 wildtype (WT) and KO cells treated with aLNN and yLNN confirmed that both lipids are directly and efficiently esterified into PE suffering limited metabolisation to longer and esterified species, likely a reflex of the loss of ACSL4 and its requirement for the efficient metabolisation of these species (Fig. 4b, c). The supplementation restored the oxidisable pool of PUFA to a similar extent as in WT cells (Fig. 4b, d). Remarkably, despite their equal abundance and propensity to undergo oxidation, yLNN appeared to be a superior ferroptosis executing substrate (Fig. 4d and Extended Data Fig. 4i), in line with its potential to generate shorter truncated phospholipid products. These results are remarkable because they indicate that the product formed determines cell death rather than solely its capacity to autoxidise. Together, these observations provide compelling evidence for the role of truncated products in contributing to ferroptosis execution and that 7-DHC and other ferroptosis inhibitors such as Lip1, directly suppress their formation.

7-DHC accumulation increase lymphoma cell fitness

Having characterised the molecular underpinnings by which 7-DHC prevents ferroptosis execution, we next asked if this protective effect could have a potential role in supporting tumour growth under conditions where ferroptosis inhibition is critical. To our initial surprise, DHCR7 mutations, despite being rare, have been described in Burkitt's Lymphoma (BL) patients³⁰. BL is a tumour entity characterised by MYC translocations and is considered the prototypic ferroptosis cancer entity. The reason for this traces back to their inherent low activity of system $x_c^{-18,31}$, likely reflecting metabolic adaptation required to spare glutamine whose dependency is increased in cell expressing high MYC levels³². Accordingly, the requirement of thiol donating compounds, such as ß-mercaptoethanol (ßMe), to support the growth of murine leukemic b-cell and human BL cell lines has been known for many decades^{18,33}. Accordingly, we first set out to explore the function of the reported mutations described for $DHCR7^{30}$ and assess if they could, in principle, increase the fitness of BL. Briefly, mutations N274K and L306R have been reported in two BL patients, and a second mutation, A24S, was reported in two different BL cell lines (Raji and BL58); we additionally included another mutation identified in a MM cell line (L317V). We created a model for the DHCR7 structure using a homologous structure (pdb id 4QUV, sequence identity 37%, similarity 51%) to gain insights into the molecular consequences of these mutations. L306R, N274K and L317V are predicted to be located in the transmembrane domain (Fig. 5a). While the substitution of the hydrophobic amino acid leucine by another hydrophobic amino acid, valine, is predicted to be tolerable in the hydrophobic membrane interior, the introduction of a positively charged amino acid (K or R) is highly

disfavoured thermodynamically in transmembrane regions of proteins and could result in misfolding of the protein. Re-expression of DHCR7-Flag-tagged version of the four corresponding mutants in the DHCR7-deficient HT1080 cell line allowed us to validate these predictions experimentally. Fig. 5b shows that except for mutation N274K all are generally well expressed compared to the WT. The potential misfolded nature of mutant N274K is likely, in addition of been thermodynamically unfavourable, a consequence of the disruption of the helix-helix interactions established between N252 and L253 (Extended Data Fig. 5a). Next, we addressed the functionality of these mutations and, in agreement with the predictions, the A24S and L317V mutations appear to lead to functional enzymes able to metabolize 7-DHC when overexpressed (Fig. 5c) and re-sensitise the DHCR7 deficient cells to ferroptosis like the WT enzyme (Fig. 5d). On the other hand, the two mutations reported in patients, N274K and the L306R are not functional, cannot metabolise 7-DHC (Fig. 5c) and cannot restore sensitivity to ferroptosis (Fig. 5d). Of notice, the A24S variant which behaved similarly to WT DHCR7 introduces a serine at the N-terminus, which has three reported phosphorylation sites, S5, S14 and S25³⁴. We, therefore, subjected the WT and A24S mutant sequence to the NetPhos server³⁵, which was able to correctly identify the three known sites with scores of 0.556, 0.606 and 0.643, respectively (typical confidence threshold set at 0.5). Intriguingly, this analysis predicted A24S as an additional phosphorylation site, with an extremely high confidence score (0.991), and further increased the confidence of the neighbour S25 to be phosphorylated (0.774). Thus, it is tentative to speculate that the A24S mutation could be a neo-phosphosite that, in context specific situation would be able to modulate DHCR7 function/levels and ultimately impact on 7-DHC levels. In-depth studies to explore this possibility are certainly warranted.

Given the lack of available cell lines with the loss of function mutations we further study the role of 7-DHC in suppressing ferroptosis by deleting DHCR7 in the BL cell line BL41 and in the multiple myeloma (MM) cell line KMS26, which shares the BL thiol dependency for growth. Following our previous results, genetic loss of DHCR7 in these cell lines conferred robust protection towards GPX4 inhibitors, and to some extent, this effect appeared to be even more pronounced (Fig. 5e). Strikingly, loss of DHCR7 abolished the characteristic thiol dependent growth as both cell lines could proliferate in the absence of thiol donating compound (Fig. 5f). Highlighting the specificity of this effect, the inhibition of DHCR7, using the highly specific DHCR7 inhibitor RB38³⁶, could also bypass the dependency on thiol donating compound as well as Lip1 (Extended Data Fig. 5b). Accordingly, the genetic and pharmacological effects were blunted by inhibiting upstream step in the biosynthesis of 7-DHC, specifically by targeting lathosterol biosynthesis using the emopamil binding protein (EBP) inhibitor Tasin-1 (Fig. 5f). Using these pharmacological tools, we expanded this observation to a larger panel of cell lines where we could show that only cells accumulating 7-DHC are able to grow in the absence of thiol donating compounds, with the exception of the BL2 cell line, which was unable to accumulate 7-DHC upon inhibition and did not show any growth limitations (Extended Data Fig. 5c, d). Having established a pro-survival role of 7-DHC in a subset of B-cell lymphomas we provide an initial assessment of the in vivo relevance of this finding using a xenograft deficient for DHCR7. For this, we used the KMS26 cell line deficient for DHCR7 and could report a significant growth advantage, suggesting that lipid peroxidation is a metabolic hurdle for these xenografts and that the accumulation of 7-DHC can mitigate this metabolic stress in vivo.

Discussion

Conclusively, our work adds to expanding biological activities of *5*,7-unsaturated sterol metabolites as recent data have suggested a potential role for this class of metabolites in mitochondrial quality control³⁷ and immunity^{38,39}. Specifically, we identify that 7-DHC is an endogenous metabolite that robustly protects cellular membranes from (phospho)lipid peroxidation and associated ferroptotic cell death. We also demonstrate that, by preventing (phospho)lipid peroxidation, 7-DHC suppresses the formation of (phospho)lipid-truncated species, which are likely the most downstream executors of ferroptosis and, upon reaching a certain threshold, could be considered as the point of "no return" in ferroptosis. Furthermore, we provide compelling evidence that the accumulation of 7-DHC increases the fitness of BL cells and could compensate for their intrinsic low system x_c^- activity and increased dependency on GPX4. This recognition is critical as recent reports have indicated that high MYCN levels, a close homologue of the BL driving oncogene *MYC*³⁰, increases cancer cells dependency on GPX4 to supress ferroptosis^{40,41}. Given the already reported multiple levels of posttranslational regulation of DHCR7 via ubiquitination and phosphorylation^{34,42-44}, our work should stimulate a better understanding of the events that can disrupt DHCR7 activity beyond the obvious loss of function identified in the mutations assayed here.

Finally, analogously to our observations, ergosterol, the major sterol found in cell membranes of fungi and protozoa and contains an indistinguishable sterol ring from 7-DHC, has been repeatedly associated with increased tolerance to oxidative stress^{36,37,45-48}. These studies suggest that the mechanism protecting membranes from (phospho)lipid peroxidation described here could be an overlooked and general tolerance mechanism kept across multiple species and highjacked by cancer cells to evade ferroptosis.

Bibliography

1 Friedmann Angeli, J. P., Krysko, D. V. & Conrad, M. Ferroptosis at the crossroads of canceracquired drug resistance and immune evasion. *Nat Rev Cancer*, doi:10.1038/s41568-019-0149-1 (2019).

2 Amaral, E. P. *et al.* A major role for ferroptosis in Mycobacterium tuberculosis-induced cell death and tissue necrosis. *J Exp Med* **216**, 556-570, doi:10.1084/jem.20181776 (2019).

3 Linkermann, A. *et al.* Synchronized renal tubular cell death involves ferroptosis. *Proc Natl Acad Sci U S A* **111**, 16836-16841, doi:10.1073/pnas.1415518111 (2014).

4 Zou, Y. & Schreiber, S. L. Progress in Understanding Ferroptosis and Challenges in Its Targeting for Therapeutic Benefit. *Cell Chem Biol* **27**, 463-471, doi:10.1016/j.chembiol.2020.03.015 (2020).

5 Dixon, S. J. *et al.* Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* **149**, 1060-1072, doi:10.1016/j.cell.2012.03.042 (2012). 6 Seiler, A. *et al.* Glutathione peroxidase 4 senses and translates oxidative stress into 12/15lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab* **8**, 237-248, doi:10.1016/j.cmet.2008.07.005 (2008).

7 Yant, L. J. *et al.* The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. *Free Radic Biol Med* **34**, 496-502 (2003).

8 Yang, W. S. *et al.* Regulation of ferroptotic cancer cell death by GPX4. *Cell* **156**, 317-331, doi:10.1016/j.cell.2013.12.010 (2014).

9 Friedmann Angeli, J. P. *et al.* Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol* **16**, 1180-1191, doi:10.1038/ncb3064 (2014).

10 Ursini, F., Maiorino, M., Valente, M., Ferri, L. & Gregolin, C. Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. *Biochim Biophys Acta* **710**, 197-211 (1982).

11 Doll, S. *et al.* ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat Chem Biol* **13**, 91-98, doi:10.1038/nchembio.2239 (2017).

12 Kagan, V. E. *et al.* Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis. *Nat Chem Biol* **13**, 81-90, doi:10.1038/nchembio.2238 (2017).

13 Zou, Y. *et al.* A GPX4-dependent cancer cell state underlies the clear-cell morphology and confers sensitivity to ferroptosis. *Nat Commun* **10**, 1617, doi:10.1038/s41467-019-09277-9 (2019).

14 Shah, R., Shchepinov, M. S. & Pratt, D. A. Resolving the Role of Lipoxygenases in the Initiation and Execution of Ferroptosis. *ACS Cent Sci* **4**, 387-396, doi:10.1021/acscentsci.7b00589 (2018).

Pedrera, L. *et al.* Ferroptotic pores induce Ca(2+) fluxes and ESCRT-III activation to modulate cell death kinetics. *Cell Death Differ* **28**, 1644-1657, doi:10.1038/s41418-020-00691-x (2021).

16 Riegman, M. *et al.* Ferroptosis occurs through an osmotic mechanism and propagates independently of cell rupture. *Nat Cell Biol* **22**, 1042-1048, doi:10.1038/s41556-020-0565-1 (2020).

17 Kandutsch, A. A. & Russell, A. E. Preputial gland tumor sterols. 3. A metabolic pathway from lanosterol to cholesterol. *J Biol Chem* **235**, 2256-2261 (1960).

18 Falk, M. H. *et al.* Apoptosis in Burkitt lymphoma cells is prevented by promotion of cysteine uptake. *Int J Cancer* **75**, 620-625, doi:10.1002/(sici)1097-0215(19980209)75:4<620::aid-ijc21>3.0.co;2-b (1998).

19 Tzelepis, K. *et al.* A CRISPR Dropout Screen Identifies Genetic Vulnerabilities and Therapeutic Targets in Acute Myeloid Leukemia. *Cell Rep* **17**, 1193-1205, doi:10.1016/j.celrep.2016.09.079 (2016). 20 Yuan, H., Li, X., Zhang, X., Kang, R. & Tang, D. Identification of ACSL4 as a biomarker and contributor of ferroptosis. *Biochem Biophys Res Commun* **478**, 1338-1343, doi:10.1016/j.bbrc.2016.08.124 (2016).

Dixon, S. J. *et al.* Human Haploid Cell Genetics Reveals Roles for Lipid Metabolism Genes in Nonapoptotic Cell Death. *ACS Chem Biol* **10**, 1604-1609, doi:10.1021/acschembio.5b00245 (2015).

Zou, Y. *et al.* Plasticity of ether lipids promotes ferroptosis susceptibility and evasion. *Nature* **585**, 603-608, doi:10.1038/s41586-020-2732-8 (2020).

23 Yin, H., Xu, L. & Porter, N. A. Free radical lipid peroxidation: mechanisms and analysis. *Chem Rev* **111**, 5944-5972, doi:10.1021/cr200084z (2011).

Xu, L., Korade, Z. & Porter, N. A. Oxysterols from free radical chain oxidation of 7dehydrocholesterol: product and mechanistic studies. *J Am Chem Soc* **132**, 2222-2232, doi:10.1021/ja9080265 (2010).

25 Shah, R., Farmer, L. A., Zilka, O., Van Kessel, A. T. M. & Pratt, D. A. Beyond DPPH: Use of Fluorescence-Enabled Inhibited Autoxidation to Predict Oxidative Cell Death Rescue. *Cell Chem Biol* **26**, 1594-1607 e1597, doi:10.1016/j.chembiol.2019.09.007 (2019).

Zhang, X., Barraza, K. M. & Beauchamp, J. L. Cholesterol provides nonsacrificial protection of membrane lipids from chemical damage at air-water interface. *Proc Natl Acad Sci U S A* **115**, 3255-3260, doi:10.1073/pnas.1722323115 (2018).

27 McLean, L. R. & Hagaman, K. A. Effect of lipid physical state on the rate of peroxidation of liposomes. *Free Radic Biol Med* **12**, 113-119, doi:10.1016/0891-5849(92)90004-z (1992).

Bacellar, I. O. L. *et al.* Photosensitized Membrane Permeabilization Requires Contact-Dependent Reactions between Photosensitizer and Lipids. *J Am Chem Soc* **140**, 9606-9615, doi:10.1021/jacs.8b05014 (2018).

29 Friedmann-Angeli, J. P., Miyamoto, S. & Schulze, A. Ferroptosis: the greasy side of cell death. *Chem Res Toxicol*, doi:10.1021/acs.chemrestox.8b00349 (2019).

30 Schmitz, R. *et al.* Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature* **490**, 116-120, doi:10.1038/nature11378 (2012).

Banjac, A. *et al.* The cystine/cysteine cycle: a redox cycle regulating susceptibility versus resistance to cell death. *Oncogene* **27**, 1618-1628, doi:10.1038/sj.onc.1210796 (2008).

Wise, D. R. *et al.* Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci U S A* **105**, 18782-18787, doi:10.1073/pnas.0810199105 (2008).

Broome, J. D. & Jeng, M. W. Growth stimulation of mouse leukemia cells by thiols and disulfides in vitro. *J Natl Cancer Inst* **49**, 579-581 (1972).

Prabhu, A. V., Luu, W., Sharpe, L. J. & Brown, A. J. Phosphorylation regulates activity of 7dehydrocholesterol reductase (DHCR7), a terminal enzyme of cholesterol synthesis. *J Steroid Biochem Mol Biol* **165**, 363-368, doi:10.1016/j.jsbmb.2016.08.003 (2017).

Blom, N., Gammeltoft, S. & Brunak, S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* **294**, 1351-1362, doi:10.1006/jmbi.1999.3310 (1999).

Horling, A., Muller, C., Barthel, R., Bracher, F. & Imming, P. A new class of selective and potent 7dehydrocholesterol reductase inhibitors. *J Med Chem* **55**, 7614-7622, doi:10.1021/jm3006096 (2012).

37 Nielson, J. R. *et al.* Sterol Oxidation Mediates Stress-Responsive Vms1 Translocation to Mitochondria. *Mol Cell* **68**, 673-685 e676, doi:10.1016/j.molcel.2017.10.022 (2017).

Xiao, J. *et al.* Targeting 7-Dehydrocholesterol Reductase Integrates Cholesterol Metabolism and IRF3 Activation to Eliminate Infection. *Immunity* **52**, 109-122 e106, doi:10.1016/j.immuni.2019.11.015 (2020).

Rodgers, M. A., Saghatelian, A. & Yang, P. L. Identification of an overabundant cholesterol precursor in hepatitis B virus replicating cells by untargeted lipid metabolite profiling. *J Am Chem Soc* 131, 5030-5031, doi:10.1021/ja809949r (2009).

40 Lu, Y. *et al.* MYCN mediates TFRC-dependent ferroptosis and reveals vulnerabilities in neuroblastoma. *Cell Death Dis* **12**, 511, doi:10.1038/s41419-021-03790-w (2021).

Floros, K. V. *et al.* MYCN-Amplified Neuroblastoma Is Addicted to Iron and Vulnerable to Inhibition of the System Xc-/Glutathione Axis. *Cancer Res* **81**, 1896-1908, doi:10.1158/0008-5472.CAN-20-1641 (2021).

Huang, E. Y. *et al.* A VCP inhibitor substrate trapping approach (VISTA) enables proteomic profiling of endogenous ERAD substrates. *Mol Biol Cell* **29**, 1021-1030, doi:10.1091/mbc.E17-08-0514 (2018).

Zou, L. & Porter, T. D. Rapid suppression of 7-dehydrocholesterol reductase activity in keratinocytes by vitamin D. *J Steroid Biochem Mol Biol* **148**, 64-71, doi:10.1016/j.jsbmb.2014.12.001 (2015).

Fitzky, B. U. *et al.* 7-Dehydrocholesterol-dependent proteolysis of HMG-CoA reductase suppresses sterol biosynthesis in a mouse model of Smith-Lemli-Opitz/RSH syndrome. *J Clin Invest* **108**, 905-915, doi:10.1172/JCI12103 (2001).

45 Dupont, S. *et al.* Antioxidant Properties of Ergosterol and Its Role in Yeast Resistance to Oxidation. *Antioxidants (Basel)* **10**, doi:10.3390/antiox10071024 (2021).

Shafi, M. T. *et al.* Mevalonate kinase of Leishmania donovani protects parasite against oxidative stress by modulating ergosterol biosynthesis. *Microbiol Res* **251**, 126837, doi:10.1016/j.micres.2021.126837 (2021).

47 Mathur, R., Das, R. P., Ranjan, A. & Shaha, C. Elevated ergosterol protects Leishmania parasites against antimony-generated stress. *FASEB J* **29**, 4201-4213, doi:10.1096/fj.15-272757 (2015).

48 Marisco, G., Saito, S. T., Ganda, I. S., Brendel, M. & Pungartnik, C. Low ergosterol content in yeast adh1 mutant enhances chitin maldistribution and sensitivity to paraquat-induced oxidative stress. *Yeast* **28**, 363-373, doi:10.1002/yea.1844 (2011).

Declarations

Acknowledgements

J.P.F.A. acknowledges the support of the Junior Group Leader program of the Rudolf Virchow Center, University of Würzburg, the Deutsche Forschungsgemeinschaft (DFG) WE 5719/2-1, FR 3746/3-1, FR 3746/5-1 and FR 3746/6-1. J.P.F.A and R.C.B also acknowledge support by the Interdisziplinäres Zentrum für klinische Forschung (IZKF), project B-424. R.C.B additionally acknowledges the DFG through grant BA 1596/7-1. M.F receives financial support from the German Federal Ministry of Education and Research (BMBF) within the framework of the e:Med research and funding concept for SysMedOS project, additional thanks goes to Prof. Ralf Hoffmann (Institute of Bioanalytical Chemistry, University of Leipzig) for providing access to his laboratory. M.C acknowledges support from the Deutsche Forschungsgemeinschaft (DFG) CO 291/7-1, 291/9-1, 291/10-1, BMBF - VIP+ program NEUROPROTEKT (03VP04260), the Ministry of Science and Higher Education of the Russian Federation (075-15-2019-1933), and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No. GA 884754). Additional support via the DFG SPP2306 is acknowledge by J.P.F.A (FR 3746/6-1), M.C, M.F and A.G.S (GA 1641/7-1). D.A.P would like to thank the Natural Sciences and Engineering Council of Canada and the Canada Foundation for Innovation for their support. S. M. and A. I. acknowledge support from the São Paulo Research Foundation (FAPESP) 2013/07937-8 (CEPID Redoxoma) and 2017/13804-1. T.C.G-M and K.M received support from the National Institutes of Health NIMH R01 MH110636. J.K.S. acknowledges National Science Foundation grants HDR: DIRSE-IL 1940169 and RAPID 2031614. We also acknowledge the technical assistance of Mrs Theresa Henninger, Mrs Zornitsa Donova and Mrs Anne Haberberger.

Author Contributions

J.P.F.A.supervised the study and conceived the experimental plan with the support of all co-authors. S.M.L performed the CRISPR based screen. F.P.F. carried most of the in vitro experiments with contributions

from H.N, T.N.X.S, Z.C and A.I. Epilipidomics analysis were performed by P.N. and M.F. FENIX assays and corresponding LC/MS/MS and UV/Vis experiments were performed by O.Z with support from D.P. L.P.P and A.G.S contributed to the study of truncated vesicles permeabilisation. T.C-G-M and K.M performed the quantification of 7-DHC oxidation products. W.S, contributed with lipidomics and sterol detections and analysis. F.P.F, V.K, K.B and R.B were responsible for performing and analysing the xenograft experiments. L.K. and J.K.S. conducted structural modelling. M.C, S.D, S.M, A.H, P.I, A.W, M.K, I.W contributed with reagent or platforms. All authors contributed with discussion, data interpretation, read and agreed on the content of the paper.

Additional Information:

Supplementary Information is available for this paper. Correspondence and requests for materials should be addressed to José Pedro Friedmann Angeli.

Competing interests

M.C. holds patents for some of the compounds described herein and is co-founder and shareholder of ROSCUE Therapeutics GmbH.

Methods

Chemicals. Lipid standards were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). HPLC grade solvents were from Thermo Fisher Scientific (Hemel Hempstead, Hertfordshire, UK). All other chemicals and reagents were from Sigma-Aldrich.

Cell Lines. 4-hydroxytamoxifen (TAM)-inducible Gpx4-/- murine immortalised fibroblasts (Pfa1) have been characterised previously¹. These cells carry two loxP-flanked *Gpx4* alleles and stably express TAM inducible Cre recombinase allowing the genetic deletion of Gpx4. Human fibrosarcoma (HT1080) cells and human melanoma MB-435S were acquired from ATCC. The multiple myeloma cell line KMS26 was purchased from JCRB. Burkitt lymphoma cell lines were a kind gift of Prof. Gilbert Lenoir (International Agency for Research on Cancer – IARC, Lyon, France). In the case of MDA-MB-435 (SAMN03151832) we confirmed it as MDA-MB-435S, which is the proven melanoma line. Cells are tested at least once a yer for mycoplasm contamination by qPCR at Eurofins Genomics.

Assessment of lipid peroxidation using C11-BODIPY (581/591). 100,000 cells per well were seeded on 6well dishes (Sarstedt) one day prior to the experiment in the presence of the tested lipid. On the next day, cells were washed and treated with the indicated concentration of RSL3 to induce ferroptosis. Cells were subsequently incubated with C11-BODIPY (581/591) (1 μ M) for 20 min at 37°C before they were harvested by trypsinisation. Subsequently, cells were resuspended in 500 μ L of fresh PBS (DPBS, Gibco) and analysed using an excitation of 488-nm (FACS Canto II, BD Biosciences). Data was collected from the FL1 detector (C11-BODIPY) with a 502LP and 530/30 BP filter. At least 10,000 events were analysed per sample. Data was analysed using FlowJo Software. Fatty acid-dependent sensitisation of ferroptosis. HT1080 cells were seeded onto 15 cm plates. After cells adhered to the cell culture dish (approximately 6 h after plating), they were treated for 16 h with different concentrations of fatty acids (γ -linoleic acid and α -linolenic acid) solved in 10% fatty acid-free BSA and collected for lipidomics analysis or subsequently treated with 100 nM RSL3 for viability assessment 4 h thereafter using PI or Alamar blue as described below.

Cell viability assays. Alamar blue method: Cells are seeded on 96-well plates at the indecated density and treated with different compounds - (dimethyl sulfoxide [DMSO], RSL3, ML210, TBOOH, L-buthionine sulfoximine [BSO] auranofin (from Sigma) and Brefeldin-A, PLX4032, Carfilzomib, Bortezomib, Docetaxel (from Sellekchem) and Atheronal B were added to the cells 6 hours after plating. Cell viability was assessed 48 h (unless stated otherwise) after treatment using Alamar Blue as an indicator of viable cells. Alamar blue solution was made by dissolving of 1 g resazurin sodium salt in 100 mL sterile PBS and sterile filtrated through a 0.22 μ m filter. Stock solutions were stored at 4°C. The working solution was made freshly by adding 200 μ L of the stock solution to 50mL growth media. After 2-4h incubation time, viability was estimated by measuring the fluorescence using a 540/35 excitation filter and a 590/20 emission on a Spark® microplate reader (Tecan, Zürich, Switzerland). Alternatively, for propidium iodide staining cells were incubated with 5 ng/ μ L of PI for 5 min, after that the cells were diluted in 250 μ L of PBs and analized on a flow cytometer.

IncuCyte measurements of lipid toxicity in cells. Kinetics of cell death were collected using the IncuCyte bioimaging platform (Essen). For this, cells were seeded in 96-well plates (10⁴ cells per well) one day before treatment. After treatment with the respective oxidized lipid specie, four images per well were captured, analyzed and averaged. Cell death was measured by the incorporation of DRAQ7. Data was collected as count of Draq 7 positive cells per total number of cells in each conditions.

Determination of cell numbers: 50,000 BL cells were seeded on a 6-well plate in triplicates at density of 25,000 per ml. The cell number was determined for a period of 28 days using a Neubauer improved chamber. The cells were kept at a constant split ration of 1 to 2 every third day.

Preparation of lentiviral particles. HEK 293T cells were used to produce replication-incompetent lentiviral particles pseudotyped with the ecotropic envelope protein of the murine leukaemia virus (MLV) or the pantropic envelope protein VSV-G. A third generation lentiviral packaging system consisting of a transfer plasmid, pEcoEnv-IRES-puro (ecotropic particles) or pMD2.G (pantropic particles), pMDLg_pRRE and pRSV_Rev was co-lipofected into HEK 293T cells using HiPerFect (Roche). Viral particle containing cell culture supernatants were harvested 48 and 72 h after transfection and used to transduce the cell line of interest by directing incubating cell with HEK293T supernatants filtered through a 0.44µM membrane.

CRISPR–Cas9 genome-wide screen. In a similar approach as used in Doll et al^{2.}, Pfa1 cells stably expressing Cas9 were transduced with a lentiviral CRISPR-guide RNA library pseudotyped with the ecotropic envelope protein of the murine leukaemia virus. This library contained 87,897 mouse sgRNAs targeting 19,150 mouse protein-coding genes. Transduction efficiency was adjusted to a multiplicity of

infection (MOI) of 0.3. Three days after infection, cells were selected with increasing concentrations of RSL3 (200 nM) for 14 days. Genomic DNA was extracted from selected and unselected cells pools. Sample preparation was performed with primers designed to bind to the pKLV2-U6sgRNA (BbsI)-PGKpuro2ABFP library generating an amplicon of 194 bp encompassing the variable region (encoding the sgRNA). Coupling different barcode sequences in the forward primer, all PCR products were combined in an equal 1:1 ratio to form the sequencing (NGS) library suitable for sequencing on an Ion Torrent P1 chip (PrimBio Research Institute, LLC). Raw sequence results are provided as separate FASTQ files for each barcode. Screen deconvolution was carried from single reads of the FASTQ file by counting the number of each sgRNA sequenced per sample using the MAGeCK algorithm³.

Primer sequences for sample preparation were as follows:

RSL3 selection:

forward,

CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCGGCTTTATATATCTTGTGGAAAGGACG; reverse,

CCTCTCTATGGGCAGTCGGTGATAGCACCGACTCGGTGCCACTTTTCAA.

Unselected control:

forward,CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACGGCTTTATATATCTTGTGGAAAGGACG; reverse, CCTCTCTATGGGCAGTCGGTGATAGCACCGACT CGGTGCCACTTTTTCAA.

Generation of knockout cell lines. Single sgRNA guides were chosen using the VBC score (https://www.vbc-score.org/)4. Guides were cloned using annealed oligonucleotides (Eurofins genomics) with specific overhangs complementary to the Bsmbl-digested pLentiCas9V2 backbone (Addgene catalog number # 52961 #83480). Cells were transduced with lentivirus expressing these constructs and selected for 7 days. Knockout efficiency was monitored by immune blotting when antibodies were available. In the case of *DHCR7* and *SC5D* the knockout was confirmed by measuring 7-DHC or lathosterol accumulation respectively. Cells were used as pools unless stated otherwise. A list of the sequences of the guides used in the study is provided below (sgRNA+NGG):

hDHCR7_sgRNA1 - CCACAAGGTATAGAGCTGGGCGG

hDHCR7_sgRNA2 - TGCGAAGGACAGGTTGATGAGGG

mDHCR7_sgRNA2 - TAGGCTGGGGAGATTGTGTGTGG

mDHCR7_sgRNA2 - AGCGAAGGACAGGTTAATGAGGG hSC5D_sgRNA1 - ACAGTAAGAATACTTATCCATGG hSC5D_sgRNA2 - TTCATCTACTGGATTCACAGAGG hACSL4_sgRNA1 - GTGAAAGAATACCTGGACTGGGG hACSL4_sgRNA2 - GGTGCTGGGACAGTTACTGAAGG EGFP_sgRNA2 - CAACTACAAGACCCGCGCCG

Immunoblotting. Immunoblot analysis of cell lysates was performed essentially as described previously¹, using antibodies to GPX4 (1:1,000; no. ab125066, Abcam), β -actin (1:10,000; no. A5441, Sigma-Aldrich), ACSL4 (1:200; no. sc-271800, Santa Cruz), Flag-Tag. Chemilumiscent images were acquired on a chemiluminscent detection system (Azure 300, Biozym, Germany).

FENIX assay (related to Figure 3). General. Phosphate buffered saline (PBS) was 12 mM phosphate, 150 mM NaCl, pH 7.4, and passed over a Chelex-100 column pre-equilibrated at pH 7.4. All purifications were completed using solvents purged with N₂ for 20 minutes. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti) was used as received. STY-BODIPY was prepared as reported in Haidasz et al.,⁵ and DTUN was prepared as reported in Shah et al.,⁶.

Purification of Sterols and SoyPC

7-DHC (Sigma, 95%) was purified before use by passing through a short silica plug with 1:4 EtOAc:hexanes. The product was protected from light and stored at -78°C under N₂. Cholesterol (Alfa Aesar, 95%) was purified by formation of cholesterol dibromide with Br₂, reduction with zinc and recrystallisation by the method of Fieser. Soy phosphatidylcholine (soy PC, Avanti) was purified by the method of Singleton⁷ to remove inhibitor. In brief, a 1.2 cm x 30 cm chromatography column was charged with a slurry of 28 g of neutral alumina in CHCl₃. A solution of 0.5 g soy PC in 10 mL CHCl₃ was then loaded onto the column followed by 50 mL CHCl₃. The product was eluted with 150 mL 1:9 MeOH:CHCl₃ while under N₂. The homogenous fractions (TLC on silica, 4:25:71 H₂O:MeOH:CHCl₃ eluant, Rf = 0.4, stained with KMnO₄) were combined and protected from light while concentrating under reduced pressure, then 5 portions of CHCl3 were used to azeotropically dry the residue. The purified soy PC was aliquoted, the residual solvent removed on high-vacuum overnight, and then stored neat at -78°C under N₂.

Preparation of SoyPC Liposome Compositions

Solutions of soy PC (31 µmol in CHCl₃) and additive (in CHCl₃) were combined to give the desired molar ratio (100% * moles of additive / total number of moles in bilayer) in 4 mL vials. The solution was concentrated to a thin film under N₂ flow, then under high-vacuum for 1 h in the dark. The residue was hydrated with N₂-purged PBS (1.03 mL), vortexed thoroughly, and the vials subjected to 10 cycles of freeze (4 mins in liquid N₂), thaw (4 mins, 30°C), and sonication (4 mins, 30°C). The lipid suspensions were then extruded (Avestin LiposoFast) 25 times across a 100 nm polycarbonate membrane, and stored under N₂ at 4°C (7-DHC treated samples were used immediately; others within 24 h).

Soy PC/STY-BODIPY Co-autoxidations

Solutions of liposomes (1.027 mM) and STY-BODIPY (1.027 μ M) were vortexed together and aliquoted (292 μ L) into a 96-well microplate (black, Nunc). PMC (3 μ L of 400 μ M in DMSO) or vehicle was added and the plate was incubated at 37°C in a plate reader (Biotek Synergy H1) for 20 mins. The reactions were initiated by addition of DTUN (5 μ L of 12 mM in EtOH), and the microplate mixed by the instrument for 3 mins before collecting sample fluorescence (λ ex = 488 nm, λ em = 518 nm) every 60 seconds. The rate of initiation was determined from PMC-inhibited (4 μ M) reactions to be R_{*i*} = (2 × [PMC]) / t_{inh} = (8.3 ± 0.26) x 10-10 Ms⁻¹ and did not vary more than ca. 10% in the various liposome compositions under these conditions. Reactions were run in analytical duplicates and the experiments repeated independently at least three times. The kinetics are reported as the mean ± standard deviation.

UPLC-MS Analysis of Soy PC Autoxidations

A 1.5 mL LC vial was equilibrated in a heating block at 37°C with PBS (483 µL) and liposomes (16.8 µL of 30 mM) for 5 mins. An aliquot (25 µL) of the sample was removed for analysis prior to initiating the reaction with DTUN (7.9 µL of 12 mM in EtOH) and gently vortexing to mix. Aliguots were then removed every 30 mins for a total of 2 hours reaction time. Each aliquot was immediately prepared for analysis by combination with chilled MeOH (75 μ L with 13.3 μ M prostaglandin B₂ as internal standard and 1 mM BHT) in a standard 200 µL LC vial insert and vortexing for 10 sec to lyse the liposome particles and solubilise the lipids. Each sample was analysed immediately on a Waters Acquity H-Class instrument fitted with a 4.6 mm x 250 mm Hypersil Gold C18 column and TQD-MS detector in ESI-positive mode (capillary voltage, 3.90 kV; cone voltage, 44 V; source temperature, 150°C; desolvation temperature, 400°C; desolvation gas, 800 L/h; collision gas flow, 0.1 mL/min; collision energy 34 V). Mobile phase (30 mins total, 1 mM NH₄OAc maintained throughout): t = 0 to 12 min, 15:85 to 1:99 H2O:MeOH; t = 12 to 24 min, hold at 1:99 H₂O:MeOH; t = 24 to 25 min, 1:99 to 15:85 H2O:MeOH; t = 25 to 30 min, hold at 15:85 H₂O:MeOH. Lipid hydroperoxides were detected by their MRM transitions: PLPC-OOH (tR = 15.5 min), 790.6 to 184.1 m/z; DLPC-OOH (tR = 14.5 min), 814.6 to 184.1; DLPC-200H (tR = 11 min), 846.5 to 184.1 m/z. The internal standard (tR = 3.8 min) was detected by SIR at 375.5 m/z. The chromatograms were processed by smoothing (scan window 2, 20 smooths, method: mean) and taking the ratio of PLPC-OOH peak integration / IS peak integration. Each reaction was repeated at least twice and is reported as the

mean ± standard deviation for the kinetic plot or mean ± standard error for relative rates derived from linear regression.

UV-Vis Analysis of Soy PC Autoxidations

A 3 mL quartz cuvette was equilibrated in a Cary 100 spectrophotometer at 37°C with PBS (2.38 mL) for 5 mins, and then baselined. Liposomes were added (83.3 μ L of 30 mM) and the cuvette inverted 5 times to mix before an initial spectrum was recorded. The reactions were then initiated with addition of DTUN (41.7 μ L of 12 mM in EtOH), the cuvette inverted 5 times to mix, and spectra from 260 to 300 nm were recorded every 10 mins. The spectra were processed by subtracting each spectrum of the 7-DHC + DTUN loaded liposomes by the first spectrum of vehicle liposomes + DTUN. A standard curve for 7-DHC in liposomes was prepared in a similar manner using the spectra obtained with liposomes prepared with non-purified soy PC that contained inhibitor to minimise 7-DHC autoxidation. The 7-DHC was quantified at 294 nm to minimise interference by lipid conjugated diene formation. The resulting kinetic traces eventually begin to increase due to these products and the formation of 7-DHC derived oxidation products. For this reason, the loss of absorbance at 294 nm plateaus at ca. 60% of the expected conversion of 7-DHC initially in the liposome sample.

Iron mediated liposomal oxidation

Lipid oxidation analysis through Ultra High Performance Liquid Chromatography (UHPLC).

All reagents and lipid standards were purchased from Sigma Aldrich (St. Louis, US) or Avanti Polar Lipids (Alabaster, US). Organic solvents were purchased from Supelco/Merck KGaA (Darmstadt, Germany).

Preparation of unilamellar liposomes

Unilamellar liposomes were prepared as described previously^{8,9}. Aliquots of L-α-phosphatidylcholine from egg yolk (egg PC), lathosterol, 7-dehydrocholesterol (7-DHC) and cholesterol (all dissolved in isopropanol or chloroform) were added to PYREX® test tubes in the following proportions: a) 100% egg PC (5 mM); b) 75% egg PC (3.75 mM) and 25% lathosterol (1.25 mM); c) 75% egg PC (3.75 mM) and 25% lathosterol (1.25 mM); c) 75% egg PC (3.75 mM) and 25% 7-DHC (1.25 mM); and d) 75% egg PC (3.75 mM) and 25% cholesterol (1.25 mM). Isopropanol and chloroform were removed with a stream of nitrogen gas and under vacuum for 1 hour, leading to the formation of dried lipid films on the test tube walls. Lipids were resuspended with 2 mL 10 mM Tris-HCl buffer (pH 7.4) and then introduced to a LiposoFast Liposome apparatus (Avestin, Ottawa, Canada), passing 21 times through a membrane of 100 nm pore size.

Iron-induced oxidation of liposomes

Each of the liposome suspensions was divided into triplicates of 600 μ L in 1.5 mL Eppendorf Tubes®. Iron (III) sulfate and L-ascorbic acid were added at the final concentrations of 40 and 400 μ M, respectively, to all triplicates except the controls. Therefore, five different incubations were prepared: a) liposomes of 100% egg PC without Fe3+/ascorbate; b) liposomes of 100% egg PC with Fe3+/ascorbate (40 μ M/400 μ M); c) liposomes of 75% egg PC and 25% lathosterol with Fe3+/ascorbate (40 μ M/400 μ M); d) liposomes of 75% egg PC and 25% 7-DHC with Fe3+/ascorbate (40 μ M/400 μ M); and e) liposome of 75% egg PC and 25% cholesterol with Fe3+/Ascorbate (40 μ M/400 μ M). Eppendorf ThermoMixer® (Eppendorf, Hamburg, Germany) was used for the incubations at 37 °C and 600 rpm. From each incubation, 60 μ L aliquots were removed at different time points (0, 20 and 40 min, in addition to 1h, 1h30min, 2h15min, 3h, 3h45min, 4h30min and 20h), in a total of 150 aliquots. Once removed, the aliquots were frozen and kept in a -80 °C freezer for subsequent HPLC analysis.

Quantification of lipid substrates and oxidation products via UHPLC coupled to UV detection

Collected aliquots were analysed through reversed-phase HPLC (Nexera UHPLC, Shimadzu, Kyoto, Japan) coupled to UV detection (scan from 190 to 370 nm) using a "Luna 5u C8(2) 100 A 250x4.60 mm" column (Phenomenex, Torrance, US). The following parameters were used for UHPLC analysis: 30 μ L sample injection, 1 mL/min isocratic flow (94% MeOH and 6% H₂O) and oven temperature of 36 °C, allowing the chromatographic separation of substrates (egg PC and sterols) and products (phosphatidylcholine hydroperoxides, PC-OOH). After running each one of the samples, integration of peak areas was performed at 205 nm for egg PC, lathosterol and cholesterol; at 275 nm for 7-DHC and 235 nm for PC-OOH. Peak area values were plotted as a function of time.

Iron oxidation-induced carboxyfluorescein (CF) release from liposomes

Membrane stability assays of CF release from liposomes were performed according to Bacellar et al.¹⁰ and specific steps described below:

Preparation of unilamellar liposomes containing encapsulated CF

Aliquots of L-α-phosphatidylcholine from egg yolk (egg PC), 7-dehydrocholesterol (7-DHC) and cholesterol (all dissolved in isopropanol or chloroform) were added to test tubes according to the following proportions (mol %): a) 75% egg PC and 25% 7-DHC; and b) 75% egg PC and 25% cholesterol. Isopropanol and chloroform were removed with a stream of nitrogen gas and under vacuum for 1 hour. Lipids were then resuspended with 500 µL of 10 mM Tris-HCl buffer (pH 8.0) containing 0.3 M NaCl and 50 mM CF (Acros Organics, Geel, Belgium). Resulting solutions went through an extrusion step of 21 times passes through a 100 nm pore diameter membrane in a LiposoFast Liposome apparatus (Avestin, Ottawa, Canada), leading to the formation of unilamellar liposomes with encapsulated CF. Liposome suspensions were submitted to a subsequent size exclusion chromatography step in a Sephadex G50 column for separation and disposal of non-encapsulated CF. Both purified liposome suspensions, containing cholesterol or 7-DHC, were stored protected from light at room temperature for use on the same day. The final concentration of phospholipids in both liposome suspensions (approx. 800 µM) was determined by a colorimetric assay with ammonium ferrothiocyanate¹¹.

Iron induced-oxidation of liposomes with encapsulated CF:

60 μ L of purified liposome suspensions were added to the wells of a 96-well plate. For quintuplicates of each liposome, oxidation was induced by addition of 20 μ M Fe2(SO4)3 and 200 μ M ascorbic acid at 37 °C in a Spark® microplate reader (Tecan, Zürich, Switzerland). The same reaction was performed in the presence of 10 μ M liproxstatin-1 (Lip-1), in addition to a control condition without oxidants. For all reactions, final volumes of 300 μ L were completed by the addition of 10 mM Tris-HCl buffer (pH 8.0) with 0.3 M NaCl. 30 μ L aliquots from extra quadruplicates were collected at five reaction time points (0, 2, 4, 8 and 16.5 h) for further analysis through liquid chromatography coupled to mass spectrometry (LC-MS).

Fluorescence monitoring of CF release by oxidised liposomes:

As the concentration of CF within liposomes is high enough to promote fluorescence self-quenching, an increase in detected fluorescence indicates liposome permeabilisation and leakage of CF from liposomes to the external solution. This phenomenon was used for indirect quantification of oxidative damage to liposomes under lipoperoxidation. Fluorescence was monitored at 517 nm (I) with excitation at 480 nm for a total of 18 hours. 20 μ L of Triton X-100 were added to each of the wells after the last reaction time point in order to completely disrupt liposomes; then fluorescence was measured again (IT). For each value of I, the percentage of CF release was calculated as presented in equation (1), and finally plotted as

% *CF* release
$$= \frac{f-f_0}{f_{max}-f_0} * 100.$$

a function of time.

Carboxyfluorescein release assays (related to figure 3)

The PL mixtures were dissolved in Carboxyfluorescein (80 mM Carboxyfluorescein, pH 7.0) in a final concentration of 5 mg/mL. Continuing, six cycles of freezing (-80 °C) and defreezing (37 °C) were conducted to ensure homogeneity of the liposomes. The PL mixtures were extruded 31 times through a polycarbonate membrane with pores of 100 nm diameter. The liposomes were filtered using a Sephadex G-50 matrix column and outside buffer (140 mM NaCl, 20 mM HEPES, 1 mM EDTA, pH 7.0) to remove the untraped CF. The liposomes (~50 µg/ml) were treated with different lipid species or with Triton X-100 as detergent positive control in a black plate with 96-wells. Fluorescence intensity of Carboxyfluorescein released by liposome rupture was measured at 488 nm emission and 520 nm extinction wavelength every minute for one hour in a Enspire plate reader. The percentage of CF release was calculated with the

$$\% CF \ release = \frac{f - f_0}{f_{max} - f_0} * 100$$

following formula:

Quantification of 7-DHC-derived oxysterols. $5x10^{6}$ per 15-cm dishes were seeded one day prior to the experiment. On the next day, medium were replaced with fresh medium with DMSO, RSL3 (200 nM), Lip-1 (500 nM) or Lip1+RSL3 and cells were treated for the indicated time points. Subsequently, cells were washed with PBS and tripsined. Medium was added to stop tripsinisation and pellet at 600g for 5 min. After this step, cells were washed with PBS (600 g for 5 min), and ressuspended in 2,2 mL of PBS. Two aliquots of 1 mL were pelleted and frozen in liquid N₂. 50 µL of cell suspension was kept for PI analysis as described previously. DHCEO, 4 α -OH-7-DHC and 4 β -OH-7-DHC were analysed by LC-MS/MS using an

APCI source in the positive ion mode as described previously¹². Briefly, lipid content from cell lysate was extracted and the neutral lipids fraction was purified by SPE chromatography. Purified content was resuspended in methanol and 10 µL was injected onto the column (Phenomenex Luna Omega C18, 1.6 µm, 100 Å, 2.1 × 100 mm) using ACN (0.1% v/v acetic acid) (solvent A) and methanol (0.1% v/v acetic acid) (solvent B) as mobile phase. The gradient was: 5% B for 2 min; 5–95% B for 0.1 min; 95% B for 1.5 min; 95–5% B for 0.1 min; 5% B for 0.5 min. The oxysterols were analysed by SRM using the following transitions: DHCEO 399 \rightarrow 381, 4 α -OH-7-DHC 383 \rightarrow 365, and 4 β -OH-7-DHC 383 \rightarrow 365. The SRM for the internal standard (D7-chol) was set to 376 \rightarrow 376 and response factors were calculated to accurately determine the oxysterol levels. Final oxysterol levels are reported as nmol/mg of protein.

Lipidomics and sterol analysis.

For the lipidomic analysis, 10E⁶ cells were extracted according to the Bligh / Dyer method¹³ with 170µl 0.1M HCl, 190µL MeOH, 190µL of CHCl3 and 20 µl external standards (0.1 mM D7-cholesterol and 0.05 mM D7-DHC in CHCl₃/MeOH (50/50, v/v)). Samples were vortexed and centrifuged. The upper phase was transferred to a fresh tube and re-extracted with 300µL CHCl₃/MeOH/H₂O (70/40/10, v/v/v). Combined lower phases are subsequently evaporated under a stream of N₂ and re-dissolved in 50µL of iPrOH. From this, 3µL are applied to the LC/MS system (Thermo Scientific Dionex Ultimate 3000 hyphenated with a Q Exactive mass spectrometer (QE-MS) equipped with a HESI probe (Thermo Scientific, Bremen, Germany); UPLC-precolumn: Acclaim 120 C8 (5 µm particles, 10 × 2 mm) (Thermo Scientific, Bremen, Germany) -UPLC-column: Acclaim RSLC 120 C8 (2.2 µm particles, 50 × 2.1 mm) (Thermo Scientific, Bremen, Germany)). Lipids were separated using a combination of mobile phase A (consisting of CH₃CN/H₂O/FA (10/89.9/0.1, v/v/v)) and mobile phase B CH₃CN/iPrOH/H₂O/FA (45/45/9.9/0.1, v/v/v). The gradient utilised was 20% solvent B for 2 min, followed by a linear increase to 100% solvent B within 5 min, then maintaining 100% B for 33 min, then returning to 20% B in 1 min and 5 min 20% solvent B for column equilibration before each injection. The flow rate was maintained at 350 µL/min at 40 °C. The eluent was directed to the ESI source of the QE-MS and analyzed from 2.0 min to 38 min after sample injection. Peaks corresponding to the calculated monoisotopic metabolite masses (MIM+/-H+ ± 3 mMU) were integrated using TraceFinder V3.3 software (Thermo Scientific, Bremen, Germany).

Epilipidomic analysis

Samples were prepared identically as described for the analysis of 7-DHC oxysterols. Lipids were extracted according to methyl-tert-butyl ether (MTBE) protocol¹⁴. All solvents contained 0.1% BHT and were cooled on ice before use. Briefly, cell pellets (5×10^6 cells; 5 experimental replicates; total of 180 samples) collected in PBS containing BHT and DTPA were washes, centrifuged, and resuspended in 40 µL of water. 4.5 µL of SPLASH® LIPIDOMIX® (Avanti Polar Lipids Inc., Alabaster, AL, USA) was added, and samples were left on ice for 15 min. Ice cold methanol (375 µL) and MTBE (1250 µL) were added, samples were vortexed and incubated for 1 h at 4°C (Orbital shaker, 32 rpm). Phase separation was induced by addition of water (375 µL), vortexed, incubated for 10 min at 4°C (Orbital shaker, 32 rpm), and

centrifuged to separate organic and aqueous phase (10 min, 4°C, 1000 x g). Organic phase was collected, dried in the vacuum concentrator, redissolved in isopropanol (100 µL), centrifuged and transferred in glass vials for LC-MS analysis. Reversed phase liquid chromatography (RPLC) was carried out on a Vanguish Horizon (Thermo Fisher Scientific, Bremen, Germany) equipped with an Accucore C30 column (150 x 2.1 mm; 2.6 µm, 150 Å, Thermo Fisher Scientific, Bremen, Germany). Lipids were separated by gradient elution with solvent A (acetonitrile/water, 1:1, v/v) and B (isopropanol/acetonitrile/water, 85:15:5, v/v) both containing 5 mM NH_4HCO_2 and 0.1% (v/v) formic acid. Separation was performed at 50°C with a flow rate of 0.3 mL/min using following gradient: 0-20 min – 10 to 86 % B (curve 4), 20-22 min - 86 to 95 % B (curve 5), 22-26 min - 95 % isocratic, 26-26.1 min - 95 to 10 % B (curve 5) followed by 5 min re-equilibration at 10% B. For relative quantification of oxidised lipids retention time scheduled parallel reaction monitoring (PRM) using elemental composition of 47 previously identified oxidised lipids was used in negative ion mode at the resolution of 17,500 at m/z 200, AGC target of $2e^5$ and a maximum injection time of 200 ms. The isolation window for precursor selection was 1.2 m/z, and normalised stepped collision energy of 20-30-40 was used for HCD. Data were acquired in profile mode. Acquired data were proceed by Skyline v. 21.1.0.146 (MacCoss Lab¹⁵) considering fragment anions of oxidised fatty acyl chains as quantifier. The obtained peak areas were normalised by appropriate lipid species from SPLASH® LIPIDOMIX® Mass Spec Standard (Avanti), e.g. by LPC(18:1(d7)), LPE(18:1(d7)), PC(15:0/18:1(d7)), or PE(15:0/18:1(d7)), and protein concentration measured for the corresponding sample. Normalised peak areas were further log-transformed and autoscaled in MetaboAnalyst online platform (https://www.metaboanalyst.ca, Xia Lab; ¹⁶). The heatmaps were created in Genesis v. 1.8.1 (Bioinformatics TU-Graz¹⁷), using mean values of log-transformed autoscaled features. The color scheme corresponds to log fold change relative to the mean log value within the samples. Shorthand notations for oxidised lipids are given using LipidLynxX system (https://www.biorxiv.org/content/10.1101/2020.04.09.033894v1).

Bioinformatics

Homology models were generated using SWISS-MODEL¹⁸ via its integrated web-based service available at https://swissmodel.expasy.org/. We used the target-template alignment function of swiss model to match the human DHCR7 sequence with the Methylomicrobium alcaliphilum sequence, and modelled the DHCR7 structure using pdb file 4QUV. Prediction of phosphorylation sites was carried out using the NetPhos 3.1 server¹⁹ using default parameters. Protein structures were visualised using PyMOL (version-2.3.4, Schrodinger, LLC). Amino acid neighbors were identified using a cut-off distance of 5Å. The DHCR7 transmembrane boundaries were predicted based on the 4QUV positioning in a lipid bilayer that had been predicted by minimising its transfer energy from water to the membrane and stored in the Orientations of Proteins in Membranes (OPM) database²⁰.

Xenograft experiments

Animal studies were approved by the district government of lower Franconia (protocol number 55.2-2532-2-335) and were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Briefly, female NOD.Cg-Prkdcscid Il2rgtm1WjI/SzJ (NSG)-mice (8 to 12 weeks old) were purchased from Charles River, Sulzfeld. A mixture of 50 μ L ECM gel (Merck, Darmstadt, Germany) and 50 μ L RPMI-1640 medium containing 5x10⁵ cells was injected, subcutaneously on the right and left flanks of the mice, genotypes of the cells were kept blinded. Four to five weeks after injections, animals were euthanized, the tumour explanted and its mass determined.

Data presentation and statistical analyses. Data are presented as mean \pm s.d. unless stated otherwise. As a general rule for cell-based experiments, graphs show the mean \pm s.d. of n = x wells (x values are given in the figure legends) representative of a single experiment performed independently y times (y value is given in figure legends) for reproducibility. Statistical analysis was performed using GraphPad Prism 5.0 software.

References

1. Seiler, A. et al. Glutathione peroxidase 4 senses and translates oxidative stress into 12/15lipoxygenase dependent- and AIF-mediated cell death. Cell Metab 8, 237-248 (2008).

2. Doll, S. et al. ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. Nat Chem Biol 13, 91-98 (2017).

3. Li, W. et al. MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens. Genome Biol 15, 554 (2014).

4. Michlits, G. et al. Multilayered VBC score predicts ssgRNAs that efficiently generate loss-offunction alleles. Nat Methods 17, 708-716 (2020).

5. Haidasz, E.A., Van Kessel, A.T. & Pratt, D.A. A Continuous Visible Light Spectrophotometric Approach To Accurately Determine the Reactivity of Radical-Trapping Antioxidants. J Org Chem 81, 737-744 (2016).

6. Shah, R., Farmer, L.A., Zilka, O., Van Kessel, A.T.M. & Pratt, D.A. Beyond DPPH: Use of Fluorescence-Enabled Inhibited Autoxidation to Predict Oxidative Cell Death Rescue. Cell Chem Biol 26, 1594-1607 e1597 (2019).

7. Fieser, L.F. Some aspects of the chemistry and biochemistry of cholesterol. Science 119, 710-716 (1954).

8. Terao, J., Piskula, M., Yao, Q. Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. Arch Biochem Biophys 308, 278-284 (1994).

9. Miyamoto, S., Kuwata, G., Imai, M., Nagao, A., Terao, J. Protective effect of phytic acid hydrolysis products on iron-induced lipid peroxidation of liposomal membranes. Lipids 35,1411-1413 (2000).

10. Bacellar, I.O.L. et al. Photosensitized Membrane Permeabilization Requires Contact-Dependent Reactions between Photosensitizer and Lipids. J Am Chem Soc 140, 9606-9615 (2018).

11. Stewart, J.C. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem 104, 10-14 (1980).

12. Genaro-Mattos, T.C. et al. Maternal cariprazine exposure inhibits embryonic and postnatal brain cholesterol biosynthesis. Mol Psychiatry 25, 2685-2694 (2020).

13. Bligh, E.G., Dyer, W.J. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37, 911–917 (1959).

14. Matyash, V., Liebisch, G., Kurzchalia, T. V., Shevchenko, A. & Schwudke, D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res 49, 1137-1146 (2008).

15. Adams, K. J. et al. Skyline for Small Molecules: A Unifying Software Package for Quantitative Metabolomics. J Proteome Res 19, 1447-1458 (2020).

16. Chong, J., Wishart, D. S. & Xia, J. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. Curr Protoc Bioinformatics 68, e86, (2019).

17. Sturn, A., Quackenbush, J. & Trajanoski, Z. Genesis: cluster analysis of microarray data. Bioinformatics 18, 207-208 (2002).

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., de Beer, T. A. P., Rempfer, C., Bordoli, L., Lepore, R., & Schwede, T. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Research, 46(W1), 296–303 (2018).

19. Blom, N., Gammeltoft, S., and Brunak, S. Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. Journal of Molecular Biology 294, 1351-1362 (1999).

20. Lomize, M.A., Pogozheva, I.D., Joo, H., Mosberg, H.I., Lomize, A.L. OPM database and PPM web server: resources for positioning of proteins in membranes. Nucleic Acids Res. 40 (Database issue), 370-376 (2012).

Figures



Figure 1

Identification and impact of DHCR7 deficiency on Ferroptosis. a, Schematic of the identification of Dhcr7 as a pro-ferroptotic gene, using CRISPR-KO library and GPX4 inhibition. b, Volcano plot of sgRNA enriched in cells selected with RSL3 compared with untreated control cells. c, Relative quantification of 7-DHC and Cholesterol levels in HT1080 cell lines stably transduced with a vector expressing Cas9 and a sgRNA targeting DHCR7 and EGFP as a control. Data are the mean \pm s.d. of n = 3 wells of a 6-well plate from one representative experiment. d, Immunoblot analysis of key ferroptosis regulators, namely FSP1, ACSL4

and GPX4 in DHCR7 deficient cells. e, Dose-dependent toxicity of the ferroptosis inducers RSL3, ML210 and FIN56 in HT1080 cell lines stably transduced with a vector expressing Cas9 and an sgRNA targeting DHCR7 and EGFP as a control. Data are the mean \pm s.d. of n = 3 wells of a 96-well plate from one representative of two independent experiments. f, Relative quantification of 7-DHC levels in HT1080 levels WT and DHCR7-Knockout cells re-expressing DHCR7 and an empty vector. Data are the mean \pm s.d. of n = 3 wells of a 6-well plate from one representative experiment. g, dose-dependent toxicity of RSL3 and FIN56 in HT1080 Cas9 DHCR7-KO clone, and overexpressing DHCR7 or mock. Cell viability was monitored using Alamar blue. Data are the mean \pm s.d. of n = 3 wells of a 96-well plate from one representative of two independent experiments; *P < 0.05; two-way analysis of variance (ANOVA).



Figure 2

7-DHC accumulation suppresses ferroptosis a, Schematic of final steps of cholesterol biosynthesis. b, Dose-dependent toxicity of RSL3 in HT1080 Cas9 WT, DHCR7 and DHCR7/SC5D knockout cell lines. Cell viability was assessed after 24 h using Alamar blue. c, Relative quantification of 7-DHC and Cholesterol levels in HT1080 Cas9 WT, DHCR7 and DHCR7/SC5D knockout cell lines. d, Dose-dependent toxicity of the ferroptosis inducers RSL3, ML210 and Erastin in the HT1080 cell lines described in (d). Cell viability was assessed after 48 h using Alamar blue. e, Effect of sterol supplementation (10µM) on RSL3 toxicity in HT1080 Cas9 WT, DHCR7 and DHCR7/SC5D knockout cell lines. Cell viability was assessed after 48 h using Alamar blue. f, Flow cytometry analysis of BODIPY 581/591 C11 oxidation in HT1080 cell line induced by RSL3 treatment (100 nM, 5h) in cells pre-treated for 16h with 10µM of different sterols. Data are the mean \pm s.d. of n = 3 wells of a 96-well plate (b, d and e) or a 6 well plate (c and f) from two (b, d and e), or one (c anf f) independent experiments; *P < 0.05; two-way analysis of variance (ANOVA).



Figure 3

7-DHC acts to suppress (phospho)lipid peroxidation. a, Soy PC liposomes supplemented with cholesterol, 7-DHC or DPPC were prepared and autoxidised using DTUN (200 µM) in PBS (12 mM, pH 7.4, 37°C). STY-BODIPY co-autoxidations are monitored by fluorescence ($\lambda ex = 488 \text{ nm}$, $\lambda em = 518 \text{ nm}$) of oxidised STY-BODIPY. b, Representative data from co-autoxidations of STY-BODIPY (1 µM) and liposomal soy PC compositions (mol% additive). c, The rates of STY-BODIPYox formation plotted as a function of additive concentration. d, The formation of PLPC-00H, DLPC-00H and DLPC-200H formed during autoxidation of soy PC can be analysed by LC-MS/MS using MRM. e, The resulting profiles of PLPC-OOH, DLPC-OOH and DLPC-200H formation over time (integrations are relative to an internal standard, prostaglandin B2). f, Rates were calculated from linear regression of the data, normalised to the uninhibited rate. g, Representative UV-Vis spectra obtained from a sample of soy PC with 8 mol% 7-DHC during autoxidation. Spectra were processed by subtracting the background trace of vehicle liposomes immediately after the addition of DTUN. Loss of 7-DHC was plotted from the 294 nm peak (inset). Each reaction (b,c,e,f) was repeated at least twice and is reported as the mean ± s.d for the kinetic plot or mean ± s.d for relative rates derived from linear regression.h, Schematic of 7-DHC oxidation. i, Quantification of DHCEO in HT1080 SC5D/DHCR7 double knockout cells expressing mock vector (black) and SC5D (red) upon 200nM RSL3 with and without 500nM Lip1 for 6h. Data are the mean ± s.d. of n = 6 wells of a 10cm plate from two independent experiments. ****P < 0.0001; two-way analysis of variance (ANOVA).



Figure 4

Phospholipid truncated species contribute to ferroptosis execution. a, Epilipidomics analysis of HT1080 Cas9 DHCR7/SC5D double KO cells overexpressing SC5D or an empty vector. Data is representative of an experiment performed five times independently (please see results from the other repetitions in the supplementary information). The heatmap overlaying critical event in ferroptosis, i.e cell rupture (PI positive), 7-DHC/Chol ratio and DHCEO values from matched experimental time points are depicted

below in grey scale. b, Lipidomics analysis of WT and ACSL4 KO HT1080 cell lines incubated for 16 hours with α LNN and γ LNN (20 μ M). Presented are the total amount of PE containing PUFA and the ratio of mono- to polyunsaturated fatty acids (MUFA/PUFA) in PE species. Mean values ± s.d. of n = 3 technical replicates (10cm plate) performed twice are presented . c, Fatty acid composition of PE species in WT and ACSL4 KO HT1080 cell lines incubated for 16 hours with α LNN and γ LNN (20 μ M). Mean values ± s.d. of n = 3 technical replicates (10cm plate) performed twice are presented twice are presented. d, Assessment of the impact of α LNN and γ LNN (20 μ M) re-sensitisation on RSL3-induced ferroptosis. Cell viability was assessed after 24 h measuring PI incorporation. Mean values ± s.d. of n = 3 technical replicates (6cm plate) performed twice are presented. *P < 0.05; two-way analysis of variance (ANOVA).



Figure 5

Impact of 7-DHC accumulation on BL growth a, Homology model of the DHCR7 structure based on the sterol reductase from M. alcaliphilum. Mutations are indicted as ball and stick. b, Immunoblot analysis of FLAG-tagged DHCR7 WT and mutant variants in a HT1080-DHCR7 knockout cell line. c, Relative quantification of 7-DHC in a HT1080-DHCR7 knockout cell line expressing FLAG-tagged DHCR7 constructs d. Dose-dependent toxicity of the ferroptosis inducer RSL3 and the combination of RSL3 and

Tasin1 (500nM) in HT1080. Data represent the mean \pm s.d. of n = 3 wells of a 96-well plate from one representative of two independent experiments. e. Left panels, dose-dependent toxicity of RSL3 in KMS26 and BL41 expressing two independent sgRNA targeting DHCR7 and EGFP. Cell viability was monitored using Alamar blue. Data are the mean \pm s.d. of n = 3 wells of a 96-well plate from one representative of two independent experiments; *P < 0.05; two-way analysis of variance (ANOVA). Right panels, relative quantification of 7-DHC levels in the KMS26 and BL41 cell lines treated with RB38 and Tasin-1. Data are the mean \pm s.d. of n = 3 wells of a 6-well plate from one representative of pharmacological inhibitors of DHCR7 (RB38) and EBP (Tasin-1) on the growth of BL41 and KMS26 cells transduced with an sgRNA targeting DHCR7 and EGFP in medium lacking thiol supplementation. g, Growth of KMS26 xenograft transduced with a vector expressing Cas9 and a sgRNA targeting DHCR7 and EGFP in NSG mice. Data are the mean \pm s.d. of n = 8 mice (KMS26), tumor have been injected on both flanks; *P < 0.05; two-way analysis of variance (ANOVA).h, graphical abstract depicting the anti-ferroptotic function of 7-DHC in the suppression of the formation of truncated-PL species.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Extendeddata1.eps
- Extendeddata2.eps
- Extendeddata3.eps
- Extendeddata4.eps
- Extendeddata5.eps