The *IGF2* mRNA binding protein p62/IGF2BP2-2 induces fatty acid elongation as a critical feature of steatosis

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Abbreviations: acetyl-CoA carboxylase alpha (ACC/ACACA), carbohydrate responsive element-binding protein (CHREBP/MLXIPL), carnitine palmitoyltransferase 1A (CPT1A), ELOVL fatty acid elongase 6 (ELOVL6), fatty acid synthase (FASN), *gas chromatography-mass spectrometry* (GC-MS), glucose infusion rate (GIR), glucose turnover (TO), hepatic glucose production (HGP), hepatocellular carcinoma (HCC), insulin-like growth factor 2 (IGF2), liver-X-receptor alpha (LXR-α/NR1H3), nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), peroxisome proliferator-activated receptor alpha (PPARA), pyruvate kinase, liver and RBC (L-PK, PKLR), sterol regulatory element binding transcription factor 1 (SREBF1/SREBP1), ultra high-performance liquid chromatography–mass spectrometry (UHPLC-MS/MS)

Abstract:

Liver-specific overexpression of the insulin-like growth factor 2 (*IGF2*) mRNA binding protein p62/IGF2BP2-2 induces a fatty liver, which highly expresses *IGF2*. Since *IGF2* expression is elevated in patients with steatohepatitis, the aim of our study was to elucidate the role and interconnection of p62 and IGF2 in lipid metabolism. Expression of *p62* and *IGF2* highly correlated in human liver disease. *p62* induced an elevated ratio of C18:C16 and increased ELOVL fatty acid elongase 6 (ELOVL6) protein, the enzyme catalyzing the elongation of C16 to C18 fatty acids and promoting nonalcoholic steatohepatitis in mice and humans. p62 overexpression induced the activation of the ELOVL6 transcriptional activator SREBF1. Recombinant IGF2 induced the nuclear translocation of sterol regulatory element binding transcription factor 1 (SREBF1) and a neutralizing IGF2 antibody reduced ELOVL6 and mature SREBF1 protein levels. Concordantly, *p62* and *IGF2* correlated with *ELOVL6* in human livers. Decreased palmitoyl-CoA levels as found in *p62* tg livers can explain the lipogenic action of ELOVL6. Accordingly, p62 represents an inducer of hepatic C18 fatty acid production *via* a SREBF1-dependent induction of ELOVL6. These findings underline the detrimental role of p62 in liver disease.

Supplemental Keywords: ELOVL6, p62/IGF2BP2-2/Imp2-2, SREBF1/SREBP1, IGF2 signalling, Hepatic steatosis

Introduction

Nonalcoholic fatty liver disease (NAFLD) is considered as the most common liver disorder in Western countries with a prevalence of 20-30% of the adult population (1, 2). There is a strong correlation between characteristics of the metabolic syndrome, such as obesity and diabetes mellitus, and NAFLD/nonalcoholic steatohepatitis (NASH) (3).

The 'two-hit' hypothesis represents a common model to describe the development and progression of fatty liver diseases. A simple steatosis can stand for the first step in early liver pathogenesis (4, 5). The progression from simple steatosis to NASH requires a 'second hit' mediated by reactive oxygen species and release of inflammatory cytokines (6). This inflammatory environment can result in hepatic cirrhosis and finally in hepatocellular carcinoma (HCC) (7).

The development of hepatosteatosis can be induced by different mechanisms. The synthesis of lipids is regulated in a complex interplay induced by a set of lipogenic transcription factors, among which liver-X-receptor alpha (LXR-α, NR1H3), sterol regulatory element binding transcription factor 1 (SREBF1, SREBP1), and carbohydrate responsive element-binding protein (ChREBP, MLXIPL) represent the most important ones (8). In this context, the fact that MLXIPL controls 50% of hepatic lipogenesis by regulating glycolytic and lipogenic gene expression (9) illustrates the importance of both insulin- as well as glucose-induced lipogenic pathways. One of the relevant inducers of lipid degradation in the liver is the peroxisome proliferator-activated receptor alpha (PPARA) (10). Most importantly, there is a close interconnection between catabolic and anabolic pathways. In this context it is important to note that the mitochondrial β-oxidation pathway is negatively regulated by high malonyl-CoA levels (11, 12).

Besides the amount of lipids, which are relevant for pathophysiological actions, there is increasing evidence that also the composition of lipids has an impact on pathophysiology. In fact, human NAFLD is characterized by numerous changes in hepatic lipid composition and relative abundance of specific fatty acids (13, 14). Also hepatitis (B/C) has been described to strongly alter hepatic lipid content and composition (15, 16). Recently, the fatty acid elongase 6 (ELOVL6), which catalyzes the elongation of C16 to C18 fatty acids (17) and is a direct target of SREBF1 (18, 19), has been shown to promote NASH in mice and humans and to be overexpressed in a murine NASH model (20, 21). Interestingly, however, there is still a lack of understanding of the upstream signaling pathways being responsible for SREBF1 activation and why elevated ELOVL6 increases total fatty acid production. Also the role of ELOVL6 in HCC is as yet poorly understood and seems to depend on disease etiology (21-23).

We recently reported that a liver-specific overexpression of the insulin-like growth factor 2 (*IGF2*) mRNA binding protein p62/IGF2BP2-2 induces steatosis in mice, coupled with high *Igf2* expression and activation of the phosphoinositide 3-kinase/AKT-signaling pathway (24). Furthermore, p62 has been shown to promote NASH development (25). Most lipid species are elevated in p62-induced steatosis, with triglycerides showing the strongest increase (26). p62 was originally identified as an autoantigen overexpressed in about one third to two thirds of HCC patients and correlates with poor outcome (27-30). Interestingly, also *IGF2* is overexpressed in NASH and HCC (31, 32), which is linked to p62 overexpression in HCC (27), and might be explained by the involvement of the *IGF2* mRNA-binding proteins in RNA localization, stability and translation (33). p62 is a splice variant of IGF2BP2 lacking exon 10, though exon 10 deletion is not affecting the six characteristic RNA binding motifs (33). Recently, Li et al. reported IGF2BP2 to bind and control the translation of c-Myc, Sp1

transcription factor and insulin-like growth factor 1 receptor (34). Binding affinities of IGF2BP2-2 to the respective mRNAs, however, are not described in the literature.

Aim of our study was to decipher the effects of p62 on lipid metabolism and to elucidate the influence of IGF2.

Materials and Methods

Animals

All animal procedures were performed in accordance with the local animal welfare committee. Mice were kept under stable conditions regarding temperature, humidity, food delivery, and 12 h day/night rhythm. *p62* transgenic mice were established as described by Tybl et al. (24). The mice were sacrificed at an age between 2.5 and 5 weeks.

Hyperinsulinemic euglycemic clamp study

Hepatic insulin sensitivity was determined by the hyperinsulinemic euglycemic clamp technique as described previously (35).

Human liver tissue

35 human liver tissues from patients undergoing surgical resection were analysed in the framework of the project, which was authorized by the ethical committees of the Medical University of Graz (Ref. Nr. 1.0 24/11/2008) and the University of Heidelberg (Prof. Bannasch). Details on patient data are given in supplemental data Table I.

Cell culture and transfection

HepG2 cells were cultured in RPMI-1640 (PAA, Cölbe, Germany) with supplementation of 10% [v/v] FCS (PAA, Cölbe, Germany), 1% [v/v] glutamine (PAA, Cölbe, Germany), and 1% [v/v] penicillin/streptomycin (PAA, Cölbe, Germany) at 37°C and 5% CO_2. HepG2 overexpression and knockdown assays were performed according to Kessler et al. (27). For the detection of IGF2-mediated SREBF1 translocation, cells were treated for the indicated

time with rhIGF-II (0.075 µg/ml, 292-G2, R&D Systems, Minneapolis, U.S.A) or with IGF2 antibody (ab9574, Abcam, United Kingdom) as previously described (27).

Fatty acid measurement by gas chromatography-mass spectrometry (GC-MS)

Murine liver samples or HepG2 cells were lyophilized, hydrolyzed by the fatty acid methyl ester (FAME) method and analysed according to Bode et al. (36). Methyl-nonadecanoate (74208, Sigma Aldrich, Taufkirchen, Germany) was used as an internal standard. The method detects both free and bound free fatty acids.

Histochemistry

Hematoxylin / eosin (HE)-staining and immunohistochemical detection of F4/80 of paraffin embedded sections were performed as previously reported (22, 24).

Real-time RT-PCR

Isolation of total RNA and reverse transcription was performed as described previously (37). RNA from human liver samples was isolated as previously described (27). Real-time RT-PCR was performed in an iQ5 cycler (Bio-Rad, Munich, Germany) or in a CFX96 cycler (Bio-Rad, Munich, Germany) with 5 x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). All samples were estimated in triplicate. Primers and conditions are listed in supplemental data Table II. Efficiency was determined for each experiment using a cDNA dilution series with a starting concentration equivalent to 0.5 µg RNA or with a standard dilution series as described previously (37). The relative gene expression was normalized to *ACTB* or *18s* mRNA values.

Preparation of nuclear extracts

Nuclear extracts from HepG2 cells were prepared as described previously (38).

Protein isolation and analysis by Western blot

Protein isolation from murine liver tissue was done according to Tybl et al. (24), whereas protein isolation from cells was according to Basirico et al. (39).

Protein separation and detection were peformed as previously described (24). Information on the used antibodies and conditions can be found in the supplemental data Table III. The primary antibodies anti-ELOVL6 and anti-α–tubulin were purchased from Sigma Aldrich (PRS4571, T9026, Taufkirchen, Germany), anti-SREBF1 and anti-PPARA from Abcam (ab3259, ab8934, Cambridge, United Kingdom), anti-FASN, anti-lamin A/C antibody from Cell Signaling Technology (#3180, #2032, Danvers, USA), anti-p62 antibody was kindly provided by Dr. Tan (TSRI, La Jolla, CA, USA) (30, 40).

Palmitoyl-CoA extraction and analyses with ultra high-performance liquid chromatography–mass spectrometry (UHPLC-MS/MS)

Fresh snap-frozen liver tissue was immediately freeze-dried and homogenized and stored at –80°C. 40 mg of freeze-dried tissue was extracted for 30 minutes in 1 ml methanol containing *n*-heptadecanoyl-CoA as internal standard (with a final concentration of 500 nM in 150 µl final volume). The extract was centrifuged at 22,000 *g* for 10 min and 4°C. The supernatant was transferred to a new vial and dried under gaseous nitrogen and reconstituted in 150 µl methanol / water (1:1 [v/v]). The standard dilution series was made in methanol / water (1:1 [v/v]). All steps were performed on ice.

The analyses were performed using a TSQ Access Max mass spectrometer equipped with an ESI source and a triple quadrupole mass detector (Thermo Finnigan, San Jose, CA). The MS

detection was carried out in heated ESI mode, at a spray voltage of 4.5 kV, a probe temperature of 400 °C, a nitrogen sheath gas pressure of 3.0×10^5 Pa, an auxiliary gas pressure of 1.0×10^5 Pa, a capillary temperature of 350 °C, and a tube lens voltage of 114 V in negative ionization mode. Palmitoyl coenzyme A lithium salt (P9716-5MG) and *n*-heptadecanoyl coenzyme A lithium salt (H1385-5MG) were purchased from Sigma Aldrich (Taufkirchen, Germany).

Xcalibur software was used for data acquisition and plotting.

The chromatographic separation was carried out on an Accela UPLC, consisting of a quaternary pump, degasser, and autosampler (Thermo Finnigan, San Jose, CA) using a Accucore RP-MS column (150x2.1, 2.6µ), with an injection volume of 25 µl.

The solvent system consisted of 10 mM ammonium acetate (A) and methanol (B).

HPLC-Method: Gradient run of initial 65% of B in A and a flow of 600 µl/min. In 0.6 min the solvent mixture was changed to 100% of B, with a flow of 700 µl/min and kept for 3.4 min.

The amounts of palmitoyl-CoA and the internal standard n-heptadecanoyl-CoA were each determined in single reaction monitoring mode using the following transitions:

Palmitoyl-CoA: precursor ion 1005.992 m/z; product ion 672.075 m/z; scan time 0.5 sec; scan width 3.000 m/z; collision voltage 44 V.

Heptadecanoyl-CoA (ISTD): precursor ion 992.228 m/z; product ion 926.442 m/z; scan time 0.5 sec; scan width 3.000 m/z; collision voltage 42 V.

Palmitoyl-CoA, with a retention time of 2.27 min, was quantified using the chromatographic peak area relative to the internal standard (RT 2.17 min). The lower limit of quantitation was

2.1 nM. The measurement was performed with wild-type livers (n=9) and livers of *p62* transgenic animals (n=10). Supplemental data Fig I shows the measurement of a representative Palmitoyl-CoA dilution series. Supplemental data Fig II shows a representative measurement of Palmitoyl-CoA extracted from mouse liver.

Statistical analysis

Results are expressed as means ± SEM. The statistical significance was determined by independent two-sample t-test. Pearson's correlation was used to test the relationship between *p62*, *IGF2, ELOVL6,* and *FASN* mRNA in human liver samples.

The results were considered as statistically significant when p value was less than 0.05.

Results

Characterization of steatosis

Liver-specific overexpression of p62 has previously been shown to induce histologically detectable steatotic features in about 60% of the animals (24) (Fig 1A).

(insert those 2 paragraphs at XX?) Previous data suggested no distinct inflammation in *p62* transgenic animals since we observed no elevated liver damage (24). Still, immunohistochemical staining and real-time RT-PCR revealed elevated levels of the macrophage marker F4/80 in *p62* transgenic livers (Fig 1B, C). 18.2% of *p62* transgenic animals exhibited distinct leukocyte infiltrates (2-3 infiltrates per microscopic field), which were not observed in wild-type animals.

Our previous data indicated slightly improved glucose tolerance of *p62* transgenic animals (24). We now performed hyperinsulinemic euglycemic clamp analysis: Both, the higher glucose infusion rate and lower hepatic glucose production with unchanged glucose turnover indicated elevated hepatic insulin sensitivity. However, these results were not statistically significant (Fig 1D).

Quantification of fatty acid composition revealed that also transgenic animals, which exhibited a normal histology, showed an increased fatty acid content (Table 1). Still, the fatty acid content was even higher in animals with a histologically proven steatosis (Table 1). Taking all *p62* transgenic animals together, we observed a 1.66 ± 0.12 fold ($p = 0.0007$) increase of the total fatty acid content compared to wild-type animals. Having a closer look at the composition of the fatty acids, we observed that the chain length of fatty acids was different in *p62* transgenic livers compared to wild-type tissue: steatotic livers exhibited an increased ratio of C18 to C16 fatty acids (Fig 1E).

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In order to study the mechanisms responsible for hepatic lipid alterations we employed HepG2 cells, which are able to develop cellular steatosis and are frequently used for respective studies (15, 41-44). The fatty acid chain length in fact depended on the presence of p62: when p62 was knocked down in HepG2 cells, increased levels of C16 fatty acids were detectable (Table 2), so that the ratio of C18 to C16 fatty acids decreased (Fig 1F). We therefore investigated the effect of p62 on ELOVL6, which catalyses the elongation of C16 to C18 fatty acids (17).

We observed that ELOVL6 is induced in a p62-dependent fashion: *p62* transgenic animals displayed significantly increased levels of ELOVL6 protein (Fig 1G). *Vice versa*, knockdown of p62 in HepG2 cells exhibited significantly reduced ELOVL6 mRNA and protein levels (Fig 1H). The dependency of ELOVL6 expression on p62 in human livers was supported by a strong correlation of *p62*/*ELOVL6* expression (Fig 1H).

Expression of lipogenic regulators

Surprisingly, *MLXIPL* mRNA expression was significantly upregulated in p62 siRNA-treated HepG2 cells (Fig 2A) and downregulated in cells overexpressing p62 (Fig 2A). We could validate this effect in *p62* tg animals, in which MLXIPL was significantly downregulated (15% ± 8%, p=0.0497, n=5 in each group). Looking at *NR1H3* mRNA expression as another lipogenic transcription factor in either p62 overexpressing or p62 siRNA cells, we did not observe any significant difference (Fig 2A). The same was true for *SREBF1c* mRNA (Fig 2A).

In addition to transcriptional regulation (45, 46), SREBF1 can be activated by cleavage from its precursor to its mature form upon insulin treatment (38). Because p62 has been demonstrated to upregulate *IGF2* in both mouse (24) as well as in human livers (27), we hypothesized that p62 regulates SREBF1 on protein level. We observed significantly increased levels of the mature isoform of SREBF1 after overexpression of p62 in HepG2 (Fig 2B). The SREBP1 mature form in *p62* transgenic mice behaved similarly, but quantified values were not statistically significant (Fig 2C). The precursor showed neither an ~~a slight tendency of~~ induction in p62 overexpressing HepG2 (Fig 2B) nor in *p62* transgenic mice (Fig 2C). Knockdown of p62 by siRNA resulted in reduced levels of both the SREBF1 precursor as well as the mature form of SREBF1 (Fig 2B).

Suggesting IGF2 to be responsible for SREBF1 activation, we treated HepG2 cells with IGF2, and indeed observed higher amounts of mature SREBF1 in their nuclei (Fig 2D). Antagonization of IGF2 activity with an IGF2 specific antibody in HepG2 reduced the levels of ELOVL6 and the nuclear form of SREBF1 compared to their respective controls (Fig 2E). In human liver tissue, in which p62 and ELOVL6 correlated, we also observed a distinct correlation of IGF2 and ELOVL6 expression levels (Fig 2F). In the same tissues ($n = 35$) we could confirm a correlation between p62 and IGF2 ($r = 0.41$, $p = 0.02$) as recently reported for a cohort of HCC patients (27).

The fatty acid synthase (FASN) as an important lipogenic enzyme displays also a target of SREBF1 activation, which is why we hypothesized that p62-induced lipid accumulation might be mediated *via* FASN induction. Interestingly, however, *FASN* mRNA and protein levels were ~~slightly decreased~~ not changed upon p62 overexpression and increased upon p62 knockdown (Fig 3A, B). Concordantly, the *p62* transgenics revealed reduced levels of FASN protein (Fig 3C). The analysis of human liver samples showed no correlation between *p62* and

FASN mRNA (r = -0.1, p = 0.58). To reassess the unforeseen behaviour of the direct SREBF1 target FASN, we had a closer look on stearoyl-CoA desaturase (delta-9-desaturase) (*SCD1*, *SCD)* and acetyl-CoA carboxylase alpha (*ACC*, *ACACA*) mRNA, which are SREBF1 targets and important enzymes in lipogenesis. Interestingly, neither p62 knockdown nor p62 overexpression revealed changes in *SCD* or *ACACA* mRNA levels (Fig 3D). Since most lipogenic genes are coordinately regulated by SREBF1 and MLXIPL (47), we speculated that inversely regulated MLXIPL action might reverse the action of activated SREBF1. Therefore, we had a closer look on pyruvate kinase, liver and RBC (L-PK, PKLR) expression, which is exclusively regulated by MLXIPL (48). Indeed, p62 regulation of *PKLR* mRNA exhibited the same expression pattern compared to *MLXIPL* mRNA: *PKLR* mRNA was increased after p62 knockdown and decreased after p62 overexpression (Fig 3E).

Regulation of lipolytic pathways

Our data as yet suggested a distinct action of p62 on fatty acid composition (i.e. chain length), but rather no effect on lipogenic enzymes. We therefore suggested that the elevated levels of lipids in p62 livers might rather be facilitated by a decreased β-oxidation. We therefore tested *PPARA* expression upon both p62 knockdown and overexpression in human hepatoma cells, and in *p62* transgenic mice. The data revealed a downregulation of *PPARA* mRNA after p62 overexpression in cells (Fig 4A) and a lack of effect in the *p62* transgenic mouse model (Fig 4B). Therefore, the investigation of PPARA expression does not provide conclusive data on a potentially decreased β-oxidation due to p62.

Mitochondrial β-oxidation is regulated by carnitine palmitoyltransferase 1A (CPT1A) activity (Fig 4C), which is controlled by malonyl-CoA. Malonyl-CoA levels depend on the palmitoyl-CoA-mediated inhibition of acetyl-CoA carboxylase alpha (ACACA). Malonyl-CoA levels

were below the detection limit of UHPLC-MS/MS analysis. Still, reduced palmitoyl-CoA levels (37 ± 12%) as found in *p62* transgenic animals suggested that attenuated mitochondrial β-oxidation is responsible for ELOVL6-induced steatosis.

Discussion

The p62-induced steatosis is characterized by an increase in almost all lipid classes with the most distinct effect on triglycerides (26). Accordingly, we here observe an elevated abundance of almost all fatty acids (Table 1). Due to unchanged serum cholesterol and triglyceride levels in *p62* transgenics compared to wild-type animals (24) it is unlikely that hepatic lipid accumulation is due to reduced lipid export from the liver or increased lipid uptake.

Excess hepatic lipid incorporation is often associated with inflammatory events, which link a simple steatosis to NASH (6). We here show that *p62* transgenic mice also display inflammatory signs, as validated by increased F4/80 and leukocyte infiltrates. The inflammation is rather mild, though, and does not result in elevated transaminase levels (24), which might also be linked to cytoprotective actions of p62 (27). Despite lipid accumulation and an inflammatory environment, development of insulin resistance is absent in *p62* transgenic mice confirming our previous data (24), which suggest slightly elevated glucose tolerance.

In addition to the general increase in lipids in *p62* transgenic livers we could observe an increased ratio of C18 to C16 fatty acids. Accordingly, ELOVL6, which catalyzes the elongation of C16 to C18 fatty acids (17), is increased in *p62* transgenic mice. Our results from HepG2 cells and human liver tissues are in line with these findings. Recently, Matsuzaka et al. reported that overexpression of ELOVL6 promotes NASH in mice and humans (20) . What is more, ELOVL6 expression specifically characterizes steatotic events being linked to inflammation (21, 22). This is in line with the finding that p62 transgenic mice both exhibit increased levels of ELOVL6 and show signs of liver inflammation. Taken together, data on ELOVL6 suggest a detrimental action of C18 fatty acids.

Pathways being responsible for the upregulation of this pathophysiological regulator of liver disease have as yet been unknown. SREBF1 is an important transcription factor that regulates lipid metabolism and contributes to the pathophysiology of the metabolic syndrome (49, 50). Our data demonstrate a p62-dependent cleavage of SREBF1 into its active form. SREBF1 gene expression as well as its cleavage-induced activation is enhanced by insulin, leading to its binding to sterol-response element, which is located in the promoters of its target genes (51). HepG2 cells, in which the transcriptional regulator of SREBF1 NR1H3 was activated, displayed increased *SREBF1c* mRNA levels (52-54), whereas SREBF1a expression, the dominant isoform in cultured hepatocytes (55), was not regulated by NR1H3 activation (54, 56, 57). Since neither p62 knockdown nor p62 overexpression showed any effect on *NR1H3* expression, it is hardly surprising that *SREBF1c* mRNA levels were not affected by p62.

IGF1 and insulin treatment have been shown to induce SREBF1 in sebocytes *via* activation of the insulin- and IGF1-receptor (58). IGF2 binds to insulin receptor, IGF1-receptor, and IGF2-receptor, with moderate to high affinity (45). Human hepatoma cell lines overexpressing p62 and *p62* transgenic animals have been shown to express high levels of *IGF2* mRNA (24, 27). We here report a p62-mediated induction of SREBF1 and ELOVL6, which depends on IGF2 expression. This causal link is supported by the correlation of *p62*, *IGF2,* and *ELOVL6* expression in human liver tissue.

Our data reveal an upregulation of MLXIPL by p62 knockdown, while SREBF1 is inactivated. *Vice versa,* a MLXIPL downregulation occurs after p62 overexpression, while SREBF1 is activated. In fact, the literature reports that SREBF1 overexpression reduces MLXIPL expression (41). Since MLXIPL knockout animals show improved plasma glucose control (48, 59), the improved glucose tolerance exhibited in *p62* transgenic animals (24) can be explained by MLXIPL downregulation and increased Igf2 levels.

Although SREBF1 can induce FASN (41), we observed that p62 decreased FASN levels, while SREBF1 was activated. The effects of p62-induced SREBF1 activation seems to be abrogated by the parallel decreased MLXIPL expression, which is in line with the finding that 50% of lipogenic gene expression is associated to MLXIPL (9). Interestingly, the p62 model is characterized by a higher sensitivity of ELOVL6 towards SREBF1 activation than to MLXIPL depletion. FASN, on the other hand, is stronger affected by attenuated MLXIPL levels. In this context, Yu et al. recently reported that human fibroblasts, in which MLXIPL was knocked down by a lentiviral short hairpin RNA plasmid showed strongly decreased levels of *FASN* mRNA expression, but rather no effect on *ELOVL6* expression (60) suggesting that MLXIPL shows stronger transcriptional activation towards FASN than to ELOVL6. PKLR, which is uniquely induced by MLXIPL (61), is reversely regulated by p62 and is therefore convergent to the MLXIPL expression.

Despite the effect of p62 on FASN in HepG2 and in the murine mouse model, we could not detect any correlation between *p62* and *FASN* expression in human liver samples. Also published data showed no elevation of FASN in human NAFLD (62). Concordantly, Donnelly et al. reported that *de novo* lipogenesis only to a minor extent contributes to elevated hepatic lipids as found in human NAFLD (63).

In line with these findings, the literature describes that liver-specific knockout of FASN did not rescue the animals from the development of a fatty liver (64) and Jones et al. recently reported the development of a steatosis in TSC22D4 overexpressing mice despite decreased *FASN* mRNA levels (65). Most interestingly, also animals overexpressing ELOVL6 show increased liver triglycerides and at the same time reduced FASN expression (20). Therefore, fatty acid synthesis appears not to be the pivotal step in p62-mediated steatosis development.

One of the important inducers of peroxisomal and mitochondrial β-oxidation pathways in the liver is PPARA (10). PPARA agonists like fenofibrate reduce steatosis in mice with a hereditary fatty liver (66). PPARA is downregulated in p62 overexpressing HepG2, but not in the *p62* transgenics, suggesting that p62 does not facilitates lipolysis *via* PPARA. We suggest that p62 induces high fatty acid levels due to elevated malonyl-CoA levels. Indeed, the mitochondrial β-oxidation pathway as the central lipolytic pathway is negatively regulated by high malonyl-CoA levels (67). ELOVL6 can elevate malonyl-CoA since it reduces the levels of its substrate palmitoyl-CoA in *p62* transgenic mice (Fig 4C). Since fatty acids in *p62* transgenic mice are mostly bound in triglycerides (26) and only free fatty acids are converted to acyl-CoAs (68), triglyceride-bound palmitic acid can not be converted into palmitoyl-CoA. Whereas high levels of palmitoyl-CoA inhibit the activity of the enzyme responsible for malonyl-CoA synthesis (69), i.e. acetyl-CoA carboxylase alpha (ACACA) (67), low levels promote the generation of malonyl-CoA *via* ACACA. Consequently, the mitochondrial β-oxidation is reduced due to malonyl-CoA-mediated attenuation in CPT1A activity (70) (Fig 4C). Pharmacological inhibition of CPT1A is associated with the development of steatosis and steatohepatitis (71, 72). Furthermore, a tamoxifen-induced steatosis in rats, which strongly inhibited *FASN* expression, was characterised by an accumulation of malonyl-CoA and therefore decreased CPT1A activity (73) (Fig 4C).

In summary, our data provide evidence of p62 as an inducer of ELOVL6, the pathophysiological promoter of NASH. ELOVL6 overexpression results in a subsequent production of a deleterious fatty acid profile, which finally induces hepatic steatosis (Fig 4C). This study underlines the detrimental role of p62 in liver disease.

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References

1. de Alwis, N. M., and C. P. Day. 2008. Non-alcoholic fatty liver disease: the mist gradually clears. *J Hepatol* **48 Suppl 1**: S104-112.
2. Erickson, S. K. 2009. Nonalcoholic fatty liver disease. *J Lipid Res* **50 Suppl**: S412-416.
3. Adams, L. A., O. R. Waters, M. W. Knuiman, R. R. Elliott, and J. K. Olynyk. 2009. NAFLD as a risk factor for the development of diabetes and the metabolic syndrome: an eleven-year follow-up study. *Am J Gastroenterol* **104**: 861-867.
4. Adams, L. A., P. Angulo, and K. D. Lindor. 2005. Nonalcoholic fatty liver disease. *Cmaj* **172**: 899-905.
5. Angulo, P. 2002. Nonalcoholic fatty liver disease. *N Engl J Med* **346**: 1221-1231.
6. Day, C. P. 2006. From fat to inflammation. *Gastroenterology* **130**: 207-210.
7. Malhi, H., and G. J. Gores. 2008. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. *Semin Liver Dis* **28**: 360-369.
8. Musso, G., R. Gambino, and M. Cassader. 2009. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Prog Lipid Res* **48**: 1-26.
9. Iizuka, K., and Y. Horikawa. 2008. ChREBP: a glucose-activated transcription factor involved in the development of metabolic syndrome. *Endocr J* **55**: 617-624.
10. Lefebvre, P., G. Chinetti, J. C. Fruchart, and B. Staels. 2006. Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis. *J Clin Invest* **116**: 571-580.
11. Wakil, S. J., and L. A. Abu-Elheiga. 2009. Fatty acid metabolism: target for metabolic syndrome. *J Lipid Res* **50 Suppl**: S138-143.
12. McGarry, J. D., S. E. Mills, C. S. Long, and D. W. Foster. 1983. Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat. *Biochem J* **214**: 21-28.
13. Puri, P., M. M. Wiest, O. Cheung, F. Mirshahi, C. Sargeant, H. K. Min, M. J. Contos, R. K. Sterling, M. Fuchs, H. Zhou, S. M. Watkins, and A. J. Sanyal. 2009. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* **50**: 1827-1838.
14. Puri, P., R. A. Baillie, M. M. Wiest, F. Mirshahi, J. Choudhury, O. Cheung, C. Sargeant, M. J. Contos, and A. J. Sanyal. 2007. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* **46**: 1081-1090.
15. Miyoshi, H., K. Moriya, T. Tsutsumi, S. Shinzawa, H. Fujie, Y. Shintani, H. Fujinaga, K. Goto, T. Todoroki, T. Suzuki, T. Miyamura, Y. Matsuura, H. Yotsuyanagi, and K. Koike. 2011. Pathogenesis of lipid metabolism disorder in hepatitis C: polyunsaturated fatty acids counteract lipid alterations induced by the core protein. *J Hepatol* **54**: 432-438.
16. Kim, K. H., H. J. Shin, K. Kim, H. M. Choi, S. H. Rhee, H. B. Moon, H. H. Kim, U. S. Yang, D. Y. Yu, and J. Cheong. 2007. Hepatitis B virus X protein induces hepatic steatosis via transcriptional activation of SREBP1 and PPARgamma. *Gastroenterology* **132**: 1955-1967.
17. Matsuzaka, T., H. Shimano, N. Yahagi, T. Kato, A. Atsumi, T. Yamamoto, N. Inoue, M. Ishikawa, S. Okada, N. Ishigaki, H. Iwasaki, Y. Iwasaki, T. Karasawa, S. Kumadaki, T. Matsui, M. Sekiya, K. Ohashi, A. H. Hasty, Y. Nakagawa, A. Takahashi, H. Suzuki, S. Yatoh, H. Sone, H. Toyoshima, J. Osuga, and N. Yamada. 2007. Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. *Nat Med* **13**: 1193-1202.
18. Kumadaki, S., T. Matsuzaka, T. Kato, N. Yahagi, T. Yamamoto, S. Okada, K. Kobayashi, A. Takahashi, S. Yatoh, H. Suzuki, N. Yamada, and H. Shimano. 2008. Mouse Elovl-6 promoter is an SREBP target. *Biochem Biophys Res Commun* **368**: 261-266.
19. Wang, C. Y., D. S. Stapleton, K. L. Schueler, M. E. Rabaglia, A. T. Oler, M. P. Keller, C. M. Kendziorski, K. W. Broman, B. S. Yandell, E. E. Schadt, and A. D. Attie. 2012. Tsc2, a positional

candidate gene underlying a quantitative trait locus for hepatic steatosis. *J Lipid Res* **53**: 1493-1501.
20. Matsuzaka, T., A. Atsumi, R. Matsumori, T. Nie, H. Shinozaki, N. Suzuki-Kemuriyama, M. Kuba, Y. Nakagawa, K. Ishii, M. Shimada, K. Kobayashi, S. Yatoh, A. Takahashi, K. Takekoshi, H. Sone, N. Yahagi, H. Suzuki, S. Murata, M. Nakamuta, N. Yamada, and H. Shimano. 2012. Elovl6 promotes nonalcoholic steatohepatitis. *Hepatology* **56**: 2199-2208.
21. Muir, K., A. Hazim, Y. He, M. Peyressatre, D. Y. Kim, X. Song, and L. Beretta. 2013. Proteomic and Lipidomic Signatures of Lipid Metabolism in NASH-Associated Hepatocellular Carcinoma. *Cancer Res* **73**: 4722-4731.
22. Kessler, S. M., Y. Simon, K. Gemperlein, K. Gianmoena, C. Cadenas, V. Zimmer, J. Pokorny, A. Barghash, V. Helms, N. van Rooijen, R. M. Bohle, F. Lammert, J. G. Hengstler, R. Mueller, J. Haybaeck, and A. K. Kiemer. 2014. Fatty acid elongation in non-alcoholic steatohepatitis and hepatocellular carcinoma. *Int J Mol Sci in Press*.
23. Kessler, S. M., S. Laggai, A. Barghash, V. Helms, and A. K. Kiemer. 2014. Lipid Metabolism Signatures in NASH-Associated HCC - Letter. *Cancer res in Press*.
24. Tybl, E., F. D. Shi, S. M. Kessler, S. Tierling, J. Walter, R. M. Bohle, S. Wieland, J. Zhang, E. M. Tan, and A. K. Kiemer. 2011. Overexpression of the IGF2-mRNA binding protein p62 in transgenic mice induces a steatotic phenotype. *J Hepatol* **54**: 994-1001.
25. Simon, Y., S. M. Kessler, R. M. Bohle, J. Haybaeck, and A. K. Kiemer. 2013. The insulin-like growth factor 2 (IGF2) mRNA-binding protein p62/IGF2BP2-2 as a promoter of NAFLD and HCC? *Gut*.
26. Laggai, S., Y. Simon, T. Ranssweiler, A. K. Kiemer, and S. M. Kessler. 2013. Rapid chromatographic method to decipher distinct alterations in lipid classes in NAFLD/NASH. *World J Hepatol* **5**: 558-567.
27. Kessler, S. M., J. Pokorny, V. Zimmer, S. Laggai, F. Lammert, R. M. Bohle, and A. K. Kiemer. 2013. IGF2 mRNA binding protein p62/IMP2-2 in hepatocellular carcinoma: antiapoptotic action is independent of IGF2/PI3K signaling. *Am J Physiol Gastrointest Liver Physiol* **304**: G328-G336.
28. Qian, H. L., X. X. Peng, S. H. Chen, H. M. Ye, and J. H. Qiu. 2005. p62 Expression in primary carcinomas of the digestive system. *World J Gastroenterol* **11**: 1788-1792.
29. Zhang, J., and E. K. Chan. 2002. Autoantibodies to IGF-II mRNA binding protein p62 and overexpression of p62 in human hepatocellular carcinoma. *Autoimmun Rev* **1**: 146-153.
30. Zhang, J. Y., E. K. Chan, X. X. Peng, and E. M. Tan. 1999. A novel cytoplasmic protein with RNA-binding motifs is an autoantigen in human hepatocellular carcinoma. *J Exp Med* **189**: 1101-1110.
31. Tovar, V., C. Alsinet, A. Villanueva, Y. Hoshida, D. Y. Chiang, M. Sole, S. Thung, S. Moyano, S. Toffanin, B. Minguez, L. Cabellos, J. Peix, M. Schwartz, V. Mazzaferro, J. Bruix, and J. M. Llovet. 2010. IGF activation in a molecular subclass of hepatocellular carcinoma and pre-clinical efficacy of IGF-1R blockage. *J Hepatol* **52**: 550-559.
32. Chiappini, F., A. Barrier, R. Saffroy, M. C. Domart, N. Dagues, D. Azoulay, M. Sebagh, B. Franc, S. Chevalier, B. Debuire, S. Dudoit, and A. Lemoine. 2006. Exploration of global gene expression in human liver steatosis by high-density oligonucleotide microarray. *Lab Invest* **86**: 154-165.
33. Christiansen, J., A. M. Kolte, T. Hansen, and F. C. Nielsen. 2009. IGF2 mRNA-binding protein 2: biological function and putative role in type 2 diabetes. *J Mol Endocrinol* **43**: 187-195.
34. Li, Z., J. A. Gilbert, Y. Zhang, M. Zhang, Q. Qiu, K. Ramanujan, T. Shavlakadze, J. K. Eash, A. Scaramozza, M. M. Goddeeris, D. G. Kirsch, K. P. Campbell, A. S. Brack, and D. J. Glass. 2012. An HMGA2-IGF2BP2 axis regulates myoblast proliferation and myogenesis. *Dev Cell* **23**: 1176-1188.

35. Lanthier, N., O. Molendi-Coste, Y. Horsmans, N. van Rooijen, P. D. Cani, and I. A. Leclercq. 2010. Kupffer cell activation is a causal factor for hepatic insulin resistance. *Am J Physiol Gastrointest Liver Physiol* **298**: G107-116.
36. Bode, H. B., M. W. Ring, G. Schwar, R. M. Kroppenstedt, D. Kaiser, and R. Muller. 2006. 3-Hydroxy-3-methylglutaryl-coenzyme A (CoA) synthase is involved in biosynthesis of isovaleryl-CoA in the myxobacterium Myxococcus xanthus during fruiting body formation. *J Bacteriol* **188**: 6524-6528.
37. Kiemer, A. K., R. H. Senaratne, J. Hoppstadter, B. Diesel, L. W. Riley, K. Tabeta, S. Bauer, B. Beutler, and B. L. Zuraw. 2009. Attenuated activation of macrophage TLR9 by DNA from virulent mycobacteria. *J Innate Immun* **1**: 29-45.
38. Azzout-Marniche, D., D. Becard, C. Guichard, M. Foretz, P. Ferre, and F. Foufelle. 2000. Insulin effects on sterol regulatory-element-binding protein-1c (SREBP-1c) transcriptional activity in rat hepatocytes. *Biochem J* **350 Pt 2**: 389-393.
39. Basirico, L., P. Morera, N. Lacetera, B. Ronchi, A. Nardone, and U. Bernabucci. 2010. Down-regulation of hepatic ApoB100 expression during hot season in transition dairy cows. *Livestock Science* **137**: 49.
40. Lu, M., R. M. Nakamura, E. D. Dent, J. Y. Zhang, F. C. Nielsen, J. Christiansen, E. K. Chan, and E. M. Tan. 2001. Aberrant expression of fetal RNA-binding protein p62 in liver cancer and liver cirrhosis. *Am J Pathol* **159**: 945-953.
41. Dubuquoy, C., C. Robichon, F. Lasnier, C. Langlois, I. Dugail, F. Foufelle, J. Girard, A. F. Burnol, C. Postic, and M. Moldes. 2011. Distinct regulation of adiponutrin/PNPLA3 gene expression by the transcription factors ChREBP and SREBP1c in mouse and human hepatocytes. *J Hepatol* **55**: 145-153.
42. Lee, H., M. T. Park, B. H. Choi, E. T. Oh, M. J. Song, J. Lee, C. Kim, B. U. Lim, and H. J. Park. 2011. Endoplasmic reticulum stress-induced JNK activation is a critical event leading to mitochondria-mediated cell death caused by beta-lapachone treatment. *PLoS One* **6**: e21533.
43. Vinciguerra, M., F. Carrozzino, M. Peyrou, S. Carlone, R. Montesano, R. Benelli, and M. Foti. 2009. Unsaturated fatty acids promote hepatoma proliferation and progression through downregulation of the tumor suppressor PTEN. *J Hepatol* **50**: 1132-1141.
44. Tripathy, S., and D. B. Jump. 2013. Elovl5 regulates the mTORC2-Akt-FOXO1 pathway by controlling hepatic cis-vaccenic acid synthesis in diet-induced obese mice. *J Lipid Res* **54**: 71-84.
45. Chao, W., and P. A. D'Amore. 2008. IGF2: epigenetic regulation and role in development and disease. *Cytokine Growth Factor Rev* **19**: 111-120.
46. Chen, G., G. Liang, J. Ou, J. L. Goldstein, and M. S. Brown. 2004. Central role for liver X receptor in insulin-mediated activation of Srebp-1c transcription and stimulation of fatty acid synthesis in liver. *Proc Natl Acad Sci U S A* **101**: 11245-11250.
47. Postic, C., and J. Girard. 2008. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest* **118**: 829-838.
48. Dentin, R., F. Benhamed, I. Hainault, V. Fauveau, F. Foufelle, J. R. Dyck, J. Girard, and C. Postic. 2006. Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice. *Diabetes* **55**: 2159-2170.
49. Ahmed, M. H., and C. D. Byrne. 2007. Modulation of sterol regulatory element binding proteins (SREBPs) as potential treatments for non-alcoholic fatty liver disease (NAFLD). *Drug Discov Today* **12**: 740-747.
50. Shimomura, I., Y. Bashmakov, and J. D. Horton. 1999. Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J Biol Chem* **274**: 30028-30032.

51. Ferre, P., and F. Foufelle. 2007. SREBP-1c transcription factor and lipid homeostasis: clinical perspective. *Horm Res* **68**: 72-82.
52. Kim, K. H., G. Y. Lee, J. I. Kim, M. Ham, J. Won Lee, and J. B. Kim. 2010. Inhibitory effect of LXR activation on cell proliferation and cell cycle progression through lipogenic activity. *J Lipid Res* **51**: 3425-3433.
53. Wang, M., S. Sun, T. Wu, L. Zhang, H. Song, W. Hao, P. Zheng, L. Xing, and G. Ji. 2013. Inhibition of LXRalpha/SREBP-1c-Mediated Hepatic Steatosis by Jiang-Zhi Granule. *Evid Based Complement Alternat Med* **2013**: 584634.
54. Yoshikawa, T., H. Shimano, M. Amemiya-Kudo, N. Yahagi, A. H. Hasty, T. Matsuzaka, H. Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, J. Osuga, K. Harada, T. Gotoda, S. Kimura, S. Ishibashi, and N. Yamada. 2001. Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter. *Mol Cell Biol* **21**: 2991-3000.
55. Shimomura, I., H. Shimano, J. D. Horton, J. L. Goldstein, and M. S. Brown. 1997. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J Clin Invest* **99**: 838-845.
56. DeBose-Boyd, R. A., J. Ou, J. L. Goldstein, and M. S. Brown. 2001. Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proc Natl Acad Sci U S A* **98**: 1477-1482.
57. Repa, J. J., G. Liang, J. Ou, Y. Bashmakov, J. M. Lobaccaro, I. Shimomura, B. Shan, M. S. Brown, J. L. Goldstein, and D. J. Mangelsdorf. 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* **14**: 2819-2830.
58. Smith, T. M., K. Gilliland, G. A. Clawson, and D. Thiboutot. 2008. IGF-1 induces SREBP-1 expression and lipogenesis in SEB-1 sebocytes via activation of the phosphoinositide 3-kinase/Akt pathway. *J Invest Dermatol* **128**: 1286-1293.
59. Iizuka, K., B. Miller, and K. Uyeda. 2006. Deficiency of carbohydrate-activated transcription factor ChREBP prevents obesity and improves plasma glucose control in leptin-deficient (ob/ob) mice. *Am J Physiol Endocrinol Metab* **291**: E358-364.
60. Yu, Y., T. G. Maguire, and J. C. Alwine. 2014. ChREBP, a glucose-responsive transcriptional factor, enhances glucose metabolism to support biosynthesis in human cytomegalovirus-infected cells. *Proc Natl Acad Sci U S A* **111**: 1951-1956.
61. Denechaud, P. D., P. Bossard, J. M. Lobaccaro, L. Millatt, B. Staels, J. Girard, and C. Postic. 2008. ChREBP, but not LXRs, is required for the induction of glucose-regulated genes in mouse liver. *J Clin Invest* **118**: 956-964.
62. Caballero, F., A. Fernandez, A. M. De Lacy, J. C. Fernandez-Checa, J. Caballeria, and C. Garcia-Ruiz. 2009. Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. *J Hepatol* **50**: 789-796.
63. Donnelly, K. L., C. I. Smith, S. J. Schwarzenberg, J. Jessurun, M. D. Boldt, and E. J. Parks. 2005. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* **115**: 1343-1351.
64. Chakravarthy, M. V., Z. Pan, Y. Zhu, K. Tordjman, J. G. Schneider, T. Coleman, J. Turk, and C. F. Semenkovich. 2005. "New" hepatic fat activates PPARalpha to maintain glucose, lipid, and cholesterol homeostasis. *Cell Metab* **1**: 309-322.
65. Jones, A., K. Friedrich, M. Rohm, M. Schafer, C. Algire, P. Kulozik, O. Seibert, K. Muller-Decker, T. Sijmonsma, D. Strzoda, C. Sticht, N. Gretz, G. M. Dallinga-Thie, B. Leuchs, M. Kogl, W. Stremmel, M. B. Diaz, and S. Herzig. 2013. TSC22D4 is a molecular output of hepatic wasting metabolism. *EMBO Mol Med*.
66. Harano, Y., K. Yasui, T. Toyama, T. Nakajima, H. Mitsuyoshi, M. Mimani, T. Hirasawa, Y. Itoh, and T. Okanoue. 2006. Fenofibrate, a peroxisome proliferator-activated receptor alpha

agonist, reduces hepatic steatosis and lipid peroxidation in fatty liver Shionogi mice with hereditary fatty liver. *Liver Int* **26**: 613-620.
67. Thupari, J. N., M. L. Pinn, and F. P. Kuhajda. 2001. Fatty acid synthase inhibition in human breast cancer cells leads to malonyl-CoA-induced inhibition of fatty acid oxidation and cytotoxicity. *Biochem Biophys Res Commun* **285**: 217-223.
68. Soupene, E., and F. A. Kuypers. 2008. Mammalian long-chain acyl-CoA synthetases. *Exp Biol Med (Maywood)* **233**: 507-521.
69. Mabrouk, G. M., I. M. Helmy, K. G. Thampy, and S. J. Wakil. 1990. Acute hormonal control of acetyl-CoA carboxylase. The roles of insulin, glucagon, and epinephrine. *J Biol Chem* **265**: 6330-6338.
70. McGarry, J. D., and N. F. Brown. 1997. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem* **244**: 1-14.
71. Anderson, N., and J. Borlak. 2008. Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. *Pharmacol Rev* **60**: 311-357.
72. Koteish, A., and A. M. Diehl. 2001. Animal models of steatosis. *Semin Liver Dis* **21**: 89-104.
73. Lelliott, C. J., M. Lopez, R. K. Curtis, N. Parker, M. Laudes, G. Yeo, M. Jimenez-Linan, J. Grosse, A. K. Saha, D. Wiggins, D. Hauton, M. D. Brand, S. O'Rahilly, J. L. Griffin, G. F. Gibbons, and A. Vidal-Puig. 2005. Transcript and metabolite analysis of the effects of tamoxifen in rat liver reveals inhibition of fatty acid synthesis in the presence of hepatic steatosis. *FASEB J* **19**: 1108-1119.

Figure legends

Figure 1. p62-induced steatosis, C18:C16 ratio, and ELOVL6 expression.

A. HE staining of wild-type (wt) and *p62* transgenic liver tissue (*p62* tg) (original magnification 100x).

B. F4/80 staining of wt and *p62* tg liver tissue (original magnification 100x).

C. F4/80 RT-qPCR of wild-type (wt) and *p62* transgenic (*p62* tg) livers (n=13, each).

D. Hyperinsulinemic euglycemic clamp study in wt (n=3) and *p62* tg (n=4) mice: glucose infusion rate (GIR), glucose turnover (TO), and hepatic glucose production (HGP). Data show mean ± SEM.

E. Hepatic C18:C16 fatty acid ratio: wt: n=10, *p62* tg: n=19.

F. C18:C16 fatty acid ratio and Western Blot of the transfection control (72 h) in HepG2 cells transfected with random siRNA (si co) or p62 siRNA (si p62) (n=3, duplicate)

G. Representative ELOVL6 Western blot of wt and *p62* tg mice (n=5, each).

H. ELOVL6 Western blot (n=3, duplicate, left) and RT-qPCR (n=2, triplicate, middle) of HepG2 transfected with si co or si p62 for ELOVL6, Right: RT-qPCR for *ELOVL6* and *p62* of 35 liver tissues normalized on *ACTB* mRNA levels.

Figure 2. Implications of p62 on lipogenic pathways.

RT-qPCR (n=3-6, triplicate, A) or Western blot (n=3, duplicate, B) of HepG2 transfected with either p62 siRNA (si p62) or p62 overexpression vector (p62) and their respective controls (si co, co-v).

A. *MLXIPL*, *NR1H3,* and *SREBF1c* mRNA.

B. Precursor (SREBF1 pre) and mature SREBF1 (SREBF1 ma).

C. SREBF1 pre and SREBF1 ma Western blot of wild-type (wt) and *p62* transgenic (*p62* tg) livers (n=6).

D. SREBF1 pre and SREBF1 ma Western blot on cytosolic and nuclear proteins of HepG2 treated with IGF2 (n=9, duplicate).

E. SREBF1 pre and SREBF1 ma and ELOVL6 Western blot on cytosolic and nuclear proteins of HepG2 (co) incubated with neutralizing IGF2 antibody (IGF2 AB, 48 h) (n=4, duplicate).

F. RT-qPCR for *ELOVL6* and *p62* of 35 liver tissues normalized on *ACTB* mRNA levels.

Figure 3. Effect of p62 on lipogenic genes.

A. *FASN* RT-qPCR of HepG2 transfected with either p62 siRNA (si p62, n=3, triplicate) or p62 overexpression vector (p62, n=5, triplicate) and their respective controls (si co, co-v) ~~(n=3, triplicate)~~.

B. FASN Western blot of HepG2 transfected with si p62 or p62 (n=3, duplicate).

C. Representative FASN Western blot of wild-type (wt) and *p62* transgenic (*p62* tg) livers (n=5, each).

D. *SCD* and *ACACA* RT-qPCR of HepG2 transfected with either p62 siRNA (n=3, triplicate) or p62 overexpression vector (n=5, triplicate) and their respective controls.

E. *PKLR* RT-qPCR of HepG2 transfected with either p62 siRNA (n=4, triplicate) or p62 overexpression vector (n=5, triplicate) and their respective controls.

Figure 4. Implications of p62 on lipolytic pathways and summary.

A. *PPARA* RT-qPCR of HepG2 transfected with either p62 siRNA (si p62, n=3, triplicate) or p62 overexpression vector (p62, n=5, triplicate) and their respective controls (si co, co-v).

B. *PPARA* RT-qPCR of wild-type (wt) and *p62* transgenic (*p62* tg) livers (n=5, each).

C. p62 overexpression decreases FASN expression by MLXIPL depletion and induces IGF2 expression. Reduced FASN levels lead to malonyl-CoA accumulation. IGF2 promotes the maturation of SREBF1, which increases ELOVL6 expression, leading to an increased C18 to C16 ratio. The reduced inhibition of palmitoyl-CoA on ACACA activity elevates malonyl-CoA levels, which inhibit the CPT1A-activity and therefore mitochondrial β-oxidation.

Table 1. p62-induced changes in murine hepatic fatty acids. Livers of wild-type (wt, n=7) and *p62* transgenic (*p62* tg, n=19, n=8 normal histology, n=11 microvesicular steatotic histology) mice were analyzed by GC-MS. p values indicate differences compared to values in livers of wt animals.

Fatty acid	wt	*p62* tg normal histology		*p62* tg microvesicular steatosis	
	[µg / mg dry tissue]	[µg / mg dry tissue]	p *vs.* wt	[µg / mg dry tissue]	p *vs.* wt
12:0	0.05±0.05	0.26±0.13	0.205	0.81±0.18	0.0016
14:0	0.32±0.11	0.65±0.29	0.344	2.08±0.38	0.0009
16:0	16.7±0.77	21.82±1.99	0.050	28.72±1.75	0.00002
16:1	0.04±0.03	0.10±0.06	0.441	0.69±0.12	0.0003
18:0	11.7±0.20	14.04±0.71	0.018	14.24±0.54	0.0008
18:1	5.54±0.47	9.98±1.84	0.061	19.34±1.76	0.000007
18:2	9.75±0.62	16.69±2.49	0.035	30.12±2.76	0.00002
20:1	n.d.	0.06±0.05	0.351	0.20±0.09	0.0455
20:2	0.34±0.07	0.43±0.14	0.611	1.11±0.17	0.0010
20:3	0.29±0.07	0.61±0.17	0.135	1.47±0.19	0.00006
20:4	10.9±0.26	10.17±0.86	0.461	13.47±0.65	0.0032
20:5	n.d.	n.d.		0.08±0.08	0.3409
22:4	0.11±0.07	0.16±0.11	0.698	0.88±0.21	0.0038
22:5	2.20±0.92	0.98±0.64	0.340	3.31±0.66	0.3781
22:6	1.55±0.52	1.00±0.30	0.413	0.83±0.22	0.2663
sum FA	59.5±2.57	76.94±8.03	0.072	117.3±9.76	0.000028

Table 2. p62-induced changes in HepG2 fatty acids. HepG2 cells treated with random siRNA (si co) and p62 siRNA (si p62) were analyzed by GC-MS. p values indicate differences compared to si co treated HepG2 cells (n=3, duplicate).

Fatty acid	si co [µg / mg lyophilized cells]	si p62 [µg / mg lyophilized cells]	p
16:0	20.06±0.91	29.21±1.93	0.03
16:1	5.40±1.32	8.01±1.53	0.12
18:0	8.98±1.3	10.04±1.88	0.71
18:1	36.08±5.17	40.58±2.52	0.55
18:2	1.63±0.21	1.31±0.25	0.57