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Mitochondrial fat oxidation is essential for lipid-induced inflammation in skeletal muscle in mice

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published on 28th November 2016 in
Nature Scientific Reports

Gerrit Lewik, 05.12.2016

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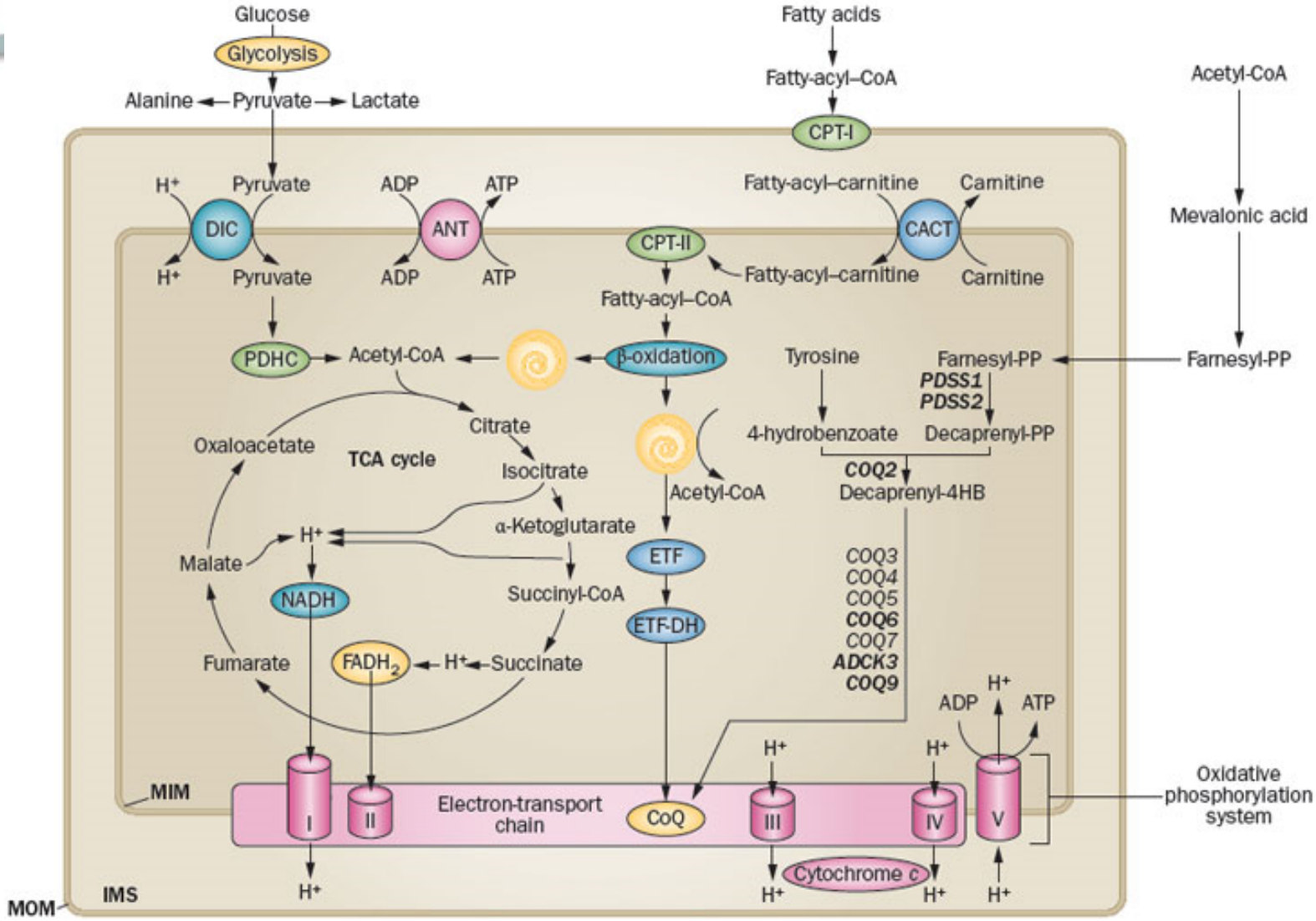


http://www.nature.com/nrneurol/journal/v9/n8/fig_tab/nrneurol.2013.126_F1.html

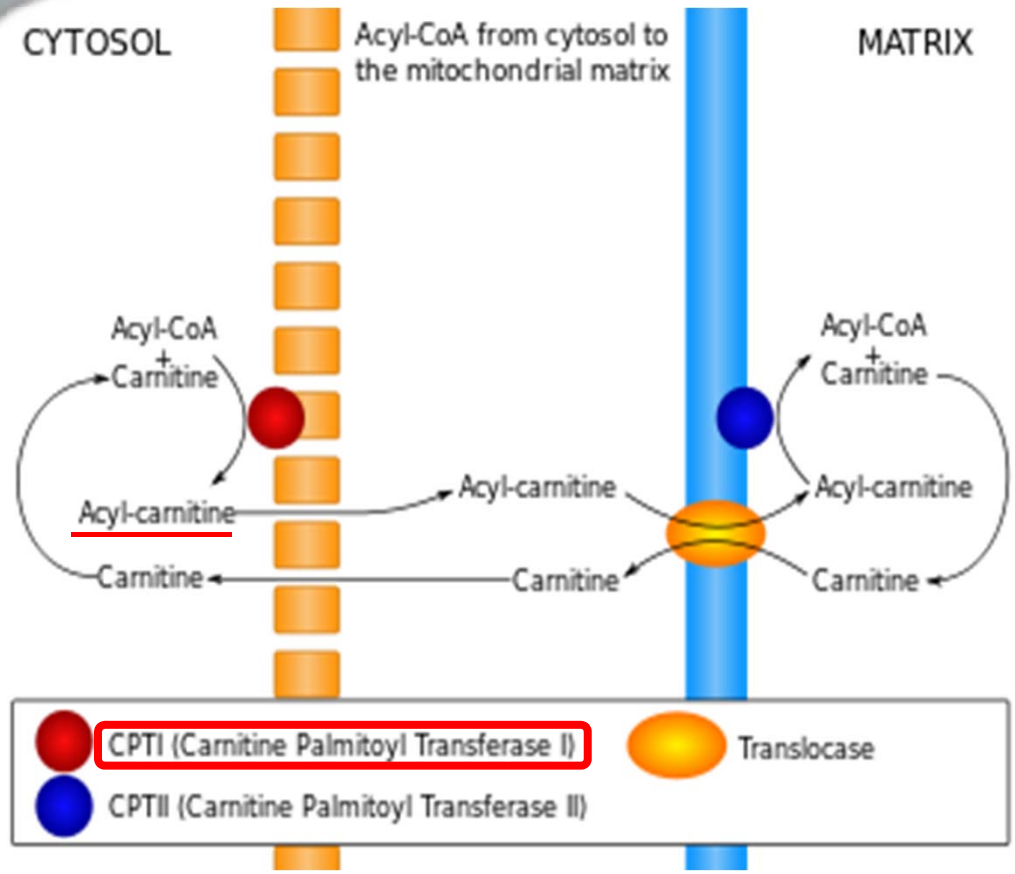
ß-Oxidation

- free fatty acids cannot penetrate any biological membrane due to their negative charge
- free fatty acids must cross the cell membrane through specific transport proteins (e.g. SLC27)→ cytosol
- processes to bring fatty acids into mitochondrion:
- 1) **activation:**

  → long-chain-fatty acid-CoA ligase: catalyzes transfer from CoA to form fatty acyl-CoA

- 2) **transport:**
  - A) <u>short</u> fatty acyl-CoA: can simply diffuse through the inner mitochondrial membrane

ß-Oxidation



https://commons.wikimedia.org/wiki/File:Acyl-CoA_from_cytosol_to_the_mitochondrial_matrix.gif

- B) long fatty acyl-CoA:
  → carnitine shuttle
  - acyl-CoA is transferred to carnitine by CPT1
  - acyl-carnitine is shuttled in by carnitine-acylcarnitine translocase (CAT) and carnitine is shuttled out
  - acyl-carnitine is converted back to acyl-CoA by CPT2
  - acyl-carnitine is shuttled into the matrix as a liberated carnitine is shuttled back to the cytosol

CPT1

- Carnitine palmitoyltransferase 1

= Carnitine acyltransferase 1

- three isoforms known in mammalian tissue:
  - CPT1a: mitochondria of all cells except skeletal muscle and brown adipose cells → liver isoform
  - CPT1b: heart and skeletal muscle cells, brown adipose tissue → muscle isoform
  - CPT1c: brain and testes → brain isoform
- exact crystal structure and mechanism not known
- location: outer membrane of mitochondrion

CPT1

- function: transfer of acyl-CoA to carnitine

  →transport of long chain fatty acid chains into mitochondrion

  → controlling the rate of FAO

  → key role in fatty acid metabolism

  →accumulation of intracellular lipids such as ceramides and diacylglycerol (DAG) are linked to impaired insulin signalling in skeletal muscle

  → connected to metabolic disorders (e.g. diabetes)

Aim of the study

- to examine the role of mitochondrial fat oxidation on lipid-induced inflammation in skeletal muscle

→ to enhance the understanding of the relationship between obesity-induced inflammation and insulin resistance

Material and Methods

- **skeletal muscle-specific Cpt1b knockout mouse model** (Cpt1bm−/−) in which mitochondrial fatty acid oxidation was inhibited
  - mice were 5–6 month old males, age was matched Cpt1bm−/− mice and control Cpt1bfl/fl mice were fed a breeder's chow diet
- **mouse primary muscle cell culture:** cultures established from gastrocnemius muscle of 1 month old *Cpt1b*m−/− and *Cpt1b*fl/fl mice
  - at least three independent cultures were performed for each gene expression analysis by qRT-PCR
- **Gene expression analysis:** total RNA from mouse tissue and total RNA from mouse primary myotubes was isolated using RNeasy Micro Kit
  - cDNA was synthesized with the iScript cDNA synthesis kit and used for qRT-PCR
- **Serial Analysis of Gene Expression (SAGE):** 1–2 ug of total RNA extracted from mouse gastrocnemius muscle was used
  - gene expression profiling was performed on an AB SOLiD 5500XL next-generation sequencing instrument

Material and Methods

- **Global gene expression analysis and Gene Set Enrichment Analysis:**
  - gene deviation significance from a uniform distribution in a priori defined gene-sets (pathways) is examined
  - visualized via row-normalized blue-red heatmaps (blue representing lower, and red representing higher gene expression levels)
- **multiplex analysis:** serum collections in mice were performed
  - analytes for IL-1β and TNFα were prepared
  - Bio-Plex Mouse Cytokine Assay kit was used
- **Western blot analysis:**
  - Gastrocnemius homogenates were prepared
  - antibodies used were pSTAT3 (Tyr705) and STAT3
- **Statistical Analyses:**
  - GraphPad Prism 5 software, *t* test and Pearson's correlation coefficient *r* were used
  - $p < 0.05$ was considered statistically significant

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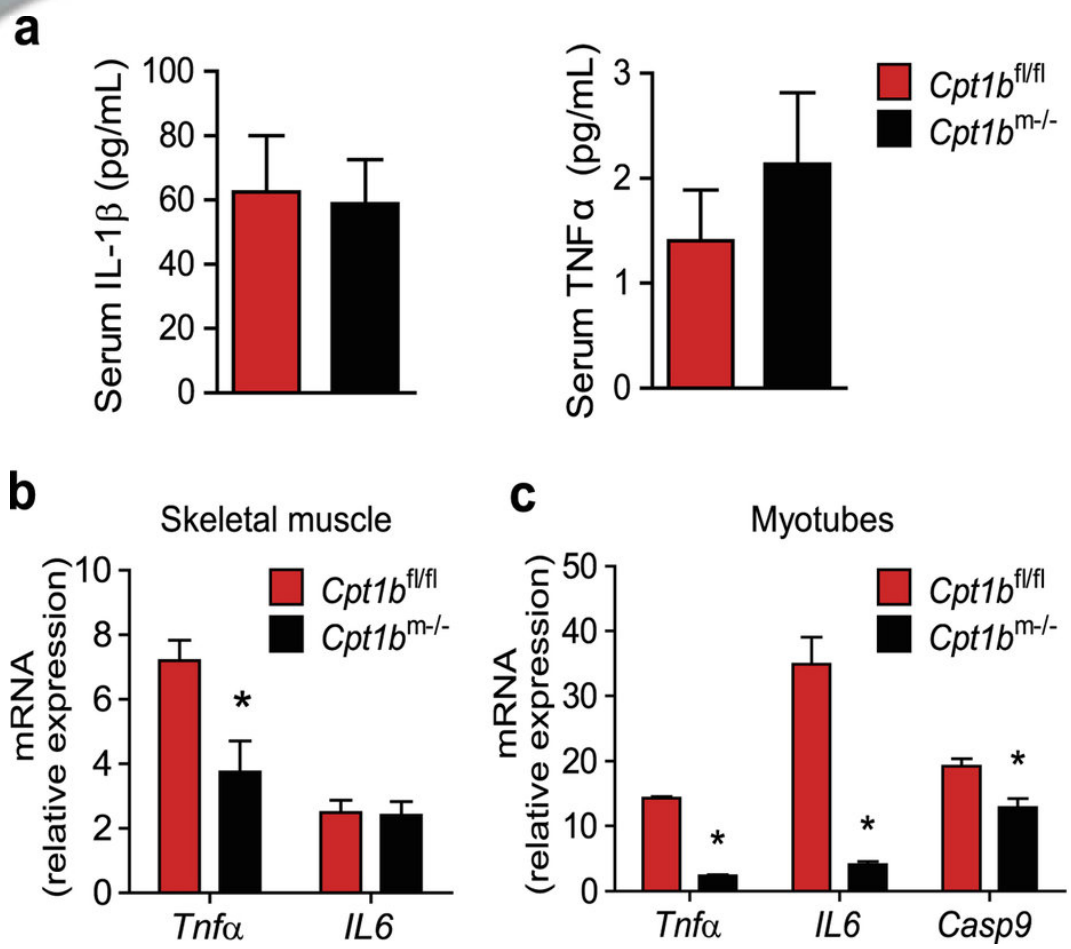
Results



*$p < 0.05$ significance for *Cpt1b*$^{m-/-}$ mice vs control *Cpt1b*$^{fl/fl}$ mice

Figure 1: Inhibition of mitochondrial fat oxidation in skeletal muscle in *Cpt1b*$^{m-/-}$ mice

a: serum levels of IL1β and TNFα did not differ ;
n = 6–8/group

b: gene expression of *TNFα* was decreased and *IL6* did not change in gastrocnemius muscle measured by q-PCR; n = 8/group

c: FA-induced inflammatory gene expression in mouse primary muscle cells is decreased; n = 3/group.

→ **inhibition does not induce inflammatory response in *Cpt1b*$^{m-/-}$ mice**

Results

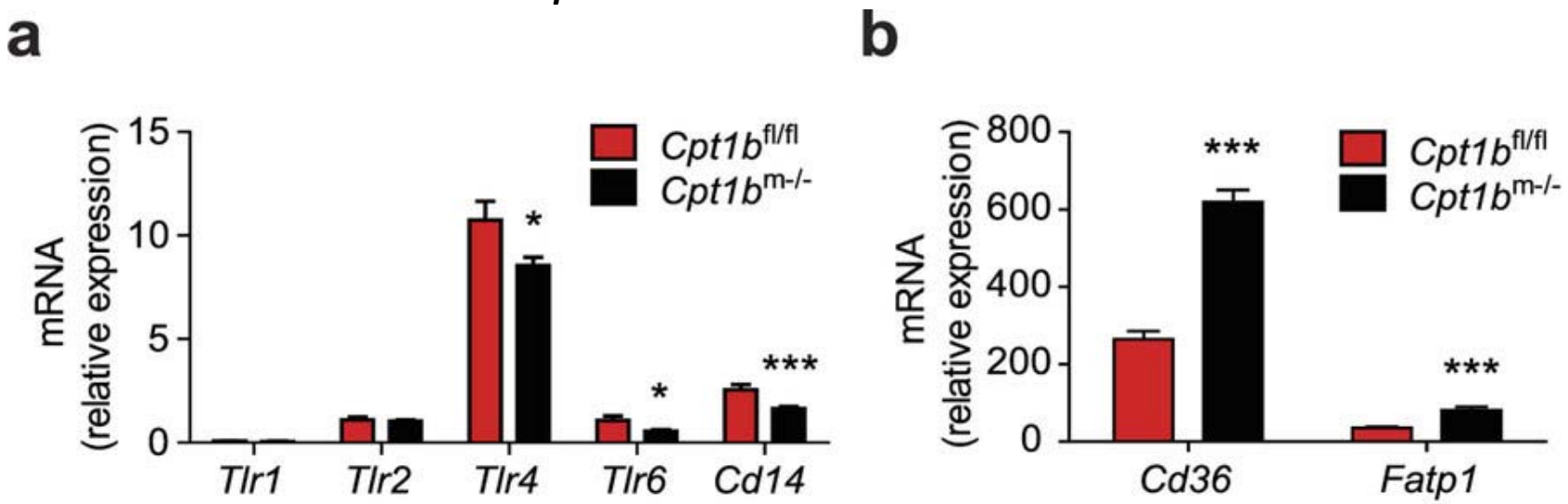
Table 1: Changes in expression of genes related to cytokine and chemokine signalling and inflammatory phenotype in *Cpt1b*$^{m-/-}$ muscle

Gene symbol	Gene name	Changes	Significance
Casp3	caspase 3	↓	0.003
Casp9	caspase 9	↓	0.015
Ccl24	chemokine (C-C motif) ligand 24	↓	<0.0001
Cd27	CD27 antigene	↓	0.005
Ltb4r1	Leukotriene B4 receptor 1	↓	0.023
Lyz2 (LyzM)	Lysozyme 2	↓	0.038
Tab1	TGF-beta activated kinase 1/MAP3K7 binding protein 1	↓	0.029
Tbkbp1	TBK1 binding protein 1	↓	0.001
Tlr6	Toll like receptor 6	↓	0.019
Traf1	TNF receptor-associated factor 1	↓	0.017

→ Gene expression datasets in gastrocnemius muscle from SAGE and GSEA;
n = 8 (for Cpt1bfl/fl mice) or n = 7 (for Cpt1bm−/− mice)
$p < 0.05$ was considered significant

Results

Figure 2: Inflammatory signaling pathways are not activated in muscle of *Cpt1b*$^{m-/-}$ mice



a,b: relative gene expression of TLR-members and *Cd14* (**a**), and fatty acid transport proteins *Cd36, Fatp1* (**b**) measured by qPCR; n = 8/group

*$p < 0.05$ and **$p < 0.01$ and ***$p < 0.005$ significances for *Cpt1b*$^{m-/-}$ vs control *Cpt1b*$^{fl/fl}$ mice

→ **several TLRs and LPS binding protein –receptor are downregulated**

→ **FA-transport proteins are increased due to FA overload**

Results

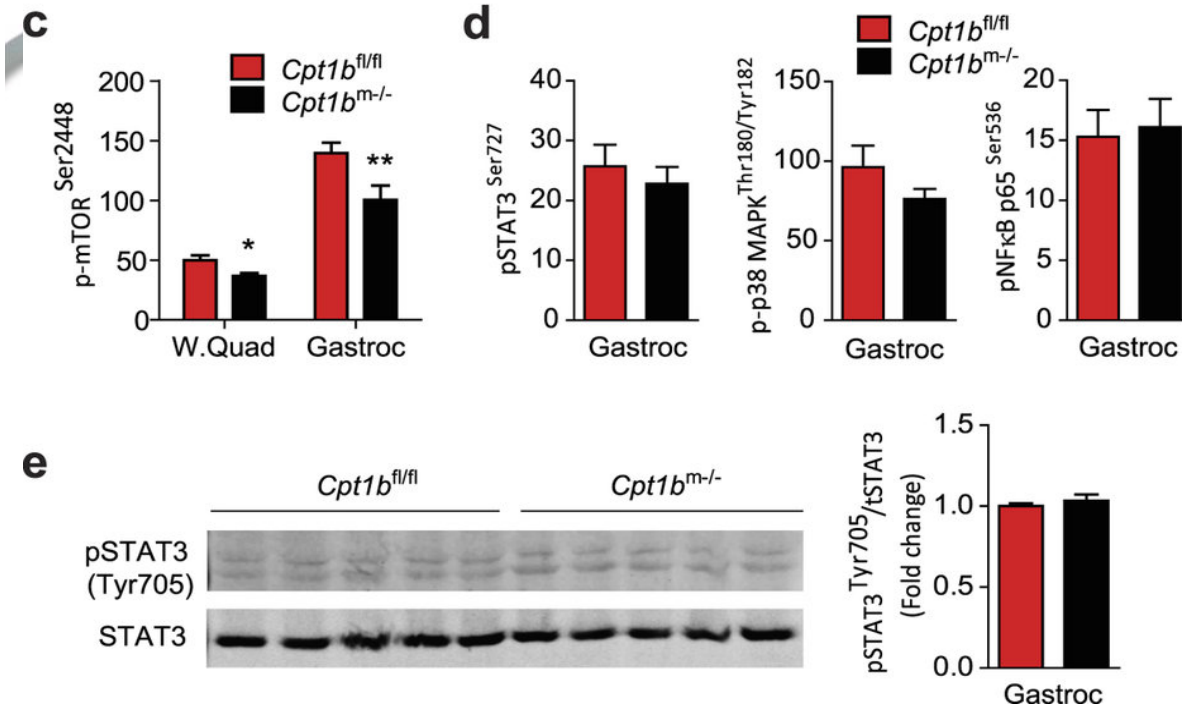


Figure 2: Inflammatory signaling pathways are not activated in muscle of *Cpt1b*$^{m-/-}$ mice

c,d: activity of mTOR (**c**), and STAT3, p38 MAPK, and NFkB (**d**) pathways evaluated by multiplex protein assay; n = 5–11/group
e: activation of STAT3 pathway evaluated by western blot analysis (left) and densitometry quantification of the immunoblots (right); n = 5/group

**p* < 0.05 and ***p* < 0.01 and ****p* < 0.005 significances for *Cpt1b*$^{m-/-}$ vs control *Cpt1b*$^{fl/fl}$ mice

→ **decreased activity of mTOR pathway in white quad and gastrocnemius muscle**
→ **no differences regarding other pro-inflammation pathways**
→ **immunoblot shows that basal activation between both groups are similar**

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Results

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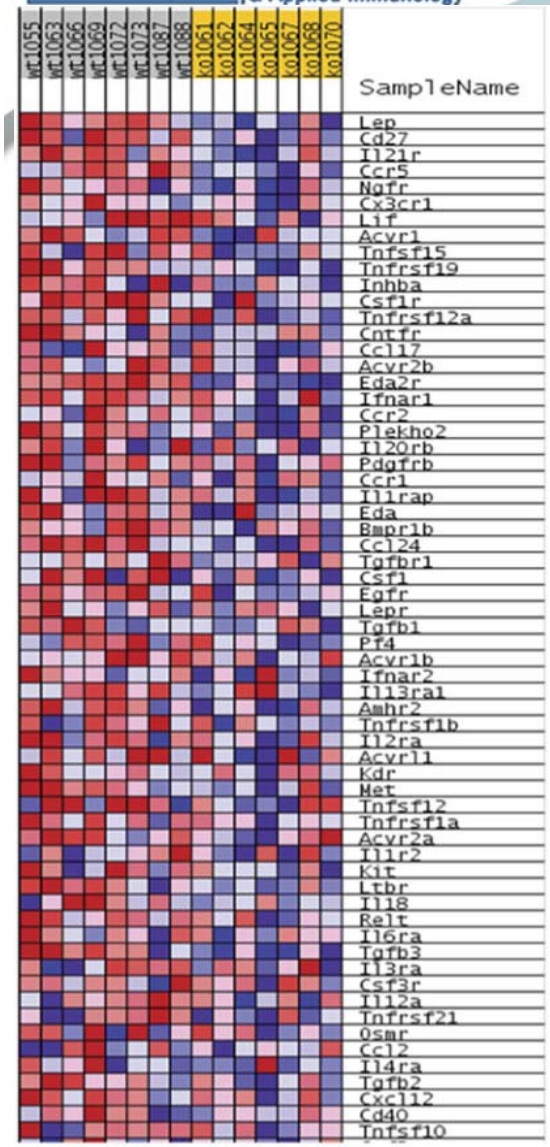

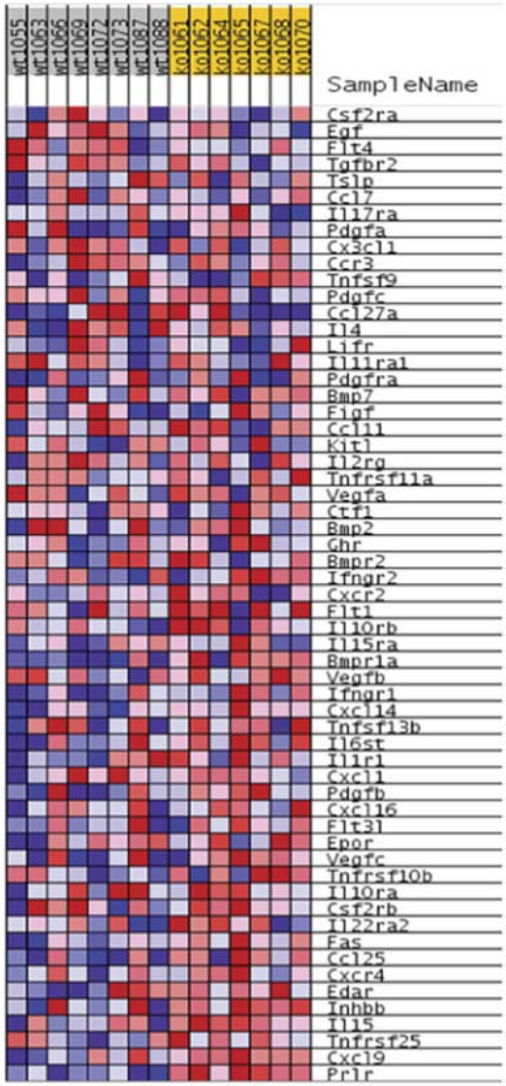

Figure 3: Cytokine-Cytokine receptor interaction

pathway related gene expression pattern determined by Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis (GSEA) in gastrocnemius muscle from *Cpt1b*$^{fl/fl}$ (wt) and *Cpt1b*$^{m-/-}$ (ko) mice;
n = 7–8/group

→ cytokine-cytokine-receptor interaction pathways are downregulated

Pathway nominal p-value: 0.0183
Pathway adjusted p-value: 0.0159
Benjamini-Hochberg (BH) – adjusted p-value: 0.0477
 # total genes in pathway: 120

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Results

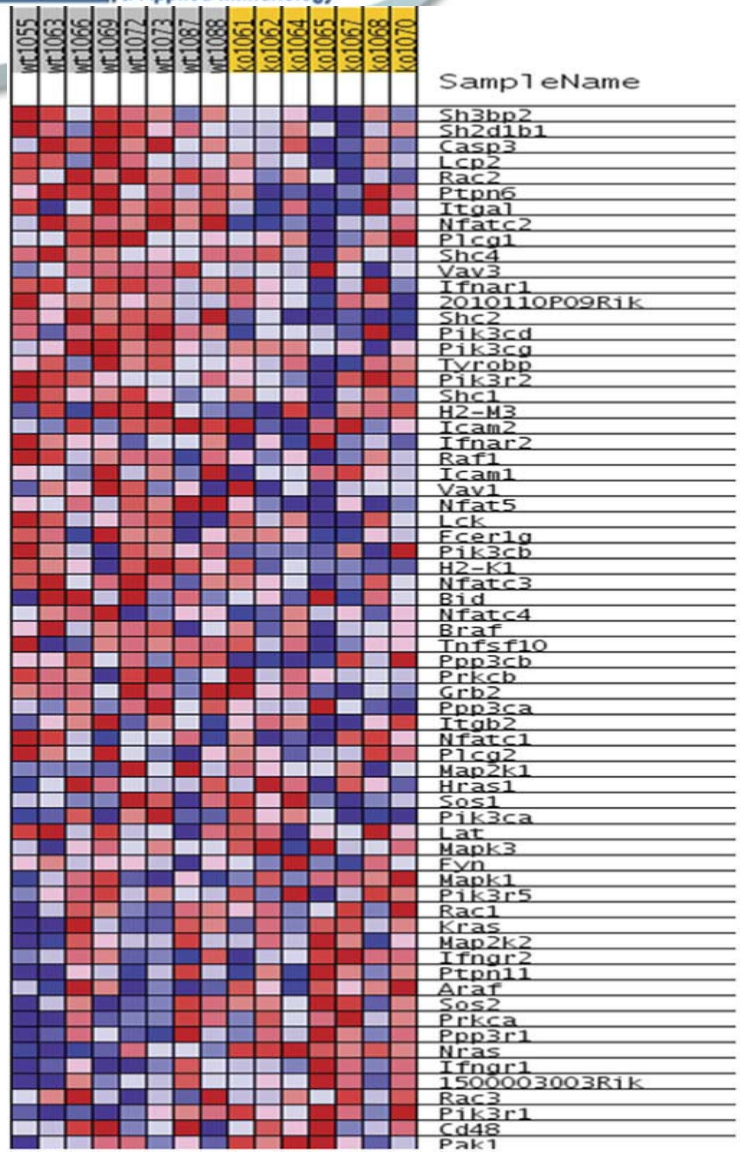

Figure 4: Natural killer cell-mediated cytotoxicity

pathway related gene expression pattern determined by IPA and GSEA in gastrocnemius muscle from *Cpt1b*$^{fl/fl}$ (wt) and *Cpt1b*$^{m-/-}$ (ko) mice; n = 7–8/group

→ **Natural-killer (NK) cell-mediated cytotoxity pathway is not significantly downregulated**

Pathway nominal p-value: 0.0
Pathway adjusted p-value: 0.0545
BH – adjusted p-value: 0.0824
 # total genes in pathway: 71

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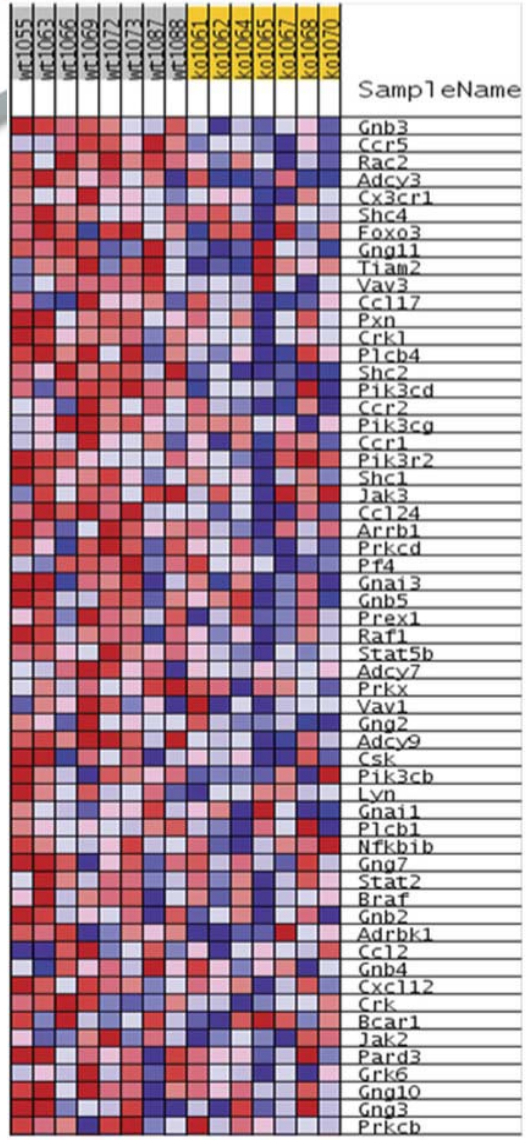


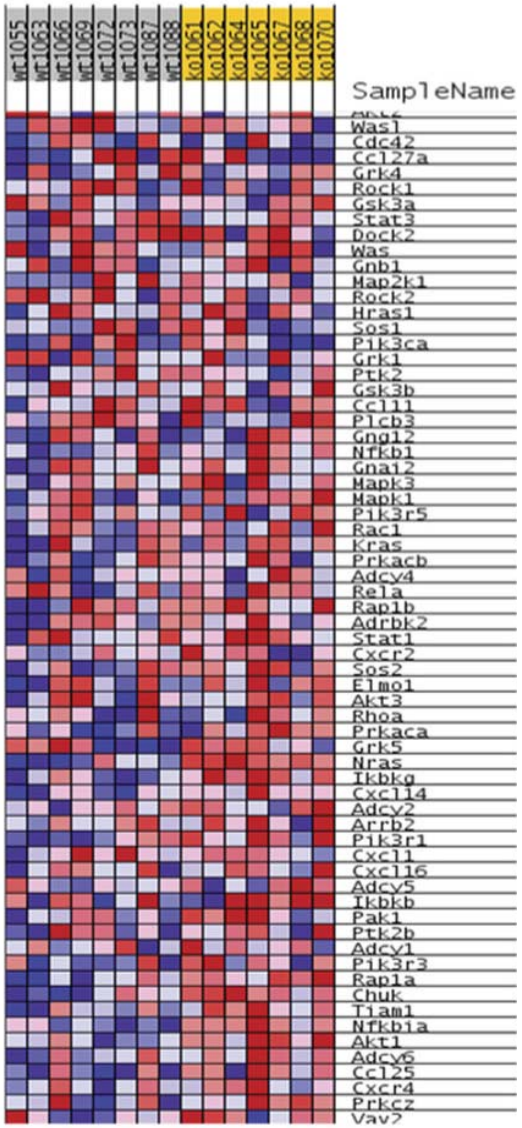


Figure 5: Chemokine signalling

related gene expression pattern determined by IPA and GSEA in gastrocnemius muscle from *Cpt1b*$^{fl/fl}$ (wt) and *Cpt1b*$^{m-/-}$ (ko) mice; n = 7–8/group

→ chemokine-pathways are decreased but not significantly

Pathway nominal p-value: 0.0128
Pathway adjusted p-value: 0.0997
BH – adjusted p-value: 0.0997
 # total genes in pathway: 127

Results

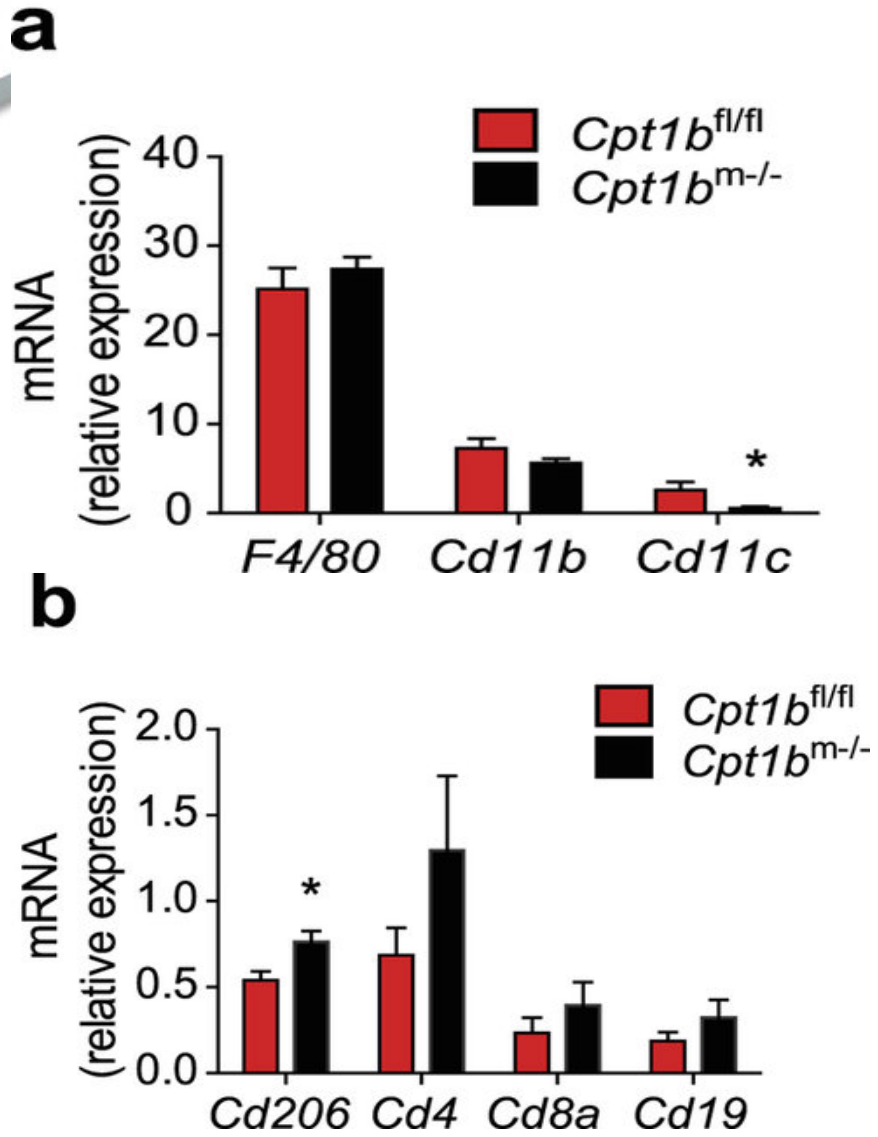


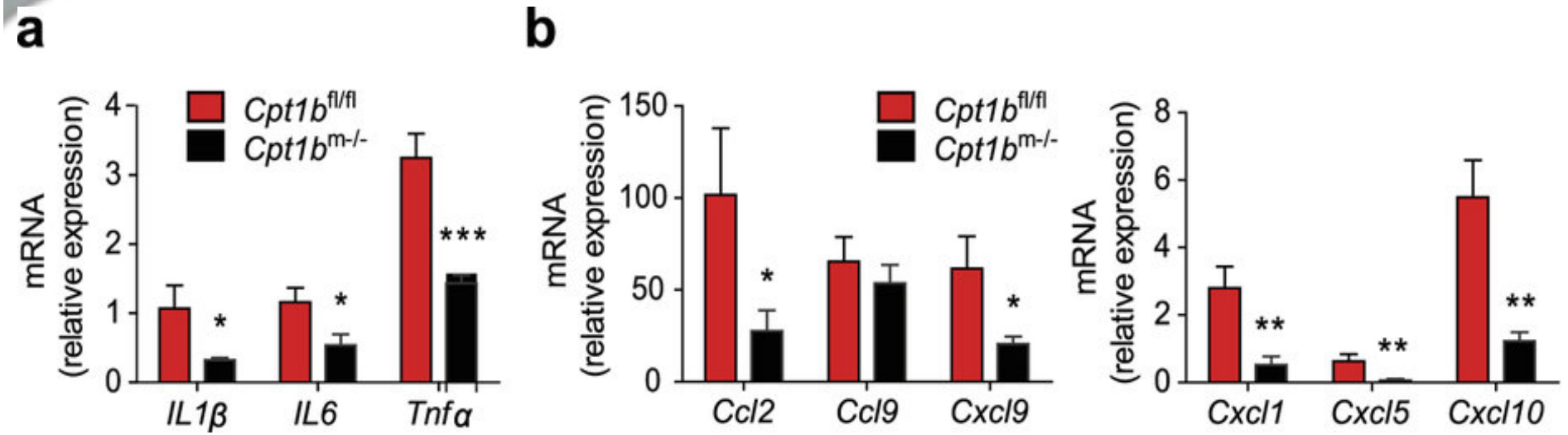
Figure 6: Immune cell markers are not elevated in skeletal muscle of *Cpt1b*$^{m-/-}$ mice

a,b: relative gene expression of *F4/80, Cd11b, Cd11c* (**a**), and *Cd206, Cd4, Cd8a, Cd19* (**b**) in gasrocnemius muscle measured by qPCR;
n = 8/group
$*p < 0.05$ and $**p < 0.01$ and $***p < 0.005$

→ **M1-macrophages are increased (pro-inflammatory)**
→ **M2-macrophages are decreased (anti-inflammtory)**
→ **infiltration of immune cells is not enhanced in knockout mice despite excess of metabolic stimuli (e.g. FA), shift from M1 to M2-macrophages?**

Results

Figure 7: Inflammatory status is improved in adipose tissue of *Cpt1b*$^{m-/-}$ mice



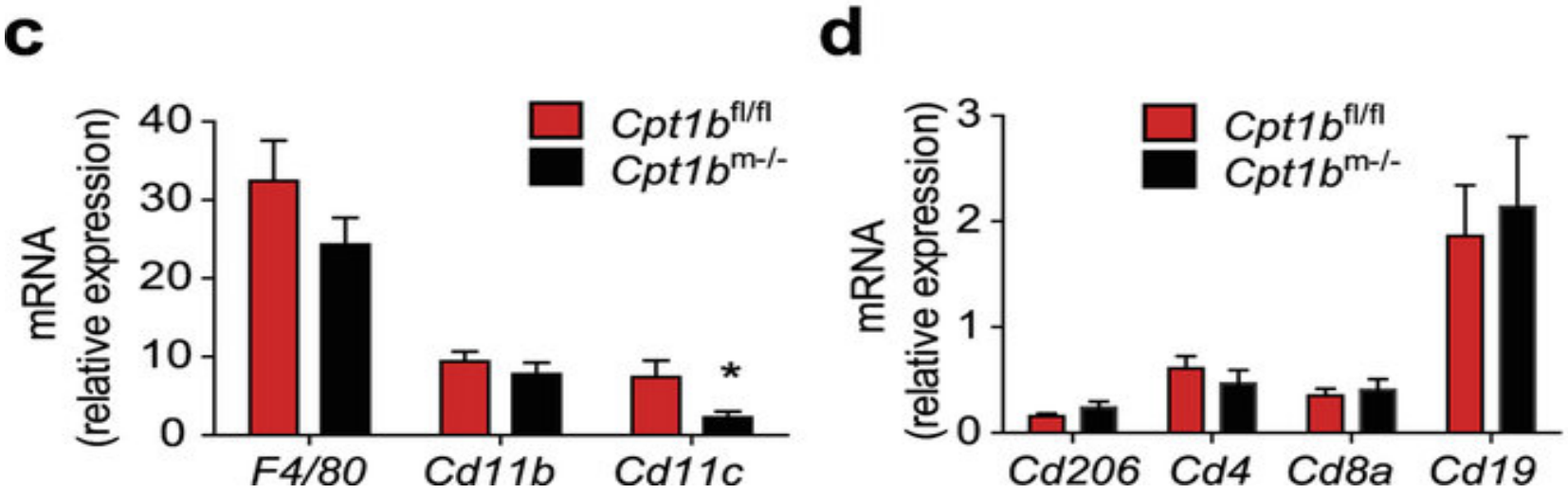
a,b: relative gene expression of *IL1β, IL6, TNFα* (**a**) and chemokines (**b**) in epididymal white adipose tissue (eWAT) measured by qPCR; n = 8/group

*$p < 0.05$ and **$p < 0.01$ and ***$p < 0.005$

→ **decreased expression of pro-inflammatory cytokines and chemokines in adipose tissue**

Results

Figure 7: Inflammatory status is improved in adipose tissue of *Cpt1b*$^{m-/-}$ mice



a,b: relative gene expression of *IL1β, IL6, TNFα* (**a**) and chemokines (**b**) in epididymal white adipose tissue (eWAT) measured by qPCR; n = 8/group

$*p < 0.05$ and $**p < 0.01$ and $***p < 0.005$

→ **expression of macrophage type 1 decreased, other pro-inflammatory and also the anti-inflammatory markers do not differ from control group**

Results - Summary

- inhibition of mitochondrial fat oxidation in skeletal muscle prevents a local inflammatory response
- TLR-signalling is downregulated in skeletal muscle from $Cpt1b^{m-/-}$ mice
- inflammatory signalling pathways are not activated in Cpt1b deficient muscle
- cytokine-cytokine receptor interaction pathways and natural killer (NK) cell-mediated cytotoxicitiy are downregulated
- CPT1b ablation shifts immune cell function towards anti-inflammatory in skeletal muscle
- inflammatory status is improved in adipose tissue of $Cpt1b^{m-/-}$ mice

Discussion

- obesity-induced chronic inflammation triggered by metabolic signals involves inflammatory responses originated within metabolic cells (e.g. adipocytes, myocytes)

  →resulting in damage to metabolic homeostasis with insulin resistance (Weisberg et al., 2003; Gregor et al., 2011; Vandanmagsar et al., 2011; Yang et al., 2010)

- previously this research team found activated NFkB pathway and increased expression of pro-inflammatory genes in insulin resistant myotubes derived from non-diabetic-lean subjetcs (Vandanmagsar et al., 2014)

  → study data suggests that excess fat is sufficient to activate inflammatory signalling pathways in skeletal muscle resulting in elevated chemokines that in turn increase infiltration of pro-inflammatory immune cells in muscle

- Cpt1b$^{m-/-}$ mice show a model of increased ectopic fat accumulation both in serum and in skeltetal muscle

- the research team has previously shown that when Cpt1b is knockt out in skeletal muscle, mice have diminished mitochondrial oxidative capacity of dietary fat (Wicks et al., 2015)

Discussion

- even at a decrease in dietary intake, knockout mice still maintain higher levels than controls of systemic and tissue lipids which have been shown to be triggers of inflammatory response (Hotamisligil et al., 2008; Dasu et al., 2008)
  - → lipid-induced inflammation would be expected in this study model
  - → but: Cpt1b$^{m-/-}$ mice do not manifest inflammation in skeletal muscle or at a systemic level and do not possess a strong inflammatory response to the presence of elevated fatty acids
- sensing and signalling mechanisms which stand at the intersection of metabolic and inflammatory pathways (TLRs, mTOR, MAPK, NFkB etc.) are activated in obesity-associated inflammation (Radin et al., 2008; Senn et a., 2006; Ivashkiv et al., 2011) were not induced in Cpt1b$^{m-/-}$ mice
- → moreover: inflammatory response is decreased in Cpt1b-deficient muscle cells; changes in chemokine production that result in a switch in knockout model from pro- to antiinflammatory function of immune cells

Discussion

- key contributor to the activation of inflammatory signalling pathways and the inflammatory response in obese state is metabolic stress of organells such as mitochondria and ER (Gregor et al., 2011)
  - → however: this reaearch team previously reported that mitochondrial and ER stress levels wer not different in muscle between Cpt1b$^{m-/-}$ and control mice (Vandanmagsar et al., 2016)
- it has been shown that skeletal muscle produces cyokines dependent upon contraction (Pederson et al., 2012; Allen et al., 2015; Pederson et al., 2012)
  - → reduced activity of Cpt1b$^{m-/-}$ mice could contribute to a reduction of contraction induced cytokine response
- adipose tissue inflammation largely contributes to obesity-induced pro-inflammatory state and insulin resistance (Welsberg et al., 2003; Lumeng et al., 2011; Hotamisligil et al., 2008; Vandanmagsar et al., 2011; Yang et al., 2010)
  - → inflammation in adipose tissue with increased pro-inflammatory cytokines and chemokines and infiltrated immune cells in obesity is absent in Cpt1b$^{m-/-}$ mice

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Discussion

- research team has recently found out that inhibition of mitochondrial oxidation in muscle induces FGF21 specifically in skeletal muscle of knockout mice (Vandanmagsar et a., 2016)
- other reports have shown that FGF21 decrease expression of IL6 and TNF alpha in adipose tissue and suppressed inflammtion in mouse model (Wang et al., 2014; Zhang et al., 2013)
- → FGF21 in Cpt1b$^{m-/-}$ mice could have anti-inflammatory action locally, on adipose or other tissues contributing to the improvement of inflammatory status
- already has been shown that there is a close connection between metabolic and inflammatory system (Wellen et al., 2005; Lackey et al., 2016; Lumeng et al., 2011; Odegaard et al., 2013)
  - → this study data shows a coordinated decrease in mitochondrial processing of fatty acids and expression of inflammatory marker genes
  - this research team previously reported that pathways like TCA cycle, pyruvate and fatty acid metabolism are upregulated in Cpt1b$^{m-/-}$ mice (Wicks et al., 2015)

Discussion - Summary

- elevated lipids within models of obesity and insulin resistance are not alone sufficient to induce chronic inflammation

  → metabolic status and homeostasis between metabolic and inflammatory mechanisms prevent lipid-induced stress

- inhibition of CPT1 in skeletal muscle is protective against muscle and systemic inflammation
  - but prescence of excess lipid stressors

  → possibly due to metabolic compensatory mechanisms developed to rescue impaired mitochondrial fat oxidation

→ further reserach is required

Personal Opinion

Pros:

- comprehensive structure
- detailed methods and statistics
- clear results
- extensive discussion part

Cons:

- reasons for difference in quantity of mice examined concerning inflammatory markers?
- statistiscs: differences in gene expression (mRNA) based on specific standard values?
- description of one figure (fig. 7) is incomplete

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Thank you for your attention!

Any questions?