The lipid peroxidation product 4-hydroxynonenal contributes to oxidative stressmediated deterioration of the ageing oocyte

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Fig. S1: 4-HNE is a by-product of lipid peroxidation in the mouse ovary. The production and localisation of 4-HNE between young (4 to 6 weeks) and aged (14 months) mouse ovaries was determined using immunofluorescence. (a) 4-HNE expression (red) was observed at basal levels within all cell types within the young ovary (scale bar = 200 μ m) including oocytes in (b) primordial (P), primary (Pr, scale bar = 20 μ m), (c) secondary (S, scale bar = 40 μ m) and (d) antral follicles (A, scale bar = 80 μ m). (e) 4-HNE generation was also observed in all cell types within the aged ovary (scale bar = 300 μ m) including oocytes in (f) primordial, primary (scale bar = 20 μ m) (g) secondary (scale bar = 40 μ m) and (h) antral follicles (scale bar = 80 μ m). Nuclei were counterstained with DAPI (blue). Immunofluorescence assays were performed in both technical and biological triplicate.



Fig. S2: **4-HNE antibody specificity.** L-Lysine-4-HNE bound anti-4-HNE antibody controls (lysine) revealed no unspecific primary or secondary antibody binding during **(a)** Immunofluorescence (scale bar = 40 μ m) or **(b)** immunocytochemistry (scale bar = 20 μ m) compared to unbound anti-4-HNE antibody (red). Nuclei were counterstained with DAPI (Immunofluorescence) or Hoechst (immunocytochemistry) (blue). Antibody specificity was also confirmed for immunoblotting, which also revealed no unspecific primary or secondary antibody binding in L-Lysine-4-HNE bound anti-4-HNE antibody controls (lysine). Immunoblotts were stripped and re-probed with GAPDH as a loading control.



Fig. S3: Acute exposure to H_2O_2 and 4-HNE at GV stage causes meiotic arrest and degeneration. Oocytes at GV stage were treated with either H_2O_2 for 1 h or 4-HNE for 2 h prior to IVM for 16 h. For the purpose of this analysis, GV oocytes were identified by the presence of a nuclear envelope and nucleolus, MI oocytes by the absence of the nuclear envelope and nucleolus and degenerative oocytes were identified *via* cytoplasmic fragmentation. (a) A dose-depended increase in GV arrest was observed after H_2O_2 (one-way ANOVA; $p \le 0.0011$) and (b) 4-HNE (one-way ANOVA; $p \le 0.0037$) treatment. (c) A dose-dependent increase following a significant decrease was also observed in MI arrest after H_2O_2 (one-way ANOVA; $p \le 0.0456$) and (d) 4-HNE (one-way ANOVA; $p \le 0.0420$) treatment. (e) Furthermore, a dose-depended increase in oocyte degeneration was observed after H_2O_2 (one-way ANOVA; $p \le 0.0037$) and (f) 4-HNE (one-way ANOVA; $p \le 0.0003$) treatment. Error bars represent SEM. IVM experiments were performed with a minimum of five replicates with each replicate containing between 20-50 oocytes pooled from a minimum of three animals.



Fig. S4: α- and β-tubulin dimer homology model with confirmed 4-HNE modifications. α- tubulin (white) 4-HNE modifications have been identified on Cys295 (green), Lys336 (red), Lys338 (yellow), Cys347 (blue) and Cys376 (orange) as well as on β-tubulin (blue) on Cys303 (purple)^{46,62,63}.

Q71U36	TBA1A_HUMAN	1	-MRECISIHVGQAGVQIGNACWELYCLEHGIQPDGQMPSDKTIGGGDDSFNTFFSETGAG	59
Q9H4B7 P23258	TBB1_HUMAN TBG1_HUMAN	1 1	-MREIVHIQIGQCGNQIGAKFWEMIGEEHGIDLAGSDRGASALQLERISVYYNEAYGR MPREIITLQLGQCGNQIGFEFWKQLCAEHGISPEGIVEEFATEGTDRKDVFFYQADDE ** : :::**.* *** *: ****. * : ::	57 58
Q71U36	TBA1A_HUMAN	60	KHVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDL	117
Q9H4B7	TBB1_HUMAN	58	KYVPRAVLVDLEPGTMDSIRSSKLGALFQPDSFVHGNSGAGNNWAKGHYTEGAELIEN	115
P23258	TBG1_HUMAN	59	HYIPRAVLLDLEPRVIHSILNSPYAKLYNPENIYLSEHGGGAGNNWASG-FSQGEKIHED :::****::**** .:: *::*::*.**:* * :: * :: :	117
Q71U36	TBA1A_HUMAN	118	VLDRIRKLADQCTGLQGFLVFHSFGGGTGSGFTSLLMERLSVDYGKKSKLEFSIYPAP-Q	176
Q9H4B7	TBB1_HUMAN	116	VLEVVRHESESCDCLQGFQIVHSLGGGTGSGMGTLLMNKIREEYPDRIMNSFSVMPSP-K	174
P23258	TBG1_HUMAN	118	IFDIIDREADGSDSLEGFVLCHSIAGGTGSGLGSYLLERLNDRYPKKLVQTYSVFPNQDE ::: : : :: . *:** : **:.*****: : *::: * .: :*: * :	177
Q71U36	TBA1A_HUMAN	177	VSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQIVS	236
Q9H4B7	TBB1_HUMAN	175	VSDTVVEPYNAVLSIHQLIENADACFCIDNEALYDICFRTLKLTTPTYGDLNHLVSLTMS	234
P23258	TBG1_HUMAN	178	MSDVVVQPYNSLLTLKRLTQNADCVVVLDNTALNRIATDRLHIQNPSFSQINQLVSTIMS :* .**:***::*: : :::* :** *: *. *.: *:: ::*:*: :*	237
Q71U36	TBA1A_HUMAN	237	SITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKA-YHEQLSVAEITNAC	295
Q9H4B7	TBB1 HUMAN	235	GITTSLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTAQGSQ-QYRALSVAELTQQM	293
P23258	TBG1_HUMAN	238	ASTTTLRYPGYMNNDLIGLIASLIPTPRLHFLMTGYTPITTDQSVASVRKTTVLDVMRRL . *::**: * :* ** :::* **:** : ::*:: :* :: .	297
Q71U36	TBA1A_HUMAN	296	FEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIATI <mark>K</mark> T <mark>K</mark> RTIQFVDW <mark>C</mark> PTGFK	352
Q9H4B7	TBB1_HUMAN	294	FDARNTMAACDLRRGRYLTVACIFRGKMSTKEVDQQLLSVQTRNSSCFVEWIPNNVK	350
P23258	TBG1_HUMAN	298	LQPKNVMVSTGRDRQTNHCYIAILNIIQGEVDPTQVHKSLQRIRERKLANFIPWGPASIQ :: * * *:: : :*.: .:*. : : ::. *: * *:	357
Q71U36	TBA1A_HUMAN	353	VGINYQPPTVVPGGDLAKVQRAV <mark>C</mark> MLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEG	412
Q9H4B7	TBB1_HUMAN	351	VAVCDIPPRGLSMAATFIGNNTAIQEIFNRVSEHFSAMFKRKAFVHWYTSEG	402
P23258	TBG1_HUMAN	358	VALSRKSPYLPSAHRVSGLMMANHTSISSLFERTCRQYDKLRKREAFLEQFRKED *.: * : ::.* *:* .: * .::. : :.**:. : *.	412
Q71U36	TBA1A_HUMAN	413	MEEGEFSEAREDMAALEKDYEEVGVDSVEGEGEEE-GEEY	451
Q9H4B7	TBB1_HUMAN	403	MDINEFGEAENNIHDLVSEYQQFQDAKAVLEEDEEVTEEAEMEPEDKGH	451
P23258	TBG1_HUMAN	413	MFKDNFDEMDTSREIVQQLIDEYHAATRPDYISWGTQEQ	451

Fig. S5: Amino acid sequence alignment between α-, β- and γ-tubulin represent a 20% sequence homology. An asterisk (*) represents residues conserved between three tubulin isoforms, a colon (:) represents conserved substitution of residues between tubulin isoforms and a full stop (.) represents semi-conserved substitutions such as amino acids with similar characteristics. α-tubulin 4-HNE modifications have been identified on Cys295 (green), Lys336 (red), Lys338 (yellow), Cys347 (blue) and Cys376 (orange) as well as on β- tubulin on Cys303 (purple)^{46,62,63}.

Table S1: LC–MS/MS protein identification (ID) of the predominant 55 kDa protein(s) targeted for adduction by 4-HNE.

Protein ID (gene symbol)	Mascot score	MW [kDa]	No. Peptides
Actin (ACTB)	152.1	41.7	3
Tubulin alpha (TBA3)	81.1	49.9	2
Tubulin beta (TBB4A)	78.1	49.9	2

Table S2: Antibodies used for immunocytochemistry (ICC), immunofluorescence (IF), proximity ligation assays (PLA), immunoprecipitation (IP) and immunoblotting (IB).

Antibody	Species	Concertation	Dilution/ concentration	Source and Cat#
4-HNE	Rabbit polyclonal	1 mg/ ml	1:50 (IF, ICC, PLA) 1:500 (IB)	Cat # HNE11-S, Jomar Diagnostics
4-HNE	Mouse monoclonal	0.1 mg/ ml	1:50 (IB)	Cat # ab48506, Abcam
α-tubulin	Mouse monoclonal	1 mg/ ml	1:400 (ICC, PLA)	Cat # A11126 ThermoFisher
α-tubulin	Rabbit monoclonal	1 mg/ ml	10 µg (IP)	Cat # ab52866, Abcam
α-tubulin	Mouse monoclonal	6 mg/ ml	1:2000 (IB)	Cat # T5168, Sigma-Aldrich
β-tubulin	Rabbit polyclonal	1 mg/ ml	10 µg (IP)	Cat # ab6046, Abcam
β-tubulin	Mouse monoclonal	0.2 mg/ ml	1:100 (ICC, PLA) 1:500 (IB)	Cat# sczsc-5274, Santa Cruz
γ-tubulin	Rabbit polyclonal	0.6 mg/ ml	10 µg (IP)	Cat # ab84355, Abcam
γ-tubulin	Mouse monoclonal	5 mg/ ml	1:1000 (ICC, PLA) 1:5000 (IB)	Cat # T6557, Sigma-Aldrich
CREST	Human monoclonal	1 mg/ ml	1:400 (ICC)	Cat # 90C-CS1058, Fitzgerald
GAPDH	Rabbit polyclonal	5 mg/ ml	1:5000 (IB)	Cat # G9545, Sigma-Aldrich