

1 **Hepatocyte nuclear factor 4-mediated lipotoxicity provokes mitochondrial damage in**
2 **peroxisome-deficient *pex19* mutants**

3

4

5

6 Margret H. Bülow^{1#}, Julia Sellin^{1#}, Christian Wingen^{1#}, Deniz Senyilmaz², Dominic

7 Gosejacob¹, Aurelio A. Teleman², Michael Hoch^{1*}

8

9

10 ¹ University of Bonn, Life & Medical Sciences Institute (LIMES), Molecular Developmental
11 Biology, Carl-Troll-Straße 31, 53115 Bonn, Germany

12

13 ² German Cancer Research Center, Signal Transduction in Cancer and Metabolism, Im
14 Neuenheimer Feld 580, 69120 Heidelberg, Germany

15

16 * Correspondence to: m.hoch@uni-bonn.de

17 # Authors contributed equally

18

19 Short title: Hnf4-mediated mitochondrial damage in *pex19* mutants

20

21

22

23

24

25

26 **Abstract**

27

28 **Peroxisomes are important metabolic organelles involved in the catabolism of several**
29 **lipid classes, e.g. very-long-chain fatty acids. Malfunction or absence of peroxisomes**
30 **leads to accumulation of educts for peroxisomal β -oxidation and mitochondrial damage,**
31 **resulting in fatal perturbation of metabolism. The impact of peroxisome deficiency on**
32 **mitochondria is not elucidated yet. Here we present a model of Hepatocyte nuclear**
33 **factor 4 (Hnf4)-induced lipotoxicity and accumulation of non-esterified fatty acids**
34 **(NEFA) as the cause for mitochondrial damage in consequence of peroxisome loss in a**
35 **Peroxin19 (*pex19*) mutant. Hyperactive Hnf4 signaling leads to upregulation of *lipase 3***
36 **and enzymes for mitochondrial β -oxidation. This results in enhanced lipolysis, elevated**
37 **concentrations of NEFA, maximal β -oxidation and mitochondrial swelling. NEFA are**
38 **ligands for Hnf4 and further enhance its activity. By genetic removal of Hnf4 in *pex19***
39 **mutants, lipotoxicity and mitochondrial swelling are reduced and their survival is**
40 **rescued.**

41

42 **Author summary**

43 Peroxisomes are cell organelles which play a major role in lipid metabolism. They interact
44 with mitochondria, the organelles which are responsible for cellular energy production. Loss
45 of peroxisomes, as it occurs in the rare, inheritable human disease class of Peroxisome
46 Biogenesis Disorders, is lethal. Over the past couple of years, a number of studies showed that
47 peroxisome loss leads to mitochondrial damage as a secondary consequence, but the
48 underlying mechanism has not been understood yet. In our study, we use a mutant of the
49 fruitfly *Drosophila melanogaster* as a model for Peroxisome Biogenesis Disorders and find
50 that a protein called Hepatocyte nuclear factor 4 is hyperactive upon peroxisome loss, which
51 provokes the mobilization of storage fat and, as a consequence, the accumulation of toxic free

52 fatty acids. These enter the mitochondria, but cannot be used for energy gain. Free fatty acids
53 are then trapped in the mitochondria and lead to their swelling and damage, which provides an
54 explanation for mitochondrial defects in Peroxisomal Biogenesis Disorders. Genetic reduction
55 of Hepatocyte nuclear factor 4 activity rescues the viability of the peroxisome mutant by
56 reducing the accumulation of free fatty acids and the subsequent mitochondrial damage,
57 which might provide a novel target for therapy development.

58

59 **Abbreviations**

60

61	4E-bp	4E-binding protein
62	Acs1	Acyl-CoA synthetase long-chain
63	Bmm	Brummer lipase
64	CPT-1	Carnitine-palmitoyl-transferase 1
65	FA	Fatty acid
66	FAME	Fatty acid methyl ester
67	Fas	Fatty acid synthase
68	FITC	Fluorescein isothiocyanate
69	HexC	Hexokinase C
70	Hnf4	Hepatocyte nuclear factor 4
71	InR	Insulin receptor
72	LCFA	Long chain fatty acid
73	Lip3	Lipase 3
74	MCFA	Medium chain fatty acid
75	NEFA	Non-esterified fatty acid
76	PBD	Peroxisomal biogenesis disorder
77	Pepck	Phosphoenolpyruvate carboxykinase

78	Pex	Peroxin
79	Pex19	Peroxin 19
80	PMP	Peroxisomal membrane protein
81	TMRE	Tetramethylrhodamine, ethyl ester
82	VLCFA	Very long chain fatty acid
83	Yip2	Yippee interacting protein 2

84

85

86 **Introduction**

87

88 Peroxisomes, while rather simply structured organelles delimited by a single membrane,
89 harbor complex metabolic functions, which are still incompletely understood. In mammalian
90 cells, they are involved in the β -oxidation of very long chain fatty acids (VLCFA), the
91 formation of ether phospholipids (like plasmalogens), the catabolism of branched chain fatty
92 acids, the production of bile acids, polyamine oxidation and amino acid catabolism.
93 Furthermore, they exhibit a functional interplay with mitochondria by employing both shared
94 and coordinated metabolic pathways [1]-[3]. They interact via mitochondria derived vesicles
95 (MDVs), which allow for the exchange of lipid as well as protein content between the two
96 compartments [4], and they share regulators controlling organelle biogenesis [5] and fission
97 [6], [7]. VLCFA and branched-chain FA have to undergo β -oxidation in peroxisomes before
98 they can be metabolized in the mitochondria [8]. The interplay between peroxisomes and
99 mitochondria is illustrated by the fact that loss of peroxisomes leads to defects in
100 mitochondrial metabolism and structure [9]-[11]. The mechanism behind these defects,
101 however, is not understood.

102 Peroxisomes primarily regulate their number by growth and division of preexisting
103 organelles, similar to mitochondria, or by de novo biogenesis via pre-peroxisomal vesicles

104 through budding off from the endoplasmic reticulum (ER) [12]-[15]. The machinery involved
105 in the inheritance, assembly, division and maintenance of peroxisomes is encoded by *peroxin*
106 (*pex*) genes, which are highly conserved in evolution from yeast to mammals [16]. Pex19 is a
107 predominantly cytoplasmic peroxisomal core factor and essential for both the import of
108 peroxisomal membrane proteins (PMPs) and the de novo formation of peroxisomes [17], [18].
109 Together with Pex3 and Pex16, it is responsible for the translocation of membrane proteins
110 and membrane vesicle assembly [19]. Mutations in *pex* genes lead to the loss of peroxisomes
111 and cause peroxisome biogenesis disorders (PBDs), and *pex19* loss of function specifically
112 leads to Zellweger Syndrome, the severest form of PBDs. Recently, Pex19 has been found to
113 be required not only for peroxisome biogenesis, but also lipid droplet formation from the ER
114 [20]. The peroxisomal biogenesis and assembly machinery as well as metabolic function are
115 well conserved in *Drosophila melanogaster* [21]-[24].

116 Hepatocyte nuclear factor 4 alpha (HNF α) is a ligand-regulated transcription factor which
117 acts as a regulator of lipid metabolism in the mammalian liver [25]. It is conserved in
118 invertebrates, and in *D. melanogaster*, the single orthologue Hnf4 acts as an important lipid
119 sensor. Hnf4 is activated by binding of non-esterified fatty acids (NEFA), which are generated
120 by lipolysis of storage fat upon starvation. Binding of NEFA leads to nuclear translocation of
121 Hnf4 and the transcription of its target genes, which further triggers lipolysis and
122 mitochondrial β -oxidation [26].

123 Here we present our study on a *D. melanogaster pex19* mutant, which recapitulates all major
124 hallmarks of Zellweger Syndrome, such as absence of peroxisomes and consequently VLCFA
125 accumulation, mitochondrial defects, neurodegeneration, and early lethality, therefore serving
126 as a model for PBDs. This simple and genetically tractable model system was used in order to
127 identify pathological factors contributing to mitochondrial dysfunction. We were able to
128 identify one major pathological cascade as a result of peroxisome loss: Hyperactive Hnf4

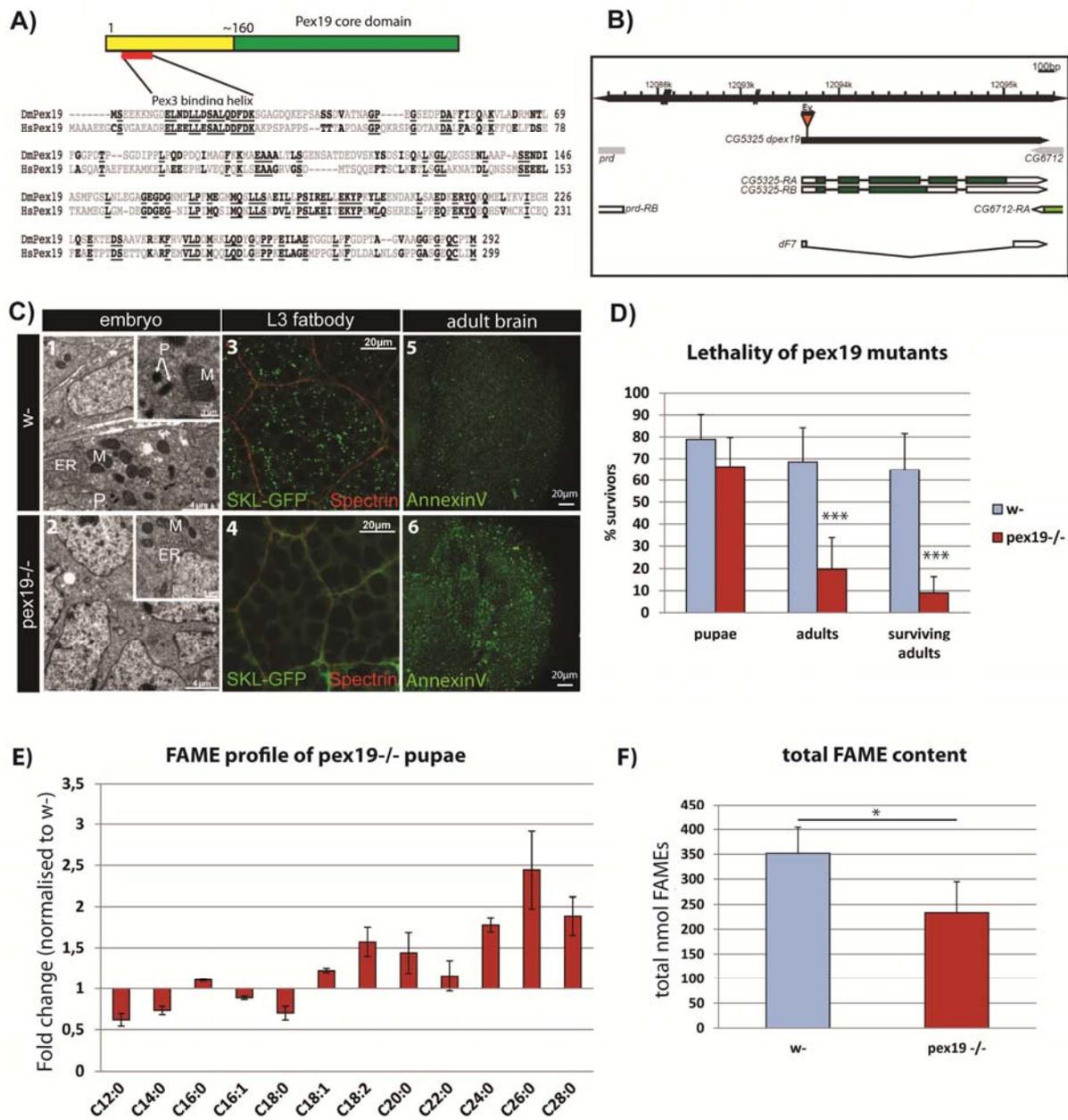
129 signaling and severely increased lipolysis in *pex19* mutants promote mitochondrial damage by
130 increasing mitotoxic NEFA levels. Our data therefore contribute to the current efforts in the
131 field.

132

133 **Results**

134 Neurodegeneration and aberrant lipid metabolism in *pex19* mutants

135 The single Pex19 orthologue in *D. melanogaster* is well conserved on the sequence and
136 structural level. It contains the typical Pex19 core domain [27] (Fig. 1a) and a highly
137 conserved Pex3 binding domain, both required for its activity as cytoplasmic shuttle receptor
138 of the peroxisome import machinery. We generated a deletion of the coding region of the
139 gene by imprecise excision (Fig. 1b), and molecularly characterized and identified the
140 resulting *pex19*^{ΔF7} flies as null mutants (subsequently referred to as *pex19* mutants). Maternal-
141 zygotic *pex19* mutants die as embryos, and ultrastructural analysis revealed the absence of
142 peroxisomes (Fig. 1c 1-2). In zygotic mutants, peroxisomes get lost during larval development
143 in these animals (Fig. 1c 3-4), with impact on their survival: while most larvae develop to
144 pupae (~70%), only 20% adults develop and most of them die during the first 24 hours (Fig.
145 1d). The few adult escapers (“surviving adults”, Fig. 1d) have high numbers of apoptotic cells
146 in their brains (Fig. 1c 5-6).



147

148 **Figure 1:**

149 **A)** Protein structure and sequence of Pex19 from human (HsPex19) and *Drosophila melanogaster*
150 (DmPex19) and their conservation. **B)** Schematic representation of the *pex19* gene locus and the
151 deletion in the *pex19*^{ΔF7} mutant. **C)** Peroxisomes are absent from *pex19*^{-/-} animals. Ultrastructural
152 analysis of maternal-zygotic *pex19*^{-/-} mutant embryos (2, compare to wildtype in 1) shows loss of
153 peroxisomes. P: peroxisomes, M: mitochondria, ER: endoplasmatic reticulum. Zygotic *pex19*^{-/-} larvae
154 do not have peroxisomes either, as shown by crossing in the peroxisome marker SKL-GFP: Loss of
155 GFP positive punctae in 4 indicates loss of peroxisomes (compare to wildtype in 3). AnnexinV-FITC
156 staining of adult optic lobes indicate neurodegeneration by high numbers of apoptotic cells in *pex19*
157 mutant brains (6, compare to 5). **D)** Lethality profile of *pex19*^{-/-} mutants, indicating the number of
158 pupae, adults including pharates and viable adults which survived for more than 24 hours. **E)** Fatty
159 acid methyl esters (FAME) from *pex19*^{-/-} pupae, normalized to w- control. **F)** Sum of lipids measured
160 in the FAME profile.

161 Scale bars as indicated. Error bars represent standard deviation (SD). *p<0,05, ***p<0,001 (Student's
162 t-test)

163

164 A genetic rescue with a Pex19 expression construct could be achieved with the ubiquitous
165 tubulin-Gal4 driver and with pumpless-Gal4, which drives expression in the fatbody and gut.
166 Expression with neuronal (*elav*-Gal4) or glia (*repo*-Gal4) drivers failed to rescue the lethality
167 of *pex19* mutants, which highlights an important role for Pex19 in metabolic organs rather
168 than the CNS (supplemental Fig. S1a).

169 Consistent with a loss of peroxisomal function, GC/MS analysis of fatty acid methylesters
170 (FAMES) prepared from pupae shows that lipids containing VLCFA are present at increased
171 levels in flies lacking peroxisomes. In addition, we found that lipids containing MCFA and
172 LCFA with C12 – C18 chain lengths are reduced (Fig. 1e). Also, the total amount of FAMES
173 is lowered (Fig. 1f). VLCFA are present at ~0,1 - 0,5 nmol per animal and their contribution
174 to energy gain is therefore negligible, while M- and LCFA are present at ~2 - 125 nmol per

175 animal (supplemental Fig. S1b) and provide fuel for energy gain by the mitochondria. The
176 shortage in MCFA and the reduction in total lipids is accompanied by a low energy status:
177 AMP kinase, a sensor for the AMP/ATP ratio, is phosphorylated and therefore indicates low
178 ATP levels [28] (supplemental Fig. S1c).

179

180 Gene expression of metabolic enzymes is altered in *pex19* mutants

181 To further characterise the lipid imbalance, we analyzed the expression of several genes
182 encoding for metabolic enzymes reportedly regulated on the transcript level [29], as well as
183 other enzymes of interest (Table 1). We suspected that *pex19* mutants are in a state of
184 starvation (low ATP levels and lack of MCFA), but instead found that insulin signaling is
185 elevated, as indicated by reduced expression of dFoxo target genes (*4E-BP* and Insulin
186 Receptor, *InR*). When analyzing the transcript levels of lipases, we found most of them
187 reduced, with the exception of *lipase 3*, which was strongly elevated (~250 fold compared to
188 w- control). Unlike *lipase 4* [30] and the ATGL homolog *brumer* [31], *lip3* is not a dFoxo
189 target gene, although it is upregulated in starved animals [32]. High expression of *lip3* is
190 therefore indicating a starvation situation in *pex19* mutants in spite of the fact that insulin
191 signaling is high. This surprising contradiction raises the question if lipid sensing is impaired
192 in *pex19* mutants, since lipolysis is upregulated in spite of high insulin signaling.

Group	Gene	fold regulation in pex19 ^{-/-} relative to w- control	fold regulation in hnf4 ^{+/-} pex19 ^{-/-} relative to w-
Insulin/TOR signaling	4E-BP	0,426 ± 0,246 p = 1,573E-05 ****	
	Insulin receptor	0,814 ± 0,165 p = 0,025 *	
lipolysis	lipase 3	249,667 ± 38,697 p = 3,776E-07 ****	93,699 ± 20,058 p = 9,546E-07 ****
	lipase 4	0,385 ± 0,006 p = 0,0047 **	
	brummer	0,732 ± 0,089 p = 0,146	
	magro	0,981 ± 0,062 p = 0,734	
	CG17192	0,272 ± 0,149 p = 0,092	
lipogenesis	Fatty acid synthase	2,196 ± 0,609 p = 1,069E-05 ****	1,379 ± 1,103 p = 0,034 *
	Acetyl-CoA carboxylase	1,392 ± 0,403 p = 0,063	0,764 ± 0,378 p = 0,019 *
glycolysis/ gluconeogenesis	Hexokinase C	29,603 ± 6,502 p = 0,0001 ***	7,234 ± 4,763 p = 5,616E-05 ****
	Gapdh2	0,201 ± 0,003 p = 0,0018 **	
	fructose-1,6- bisphosphatase	1,565 ± 0,525 p = 0,019 *	
	pepck-c	0,473 ± 0,147 p = 0,0003 ***	
	pyruvate carboxylase	1,230 ± 0,530 p = 0,531	
acyl-CoA transport	carnOpalm (CPT1)	1,705 ± 0,886 p = 0,008 **	1,403 ± 0,812 p = 0,47
	colt (CPT2)	1,468 ± 0,559 p = 0,007 **	1,103 ± 0,380 p = 0,055
	carnOoct (CROT)	3,440 ± 0,528 p = 1,426E-07 ****	3,462 ± 0,08 p = 0,9
mitochondrial metabolism	yip2 (acetyl-CoA acyltransferase)	19,897 ± 2,934 p = 1,037E-06 ****	0,940 ± 0,369 p = 3,658E-08 ****
	Acs1 (Long-chain-fatty-acid CoA ligase)	14,890 ± 5,627 p = 0,002 **	9,415 ± 4,884 p = 0,078
	spargel (PGC1-α)	2,553 ± 0,777 p = 0,0007 ***	
	Isocitrate dehydrogenase	2,721 ± 0,463 p = 9,329E-05 ****	2,770 ± 1,584 p = 0,915

193

194 **Table 1:**

195 Real-time qPCR analysis of genes encoding for metabolic enzymes. Δ_{cq} values are normalized to w-
196 ($\Delta\Delta C_q$ or fold regulation). Putative Hnf4 target genes are typed in blue. CPTI: carnitine
197 palmitoyltransferase, CROT: carnitine octanoyltransferase, PGC1- α : Peroxisome proliferator-activated
198 receptor gamma coactivator 1-alpha. Significance for *pex19*^{-/-} values tested against w- values and for
199 *hnf4*^{+/-} *pex19*^{-/-} values against *pex19*^{-/-}. \pm indicates SD. **p*<0,05, ***p*<0,01, ****p*<0,001,
200 *****p*<0,0001

201

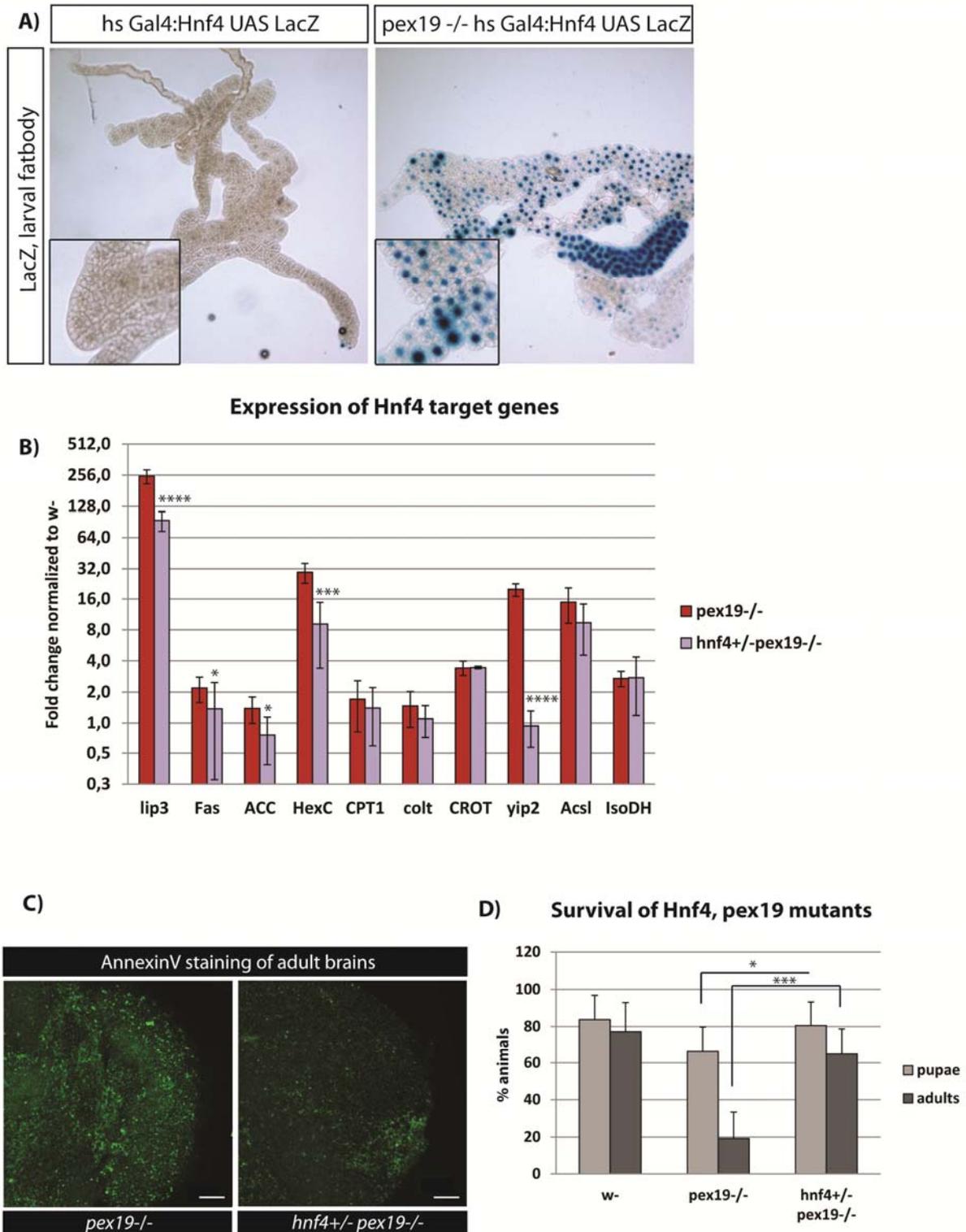
202 Among the glycolytic and gluconeogenic enzymes, we found *hexokinase c*, which catalyzes
203 the initial step of glycolysis (conversion of glucose to glucose-6-phosphate), ~30 fold
204 upregulated. We analyzed 3 genes involved in mitochondrial acyl-CoA import, carnitine-O-
205 octanoyltransferase (CROT) and the carnitine-palmitoyltransferases 1 and 2, and found all of
206 them upregulated in *pex19* mutants. Other genes for enzymes involved in mitochondrial
207 metabolism (β -oxidation, TCA cycle), most prominently the acetyl-CoA acyltransferase *yip2*
208 (*yippee* interacting protein 2, ~20 fold) and the long-chain fatty acid CoA ligase (*Acsl*, ~15
209 fold), were also upregulated. Strikingly, most of the upregulated genes in *pex19* mutants are
210 targets of the Hepatocyte nuclear factor 4 (Hnf4), a transcription factor which regulates
211 metabolic processes such as lipolysis and mitochondrial fatty acid β -oxidation in response to
212 starvation [26], and which stimulates insulin secretion [33]. This prompted us to investigate
213 Hnf4 in the context of peroxisome deficiency.

214

215 Hnf4 is hyperactive in *pex19* mutants and responsible for their reduced viability

216 We used a LacZ reporter under the control of a heat-shock inducible Hnf4:Gal4 fusion protein
217 [26], which did not show Hnf4 activity in fatbodies of 3rd instar wild type larvae. By contrast,
218 Hnf4 activity was observed in *pex19* mutants (Fig. 2a). We reanalyzed the Hnf4 target genes
219 among the metabolic enzymes displayed in Table 1 and found that *lip3*, *fas*, *acc*, *hexC* and

220 *yip2* are indeed significantly downregulated in *hnf4*, *pex19* mutants in comparison to *pex19*
221 mutants (Fig. 2b). We concluded that counteracting Hnf4 hyperactivity by introducing a
222 heterozygous mutation in *pex19* mutants normalizes the high expression of important Hnf4-
223 regulated metabolic enzymes.



224

225 **Figure 2:**

226 **A)** An HNF4 reporter, inducible by heat-shock, which expresses LacZ, was crossed into the *pex19*
227 mutant background. HNF4 is induced in fatbodies of 3rd instar larvae in *pex19* mutants. **B)**
228 Representation of the putative Hnf4 target genes: all of the measured Hnf4 target genes are
229 upregulated in *pex19* mutants and most are downregulated in *hnf4+/- pex19-/-*. **C)** AnnexinV-FITC
230 staining of adult optic lobes. Scale bars represent 20 μ m. **D)** Lethality profile of *hnf4+/- pex19-/-*
231 mutants, indicating the number of pupae, adults including pharates, and viable adults which survived
232 for more than 24 hours. Error bars indicate SD. * $p < 0,05$, *** $p < 0,001$, **** $p < 0,0001$

233

234 Furthermore, we assessed the neurodegeneration of *hnf4*, *pex19* mutants by staining brains of
235 5 day old adults with AnnexinV-FITC and found that the number of apoptotic cells decreases
236 in comparison to *pex19* mutants (Fig. 2c).

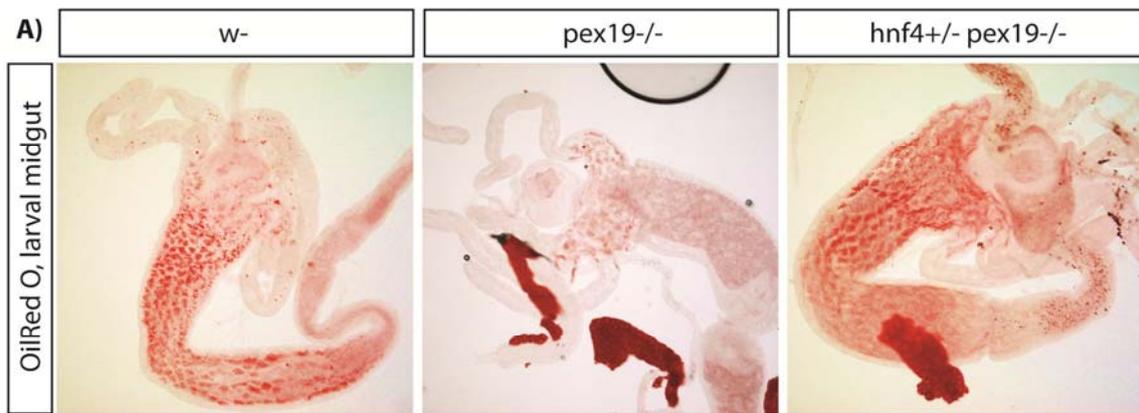
237 Next, we tested the lethality of *hnf4*, *pex19* mutants and found that *pex19* mutants can be
238 rescued to adulthood by removal of one copy of Hnf4. The resulting *hnf4*, *pex19* mutants
239 showed a rate of hatched adults of >60% (Fig. 2d). Adult *hnf4*, *pex19* mutants were viable and
240 fertile, but died 2-3 weeks after hatching from the pupa. This result indicates that elevated
241 Hnf4 signaling has a major contribution to the lethality of *pex19* mutants.

242

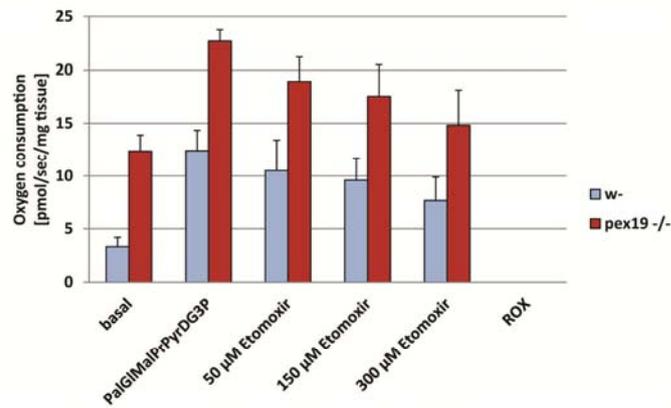
243 Hnf4 induces lipolysis and β -oxidation in *pex19* mutants

244 Hnf4 is an important lipid sensor in *D. melanogaster*. It acts as a receptor for non-esterified
245 fatty acids (NEFA) and, in consequence of NEFA-binding, regulates the transcription of
246 lipolytic and β -oxidation enzymes [26]. Intriguingly, the lipolytic enzyme *lipase 3* is
247 extremely highly upregulated in *pex19* mutants, which prompted us to investigate the gut lipid
248 stores. We stained neutral lipids with OilRed O in gut tissue of 3rd instar larvae and found
249 that gut lipid stores are empty in *pex19* mutants while their oenocytes are filled with lipid

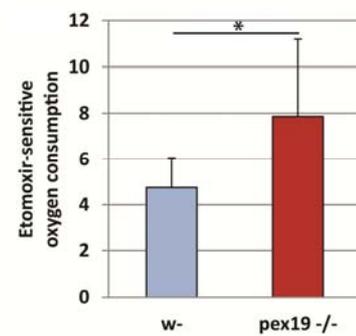
250 droplets (supplemental Fig. S1d), which indicates lipid mobilization [34], consistent with
251 lowered amounts of M- and LCFA and overall FAME content. Gut lipid filling is restored in
252 *hnf4*, *pex19* double mutants, indicating that the lipolytic phenotype of *pex19* mutants is indeed
253 connected to Hnf4 (Fig 3a).



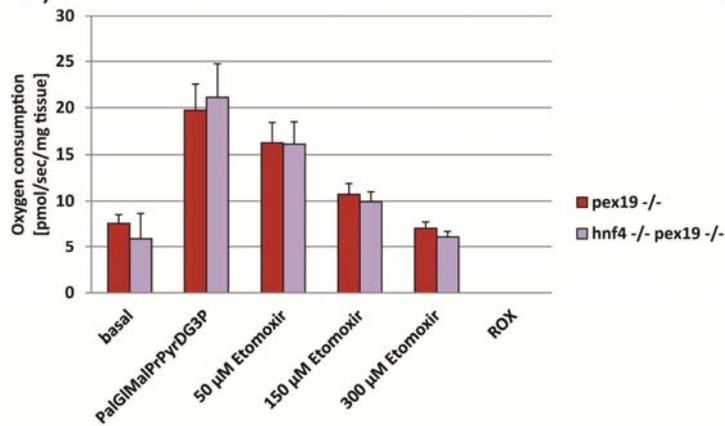
B) Oxygen consumption of larval tissue



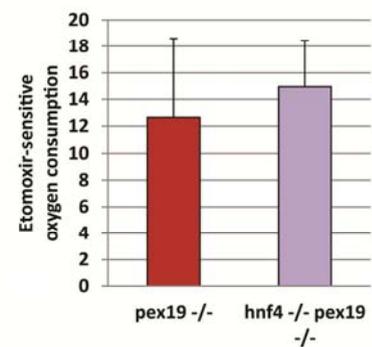
C) mitochondrial beta-oxidation



D) Oxygen consumption of larval tissue



E) mitochondrial beta-oxidation



254

255 **Figure 3:**

256 **A)** OilRed O staining of neutral lipids in anterior midguts of 3rd instar larvae. **B)** Oxygen consumption
257 levels of wildtype and *pex19*^{-/-} larval tissue (Pal: palmitate, Gl:glutamate, Mal:malate, Pr:proline,
258 Pyr:pyruvate, D:ADP, G3P:glycerol-3-phosphate). **C)** Etomoxir-sensitive mitochondrial β -oxidation
259 rate. **D)** Oxygen consumption levels of *pex19*^{-/-} and *hnf4*^{-/-} *pex19*^{-/-} larvae. **E)** mitochondrial β -
260 oxidation rate. Error bars represent SD. * $p < 0,05$

261

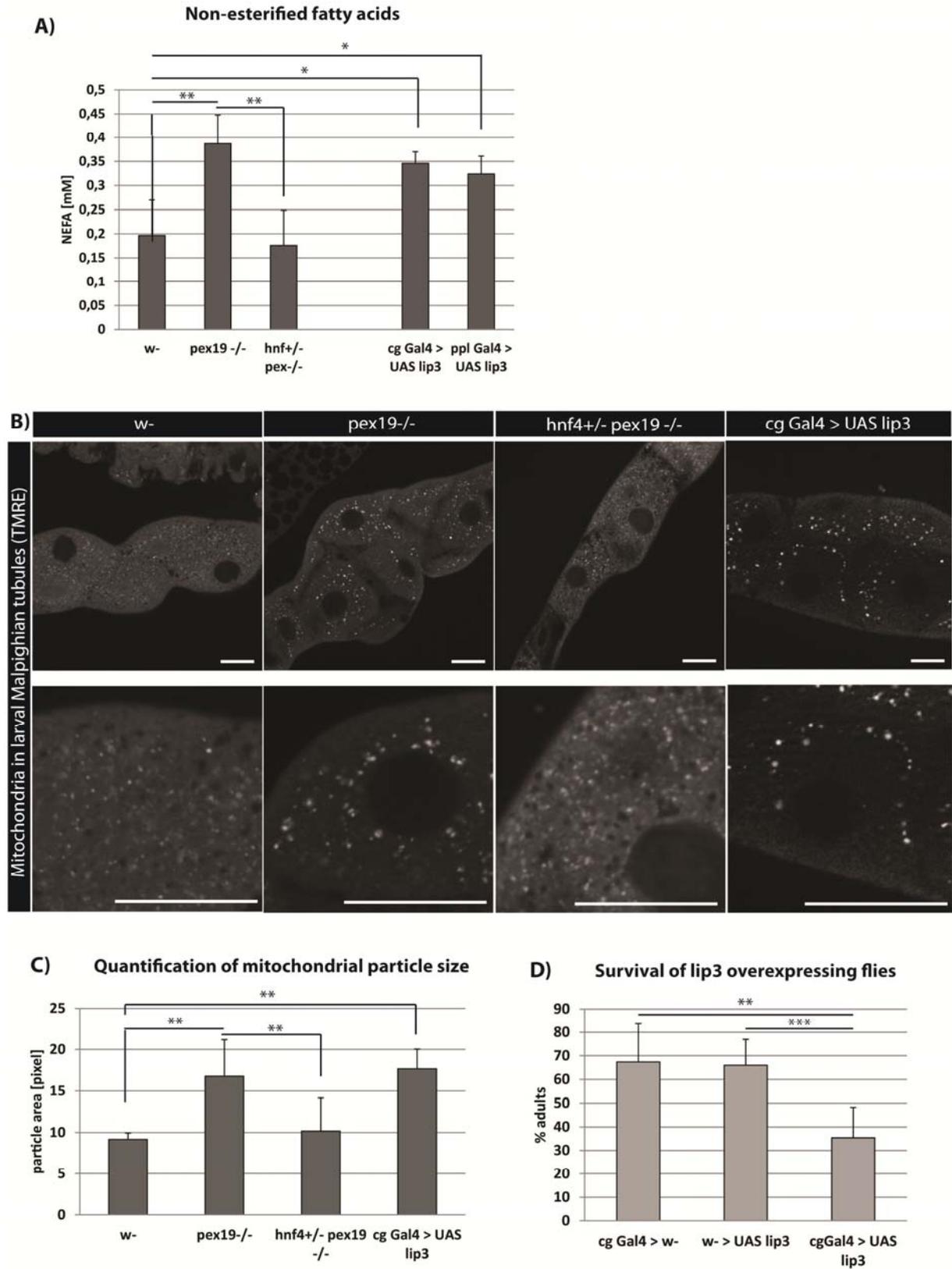
262 Next, we analyzed mitochondrial β -oxidation in permeabilized third instar larvae using a
263 Clark electrode (Oxygraph). β -oxidation levels were directly quantified as the amount of
264 oxygen consumption (in the presence of palmitoyl-CoA substrate, the necessary TCA
265 intermediates, and ADP) that is sensitive to etomoxir, a standard carnitine palmitoyl
266 transferase (CPT-I) inhibitor [35], which has been used successfully in *D. melanogaster*
267 before [36], [37]. We found that *pex19* mutants have elevated levels of etomoxir-sensitive
268 oxygen consumption as a measure for mitochondrial β -oxidation, which is in line with high
269 Hnf4 activity, increased lipolysis and reduction of M- and LCFA (Fig. 3b, c). To exclude that
270 this is due to an increase of mitochondrial abundance, we measured mtDNA and citrate
271 synthase activity, which are similar in *pex19* mutant and wildtype larvae (supplemental Fig.
272 S2a, b), indicating that the increased oxygen consumption is due to increased mitochondrial
273 flux, and not increased amounts of mitochondria. We measured the oxygen consumption in
274 *hnf4*, *pex19* double mutants and found that removal of Hnf4 leaves mitochondrial β -oxidation
275 at high levels (Fig. 3d, e). Since the expression of genes relevant for β -oxidation is lowered in
276 *hnf4*, *pex19* compared to *pex19* mutants, this result suggests that the β -oxidation machinery is
277 at maximal capacity.

278

279 Hnf4-induced lipase 3 leads to mitochondrial swelling and high amounts of NEFA

280 We hypothesized that high Hnf4 signaling in *pex19* mutants leads to enhanced *lipase 3*
281 activity and lipolysis, resulting in too high levels of free fatty acids (non-esterified fatty acids,
282 NEFA) and maximum mitochondrial β -oxidation as a compensatory mechanism. When too
283 many fatty acids are set free from the lipid stores by lipases, they can enter the mitochondrion
284 without being activated with CoA, which prevents their β -oxidation and leads to
285 mitochondrial swelling and damage. To analyze the NEFA content of tissue of *pex19* mutant
286 larvae, we developed an adapted protocol of the copper-triethanolamine method [38] suitable
287 for *D. melanogaster* tissue. We found that the NEFA content is about twice as high in *pex19*
288 samples compared to w-, while *hnf4*, *pex19* mutants have wildtypic levels of NEFA (Fig. 4a).

289 To test whether NEFA accumulation was due to high *lipase 3* expression, we measured the
290 NEFA levels in larvae overexpressing *lipase 3* under the control of the fatbody drivers
291 *pumpless* (*ppl*)-Gal4 or *combgap* (*cg*)-Gal4. These animals show high expression of *lipase 3*
292 relative to wildtype levels, comparable to *pex19* mutants (100-150fold, supplemental Fig.
293 S2c). With both drivers, *lipase 3* overexpression leads to elevated NEFA levels comparable to
294 those in *pex19* mutants (Fig. 4A).



295

296 **Figure 4:**

297 **A)** Amount of non-esterified fatty acids (NEFA) in whole 3rd instar larvae. **B)** TMRE staining to
298 visualize active mitochondria in Malpighian tubules of 3rd instar larvae. Scale bars represent 20 μm .
299 **C)** Quantification of the average particle area of TMRE-positive compartment. **D)** Flies
300 overexpressing lipase 3 in the fatbody show reduced survival in comparison to controls. Error bars
301 represent SD. * $p < 0,05$, ** $p < 0,01$

302

303 To further strengthen the hypothesis that NEFA accumulation in *pex19* mutants leads to
304 impaired mitochondrial function, we stained mitochondria with tetramethylrhodamine
305 ethylester (TMRE), a dye that is readily incorporated by active mitochondria and indicative of
306 their membrane potential. In Malpighian tubules of 3rd instar larvae, mitochondria were
307 reduced in number and enlarged in size in *pex19* mutants. This mitochondrial swelling was
308 ameliorated in *hnf4,pex19* mutants (Fig. 4B, C). To test whether mitochondrial swelling was
309 due to high NEFA levels in response to elevated *lipase 3* expression, we stained tissue of
310 larvae expressing *lip3* under the control of the *cg-Gal4* fatbody driver, and found that
311 mitochondrial swelling occurs also in these animals. Furthermore, they show significantly
312 reduced viability compared to control flies (Fig. 4D), although not to the same extent as *pex19*
313 mutants.

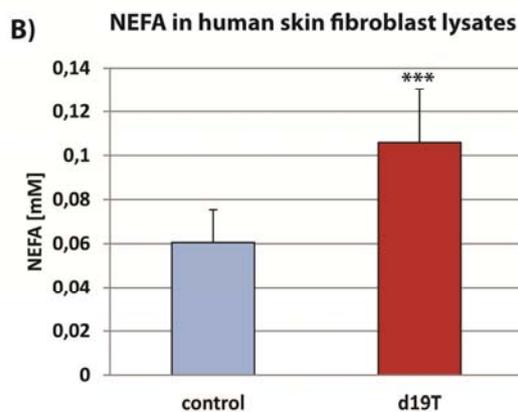
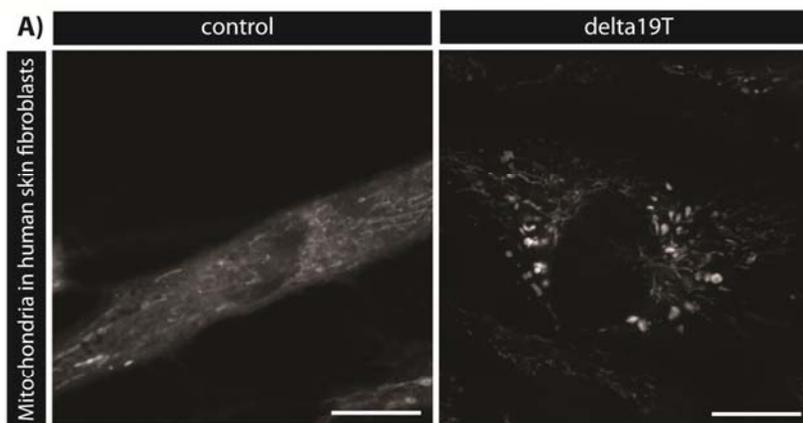
314 Taken together, our data show that elevated Hnf4 signaling leads to high expression levels of
315 *lipase 3* and enhanced lipolysis, resulting in accumulation of NEFA, which enter the
316 mitochondria where they lead to mitochondrial swelling and dysfunction. A MitoSOX Red
317 staining confirmed high ROS production by mitochondria in *pex19* mutants (supplemental
318 Fig. S2E). NEFA act as ligands for Hnf4 and thus activate it further, thereby causing a self-
319 sustained downward spiral culminating in energy deficit due to mitochondrial damage and
320 ultimately death of the organism.

321

322 NEFA accumulation in response to peroxisome loss is conserved between flies and humans

323 In order to assess the cross-species portability of our findings, we analyzed the mitochondrial
324 morphology and NEFA content in a human fibroblast line from a Zellweger Syndrome patient
325 with a mutation in *pex19* [39] ($\Delta 19T$). While mitochondria appear small and network-like in a
326 control cell line, they were fragmented and swollen in the $\Delta 19T$ cells (Fig. 5A). Furthermore,
327 NEFA were increased in these cells in comparison to the control cell line (Fig. 5B). Our
328 results suggest a similar mechanism of the impact of *pex19* mutation on mitochondria and the
329 pathology of the disease as observed in our *D. melanogaster* mutant.

330



331

332 **Figure 5:**

333 **A)** TMRE staining to visualize active mitochondria in human skin fibroblasts from a healthy person
334 (control) and a patient with a *pex19* mutation ($\Delta 19T$). **B)** Amount of non-esterified fatty acids (NEFA)
335 in human skin fibroblasts. Error bars represent SD. *** $p < 0,001$

336

337 **Discussion**

338 The tight interconnection of peroxisomes and mitochondria has long been established, and an
339 impact of peroxisome loss on mitochondria has been proposed before. However, the reason
340 for mitochondrial damage as a result of peroxisome deficiency has remained elusive. Here we
341 propose a mechanism by which peroxisome loss due to *pex19* mutation leads to mitochondrial
342 swelling through dysregulated lipolysis. The lipid sensor Hnf4 accounts for upregulation of
343 *lipase 3* and enzymes for mitochondrial acyl-CoA import and β -oxidation. Subsequently,
344 through a vicious cycle of increased lipolysis and NEFA amounts, which in turn can further
345 activate Hnf4 as its ligands, extreme upregulation of *lipase 3*, depletion of the lipid stores, and
346 NEFA accumulation occur, while mitochondrial β -oxidation runs at maximal capacity. This
347 ultimately causes mitochondrial swelling and damage, leading to energy deficiency and
348 lethality. Thus, Hnf4 hyperactivity and dramatic Hnf4-induced *lipase 3* overexpression play
349 major and yet unrecognized roles in the pathophysiology of peroxisome loss.

350 While *lipase 3* overexpression alone leads to accumulation of toxic NEFA and mitochondrial
351 swelling, which we consider a major contribution to the lethality of the mutant, it does not
352 phenocopy the lethality of *pex19* mutants fully (although it does reduce viability to some
353 extent). This might hint that the peroxisomes, which are still present in *lipase 3*
354 overexpressing animals, but not in *pex19* mutants, play a role in NEFA clearance or
355 mitochondrial turnover rate, thereby counteracting the detrimental effects of dysregulated
356 lipolysis. This hypothesis fits to the fact that peroxisomes and mitochondria share fission and
357 fusion factors and thereby might influence each other's quality control.

358 The exact link between Hnf4 and Pex19 remains to be determined. Interestingly, among the
359 putative target genes of Hnf4, several peroxins (*pex2*, *pex12* and *pex16*) can be found [26].
360 This opens up the possibility of a regulatory feedback between peroxisome activity or
361 abundance and Hnf4 activity, which could explain the fact that, in the absence of
362 peroxisomes, Hnf4 activity gets out of hand and initiates a program of maximal lipolysis and
363 beta-oxidation with detrimental results for mitochondria and the whole organism.

364

365 **Methods**

366 Flywork

367 The *pex19* mutant was generated by imprecise excision following *D. melanogaster* standard
368 techniques. The line *pex19^{AF7}* was chosen from a jump-out screen and tested as a transcript
369 null. To detect homozygous animals, they were crossed with a CyO-twi-GFP marker. As
370 control flies we used the strain w¹¹¹⁸ (Bloomington stock #3605). Wildtype and heterozygous
371 *pex19^{AF7}* flies were reared on standard fly food. *hnf4^{A33}* and *hnf4^{A17}* flies were kindly provided
372 by Carl Thummel. *hnf^{A17} pex19^{AF7}* and *hnf4^{A33} pex19^{AF7}* double mutants were generated by
373 genomic recombination, and *hnf4^{A33/+} pex19^{AF7/ΔF7}* mutants by crossing the double mutant
374 with *pex19^{AF7}*. For survival assays, larvae were collected as 1st instars and transferred to fresh
375 plates with yeast paste. 25 larvae were collected for each condition, and at least 5 independent
376 experiments were conducted. The number of surviving pupae, adults including pharates and
377 viable adults (survivors), which were able to move and lived at least 24h, was counted.

378

379 Cell Culture

380 Human fibroblast control and $\Delta 19T$ cells were kept in Dulbecco's modified eagle medium
381 (DMEM, Gibco) with 10% FBS, 10000 units of Penicillin and 10 mg Streptomycin per ml.
382 For NEFA measurement, 1×10^5 cells were seeded in 6 well plates and harvested after 48 h.
383 Cells were pelleted and cell pellets were treated like larval tissue (see NEFA section). For
384 stainings, cells were seeded into 8-well-slides for microscopy and stained after 48 h with
385 TMRE and analyzed immediately.

386

387 Imaging

388 Antibodies used were anti-GFP (Sigma) and anti-Spectrin (DSHB). Secondary antibodies
389 coupled to Alexa dyes were from Molecular probes. Stainings were analyzed using a Zeiss
390 LSM 710 confocal microscope. For ultrastructural analysis, embryos were embedded in 2 %
391 agarose and post fixed with 5 % glutaraldehyde for 2 h at room temperature. Ultra-thin
392 sections were prepared and analyzed using a Zeiss Libra 120 electron microscope. For
393 apoptosis assays, brains of adult flies were dissected and stained using an AnnexinV-FITC
394 apoptosis detection kit (Sigma Aldrich) according to the manufacturer's instructions.

395 For immunohistochemistry, we dissected tissue of interest from 3rd instar larvae. Tissue was
396 fixed for 30 minutes in 3,7 % formaldehyde and washed with PBT before and after incubation
397 with primary antibody and Alexa dye-coupled secondary antibody. Tissue was mounted in
398 Fluoromont G and analyzed using a Zeiss LSM 710 confocal microscope. For stainings of
399 neutral lipids with OilRed O, larval tissue was dissected in PBS by inverting the cuticle to
400 provide access to the oenocytes. Tissue was fixed for 20 min in 3,7 % formaldehyde and
401 washed with PBS. Before and after staining with a 60 % OilRed O solution for 30 min, tissue
402 was incubated for 5 min with 60 % isopropanol. Tissue was washed with PBS, mounted in
403 glycerol and immediately analyzed using an Olympus AX70 microscope.

404 For staining of mitochondria, 96 h old larvae or 5 day old adults were dissected in ice cold
405 PBS, and their Malpighian tubules were stained for 20min at RT with 50nM TMRE (Sigma-
406 Aldrich) in PBS or MitoSOXTM (Molecular Probes) according to the manufacturer's protocol.
407 The Malpighian tubules were then directly mounted in Fluoromount G and analyzed with a
408 Zeiss LSM 710.

409

410 Lipid profile

411 For quantification of fatty acid methyl esters (FAME), 15 3rd instar larvae were homogenized
412 in 1N MeHCl in a Precellys 24 homogenizer (peqlab). A minimum of n=7 was analyzed for
413 each condition. C15:0 and C27:0 standards were added and samples were incubated for 45
414 min at 80 °C. Methyl esters were collected by addition of hexane and a 0,9 % NaCl solution.
415 The hexane phase was collected in a new glass vial and concentrated by vaporization.
416 Samples were analyzed by gas chromatography / mass spectrometry using an Agilent HP
417 6890 with a HP-5MS column.

418

419 Free Fatty Acids

420 Non-esterified fatty acids (NEFA) were measured by an adaptation of the copper-soap
421 method[38]. In brief, three 3rd instar larvae were weighed and homogenized in 20 µl of 1M
422 phosphate buffer per mg tissue. 25 µl of the supernatant were transferred to 500 µl of
423 Chloroform/Heptane 4:3, and lipids were extracted by shaking the vial for 5 min. Unspecific
424 background provoked by phospholipids was circumvented by addition of 23 mg of activated
425 silicic acid. 300 µl of the chloroform phase were transferred to 250 µl of Cu-TEA (copper-
426 triethanolamine). After shaking and centrifuging, 150 µl of the organic phase were transferred
427 to fresh cups. Liquid was evaporated in a 60 °C heat block, and lipids were dissolved in 100

428 μ l of 100% ethanol. Copper was detected by complexation with a mixture of dicarbazone -
429 dicarbazide, and the colour intensity was measured in a 96 well plate at 550 nm in a TECAN
430 platereader.

431

432 β -oxidation measurements

433 6 larvae per genotype were washed with PBS and their weight was recorded for normalization
434 purposes. The larvae were inverted in ice cold PBS and permeabilized in ice cold BIOPS
435 buffer (2.77mM CaK₂EGTA, 7.23mM K₂EGTA, 5.77mM Na₂ATP, 6.56mM MgCl₂.6H₂O,
436 20mM taurine, 15mM Na₂.phosphocreatine, 20mM imidazole, 0.5mM DTT, 50mM MES)
437 containing 100ug/mL saponin (fresh) at 4°C with gentle rocking for 10 mins. Then the larvae
438 were equilibrated in respiration medium (MiR05, 0.5mM EGTA, 3mM MgCl₂.6H₂O, 60mM
439 K-Lactobionate (lactobionic acid is dissolved in H₂O and pH is adjusted to pH 7.4 with
440 KOH), 20mM Taurine, 10mM KH₂PO₄, 20mM HEPES, 110mM sucrose, 1g/L fatty acid free
441 BSA) supplemented with 0.5mM carnitine. The larvae were added into the oxygraph
442 chambers and oxygen concentration was brought to around 500uM by using catalase and
443 H₂O₂. After basal respiration was recorded, 5uM palmitoylCoA was added to the chamber.
444 Fatty acid β -oxidation was induced by adding complex I substrates, ETF (electron transfer
445 flavoprotein) substrates, and ADP (10mM proline, 10mM pyruvate, 5mM malate, 5mM
446 glutamate, 2mM ADP and 15mM glycerol-3- phosphate). After that, etomoxir was added at
447 the indicated concentrations to block fatty acid transfer into mitochondria via CPT1, thereby
448 blocking β -oxidation and leaving complex I-dependent respiration. Finally, residual oxygen
449 consumption (ROX) was measured by inhibiting complex III with antimycin A. All values
450 were corrected for ROX. β -oxidation was calculated by subtracting etomoxir-resistant
451 respiration from respiration in the presence of all substrates.

452

453 Realtime qPCR

454 Whole RNA of 3rd instar larvae was isolated using TriFast reagent (peqlab). Tissue was
455 homogenized using a Precellys 24 homogenizer (peqlab). Transcription to cDNA was
456 performed using the Quantitect Reverse Transcription Kit (Quiagen). Quantitative PCR was
457 performed with a CFX Connect cycler (biorad). Each experiment was repeated at least 5
458 times.

459

460 **Author contribution**

461 Conceptualization, M.H.B., J.S., C.W. and M.H.; Methodology, M.H.B., J.S., C.W., D.G.;
462 Investigation, M.H.B., J.S., C.W., D.G., D.S.; Writing – Original Draft, M.H.B. and J.S.;
463 Writing – Review & Editing, M.H.B., J.S., M.H. and A.A.T.; Visualization: M.H.B. and J.S.;
464 Funding Acquisition, M.H. and A.A.T., Supervision, M.H. and A.A.T.

465

466 **Acknowledgments**

467 We thank the Bloomington stock center for fly strains, Carl Thummel for providing Hnf4 fly
468 lines and Ingo Zinke and Michael Pankratz for the ppl-Gal4 and UAS lip3 line. We thank
469 Christoph Thiele for input and help with the NEFA measurements. We thank Gabriele Dodt,
470 Ronald Wanders, Heike Schulze and Konrad Sandhoff for human fibroblast lines, Mélisande
471 Richard for help with the ultrastructural analysis, Fatmire Bujupi for help with the
472 characterization of the *pex19* mutant, the group of Peter Dörmann for help with mass
473 spectrometry analysis of FAMES, and members of the Hoch group for discussion.

474 The work was funded by grants to M.H. (*SFB 645, TPB1 and TR 83, TP A7*) and by the
475 Helmholtz Portfolio grant “*metabolic dysfunction*” to M.H. and A.A.T. M.H. is a member of
476 the Bonn Excellence Cluster *ImmunoSensation*.

477

478 **References**

479

- 480 (1) Wanders RJ, Waterham HR, Ferdinandusse S. Metabolic Interplay between
481 Peroxisomes and Other Subcellular Organelles Including Mitochondria and the
482 Endoplasmic Reticulum. *Front Cell Dev Biol*. Jan 28 2009;3:83.
483
- 484 (2) Thoms S, Grønborg S, Gärtner J. Organelle interplay in peroxisomal disorders. *Trends*
485 *Mol Med*. 2009 Jul;15(7):293-302. doi: 10.1016/j.molmed.2009.05.002. Epub 2009
486 Jun 26.
487
- 488 (3) Mohanty A, McBride HM. Emerging roles of mitochondria in the evolution,
489 biogenesis, and function of peroxisomes. *Front Physiol* 2013. 4:268.
490
- 491 (4) Neuspiel M, Schauss AC, Braschi E., Zunino R, Rippstein P, Rachubinski RA et al.
492 Cargo-selected transport from the mitochondria to peroxisomes is mediated by
493 vesicular carriers. *Curr Biol* 2008. Jan 22;18(2):102-8
494
- 495 (5) Bagattin A, Hugendubler L, Mueller E. Transcriptional coactivator PGC-1alpha
496 promotes peroxisomal remodeling and biogenesis. *Proc Natl Acad Sci U S A* 2010.
497 107(47):20376-81
498

- 499 (6) Koch A, Yoon Y, Bonekamp NA, McNiven MA, Schrader M. A role for Fis1 in both
500 mitochondrial and peroxisomal fission in mammalian cells. *Mol Biol Cell* 2005.
501 16(11):5077-86
502
- 503 (7) Pan R, Hu J. The conserved fission complex on peroxisomes and mitochondria. *Plant*
504 *Signal Behav* 2001. 6(6):870-2
505
- 506 (8) Wanders RJ. Metabolic functions of peroxisomes in health and disease. *Biochimie*
507 2014. 98:36-44
508
- 509 (9) Baes M, Gressens P, Baumgart E, Carmeliet P, Casteels M, Fransen M. A mouse
510 model for Zellweger syndrome. *Nat Genet.* 1997 Sep;17(1):49-57.
511
- 512 (10) Peeters A, Swinnen JV, Van Veldhoven PP, Baes M. Hepatosteatosi in peroxisome
513 deficient liver despite increased β -oxidation capacity and impaired lipogenesis.
514 *Biochimie* 2011. Oct;93(10):1828-38
515
- 516 (11) Peeters A, Shinde AB, Dirkx R, Smet J, De Bock K, Espeel M, et al. Mitochondria in
517 peroxisome-deficient hepatocytes exhibit impaired respiration, depleted DNA, and
518 PGC-1 α independent proliferation. *Biochim Biophys Acta.* 2015 Feb;1853(2):285-98.
519 doi: 10.1016/j.bbamcr.2014.11.017. Epub 2014 Nov 20.
520
- 521 (12) Hoepfner D. et al. Contribution of the endoplasmic reticulum to peroxisome
522 formation. *Cell* 2005. 122(1):85-95
523

- 524 (13) Kim PK et al. The origin and maintenance of mammalian peroxisomes involves a de
525 novo PEX16-dependent pathway from the ER. *J. Cell Biol.* 2006 173, 521–532
526
- 527 (14) Platta HW, Erdmann R. Peroxisomal dynamics. *Trends Cell Biol.* 2007 17(10):474-
528 84.
529
- 530 (15) Ma C, Agrawal G, Subramani S. Peroxisome assembly: matrix and membrane protein
531 biogenesis. *J Cell Biol.* 2011 193(1):7-16.
532
- 533 (16) Pieuchot L, Jedd G. Peroxisome assembly and functional diversity in eukaryotic
534 microorganisms. *Annu. Rev. Microbiol.* 2012 66, 237–263
535
- 536 (17) Léon S, Goodman JM, Subramani S. Uniqueness of the mechanism of protein import
537 into the peroxisome matrix: transport of folded, co-factor-bound and oligomeric
538 proteins by shuttling receptors. *Biochim Biophys Acta.* 2006 Dec;1763(12):1552-64.
539 Epub 2006 Aug 30.
540
- 541 (18) Brown LA. & Baker A. Shuttles and cycles: transport of proteins into the peroxisome
542 matrix. *Mol. Membr. Biol.* 2008 25:363-75
543
- 544 (19) Fujiki Y. et al. Peroxisome biogenesis in mammalian cells. *Front Physiol.* 2014 Aug
545 15;5:307.
546
- 547 (20) Schrul B, Kopito RR. Peroxin-dependent targeting of a lipid-droplet-destined
548 membrane protein to ER subdomains. *Nat Cell Biol.* 2016 Jul;18(7):740-51.
549

- 550 (21) Mast FD. et al. A Drosophila model for the Zellweger spectrum of peroxisome
551 biogenesis disorders. *Dis Model Mech.* 4(5):659-72. (2011)
552
- 553 (22) Faust JE, Manisundaram A, Ivanova PT, Milne SB, Summerville JB, et al.
554 Peroxisomes are required for lipid metabolism and muscle function in Drosophila
555 melanogaster. *PLoS One* 2014 Jun 19;9(6):e100213.
556
- 557 (23) Faust JE, Verma A, Peng C, McNew JA. An inventory of peroxisomal proteins and
558 pathways in Drosophila melanogaster. *Traffic* 2012 13(10):1378-92.
559
- 560 (24) Sivachenko A, Gordon HB, Kimball SS, Gavin EJ, Bonkowsky JL, Letsou A.
561 Neurodegeneration in a Drosophila model of Adrenoleukodystrophy: the roles of the
562 bubblegum and double bubble acyl-CoA synthetases. *Dis Model Mech.* Feb 18 2016.
563 pii: dmm.022244.
564
- 565 (25) Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepatocyte nuclear factor
566 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression
567 and lipid homeostasis. *Mol Cell Biol.* 2001 Feb;21(4):1393-403
568
- 569 (26) Palanker L, Tennessen JM, Lam G, Thummel CS. Drosophila HNF4 regulates lipid
570 mobilization and beta-oxidation. *Cell Metab.* 2009 9:228-39
571
- 572 (27) Sato Y et al., Structural basis for docking of peroxisomal membrane protein carrier
573 Pex19p onto its receptor Pex3p. *EMBO J.* 2010 29:4083-93
574

- 575 (28) Pan DA, Hardie DG. A homologue of AMP-activated protein kinase
576 in *Drosophila melanogaster* is sensitive to AMP and is activated by ATP depletion.
577 *Biochem J.* 2002 Oct 1;367(Pt 1):179-86
578
- 579 (29) Chambers MC, Song KH, Schneider DS. *Listeria monocytogenes* infection causes
580 metabolic shifts in *Drosophila melanogaster*. *PLoS One* 2012 7(12):e50679.
581
- 582 (30) Vihervaara T, Puig O. dFOXO regulates transcription of a *Drosophila* acid lipase. *J*
583 *Mol Biol.* 2008 Mar 7;376(5):1215-23.
584
- 585 (31) Grönke S, Mildner A, Fellert S, Tennagels N, Petry S, Müller G, Jäckle H, Kühnlein
586 RP. Brummer lipase is an evolutionary conserved fat storage regulator in *Drosophila*.
587 *Cell Metab.* 2005 May;1(5):323-30.
588
- 589 (32) Becker T, Loch G, Beyer M, Zinke I, Aschenbrenner AC, Carrera P, Inhester T,
590 Schultze JL, Hoch M. FOXO-dependent regulation of innate immune homeostasis.
591 *Nature* 2010 Jan 21;463(7279):369-73.
592
- 593 (33) Barry WE, Thummel CS. The *Drosophila* HNF4 nuclear receptor promotes glucose-
594 stimulated insulin secretion and mitochondrial function in adults. *Elife* 2016 May
595 17;5. pii: e11183
596
- 597 (34) Gutierrez E, Wiggins D, Fielding B, Gould AP. Specialized hepatocyte-like cells
598 regulate *Drosophila* lipid metabolism. *Nature* 2007 Jan 18;445(7125):275-80.
599

- 600 (35) Kuznetsov AV. et al. Analysis of mitochondrial function in situ in permeabilized
601 muscle fibers, tissues and cells. *Nat. Protoc.* 2008 3:965-76.
602
- 603 (36) Xu X. et al. Insulin signaling regulates fatty acid catabolism at the level of CoA
604 activation. *PLoS Genet.* 2012 8(1):e1002478
605
- 606 (37) Banerjee KK. et al. Fat body dSir2 regulates muscle mitochondrial physiology and
607 energy homeostasis nonautonomously and mimics the autonomous functions of dSir2
608 in muscles. *Mol Cell Biol.* 2013 33(2):252-64.
609
- 610 (38) Tinnikov AA, Boonstra R. Colorimetric micro-determination of free fatty acids in
611 plasma using microplate readers. *Clin Chim Acta* 1999 Mar;281(1-2):159-62
612
- 613 (39) Munteau AC, Roscher AA, Kunau WH, Dodt G. The interaction between human PEX3
614 and PEX19 characterized by fluorescence resonance energy transfer (FRET) analysis.
615 *Eur J Cell Biol.* 2003 Jul;82(7):333-42.
616
617