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Research paper

Age-dependent regulation of antioxidant genes by p38 α MAPK in the liverSalvador Pérez^a, Sergio Rius-Pérez^a, Ana M. Tormos^a, Isabela Finamor^a, Ángel R. Nebreda^{b,c}, Raquel Taléns-Visconti^d, Juan Sastre^{a,*}^a Department of Physiology, School of Pharmacy, University of Valencia, Burjassot, 46100 Spain^b Institute for Research in Biomedicine (IRB Barcelona), Barcelona Institute of Science and Technology, 08028 Barcelona, Spain^c ICREA, Pg. Lluís Companys 23, 08010, Barcelona, Spain^d Department of Pharmacy and Pharmaceutical Technology and Parasitology, School of Pharmacy, University of Valencia, Burjassot, 46100 Spain

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ABSTRACT

p38 α is a redox sensitive MAPK activated by pro-inflammatory cytokines and environmental, genotoxic and endoplasmic reticulum stresses. The aim of this work was to assess whether p38 α controls the antioxidant defense in the liver, and if so, to elucidate the mechanism(s) involved and the age-related changes. For this purpose, we used liver-specific p38 α -deficient mice at two different ages: young-mice (4 months-old) and old-mice (24 months-old). The liver of young p38 α knock-out mice exhibited a decrease in GSH levels and an increase in GSSG/GSH ratio and malondialdehyde levels. However, old mice deficient in p38 α had higher hepatic GSH levels and lower GSSG/GSH ratio than young p38 α knock-out mice. Liver-specific p38 α deficiency triggered a dramatic down-regulation of the mRNAs of the key antioxidant enzymes glutamate cysteine ligase, superoxide dismutase 1, superoxide dismutase 2, and catalase in young mice, which seems mediated by the lack of p65 recruitment to their promoters. Nrf-2 nuclear levels did not change significantly in the liver of young mice upon p38 α deficiency, but nuclear levels of phospho-p65 and PGC-1 α decreased in these mice. p38 α -dependent activation of NF- κ B seems to occur through classical I κ B Kinase and via ribosomal S6 kinase1 and AKT in young mice. However, unexpectedly the long-term deficiency in p38 α triggers a compensatory up-regulation of antioxidant enzymes via NF- κ B activation and recruitment of p65 to their promoters. In conclusion, p38 α MAPK maintains the expression of antioxidant genes in liver of young animals via NF- κ B under basal conditions, whereas its long-term deficiency triggers compensatory up-regulation of antioxidant enzymes through NF- κ B.

1. Introduction

p38 α is a redox sensitive mitogen activated protein kinase (MAPK) activated by environmental, genotoxic and endoplasmic reticulum stresses, and by pro-inflammatory cytokines, in addition to oxidative stress [1,2]. It is one of the major MAPKs critically involved in the regulation of cell proliferation, differentiation, migration, apoptosis, and senescence as well as in inflammation [2,3]. The family of p38 MAPKs comprises four isoforms (p38 α , p38 β , p38 γ , and p38 δ), and p38 α is ubiquitously expressed in mammalian cells [2,4–6]. It is activated by the upstream MAPKs MKK3 and MKK6, and under certain conditions by MKK4 [7]. It may be also activated by

autophosphorylation [8] or by oxidative-induced inactivation of certain protein phosphatases [9]. Substrates of p38 α comprise transcription factors, such as p53 and activating transcription factor 2 (ATF2), and protein kinases, such as MAPK-activated protein kinase 2 (MK2) [10,11]. There is evidence that p38 α may activate nuclear factor kappa B (NF- κ B), being involved in the up-regulation of pro-inflammatory cytokines, such as TNF- α and IL-1 β [12].

Hydrogen peroxide activates p38 α in endothelial cells and alveolar epithelial cells [13–15]. Under pathological conditions, an excess of reactive oxygen species (ROS) may activate p38 α leading either to cell dysfunction or cell death by apoptosis [7,16–19]. In contrast oxidative stress triggers dephosphorylation of p38 α in the metabolic syndrome by

Abbreviations: Akt, Protein kinase B; AP-1, Activator protein-1; ASK1, Apoptosis signal-regulating kinase 1; ATF2, activating transcription factor 2; ChIP, Chromatin immunoprecipitation; DEN, Diethyl nitrosamine; EGFR, Epidermal growth factor receptor; G6PDH, Glucose-6-phosphate dehydrogenase; GCLC, Glutamate cysteine ligase catalytic subunit; GSH, Reduced glutathione; GSSG, Oxidized glutathione; Gstm1, Glutathione S-transferase mu 1; HPLC, High-performance liquid chromatography; Hsp, Heat shock protein; IKK, I κ B Kinase; IL, Interleukin; JNK, c-Jun N-terminal kinase; LPS, Lipopolysaccharide; MAPK, mitogen activated protein kinase; MEF, Mouse embryonic fibroblasts; MK2, MAP-activated protein kinase 2; MKK, MAPK kinase; MKP-1, MAPK phosphatase-1; NEM, N-ethyl maleimide; NF- κ B, Nuclear factor kappa B; Nrf2, Nuclear factor erythroid 2-related factor-2; PGC-1 α , Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS, Reactive oxygen species; RSK1, Ribosomal S6 kinase1; SOD1, Cu/Zn-superoxide dismutase; SOD2, Mn-superoxide dismutase; TBP, TATA-binding protein; TNF- α , Tumor necrosis factor-alpha; Trx, Thioredoxin

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JNK-induced upregulation of phosphatase MKP-1, which exhibits a great affinity for p38 α as substrate [20].

The major mechanism responsible for the activation of p38 α MAPK by oxidative stress is through the MAP3K5 apoptosis signal-regulating kinase 1 (ASK1) [21], which dissociates from its inhibitory binding protein thioredoxin (Trx) upon thiol oxidation [21,22]. Moreover, mitochondrial ROS may activate p38 MAPK by inducing dissociation of the complex Trx-ASK1 [23]. Aging is associated with chronic oxidative stress and over-activation of p38 α [24,25]. Indeed, mitochondrial chronic oxidative stress is considered a hallmark of cellular aging [24,26–28]. The ASK1/Trx-ASK1 ratio increases with age in mouse liver, which would explain the enhanced age-related p38 MAPK activity in the liver [23,29]. The intracellular redox state is essential for the control of cell fate and seems to be tightly regulated during aging. It has been reported that p38 α inhibition down-regulates the expression of two anti-oxidant enzymes, glutathione peroxidase and thioredoxin, in breast and colon cancer cells *in vitro* [30], but the regulation of the antioxidant defense system by p38 α under physiological conditions has been scarcely studied.

p38 α mediates the increase in peroxiredoxin I activity induced by lipopolysaccharide (LPS) in microglial cells [31]. On the other hand, liver-specific p38 α deficiency leads to decreased phosphorylation of Hsp27 (Hsp25 in the murine model) in old mice triggering severe impairment of the actin cytoskeleton [32], and to decreased Hsp25 expression in diethyl nitrosamine (DEN)-treated mice, which was responsible for glutathione depletion and accumulation of ROS in this experimental model [33]. Although p38 α lowers ROS accumulation during liver injury, the role of p38 α in the regulation of the antioxidant defense in absence of liver injury has not been investigated yet. Hence, our aim was to assess whether p38 α controls the antioxidant defense in the liver; and if so to elucidate the mechanism(s) involved and the age-related changes.

2. Materials and methods

2.1. Animals

Liver-specific p38 α -deficient mice were generated by combining p38 α floxed alleles [34] and the Afp-Cre transgene as described [35]. Animals were distributed in wild-type and knock-out mice at two different ages: young (4 months-old) and old (24 months-old). Four to six mice were used for each experimental group.

All mice received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication 86–23 revised 1985). The study was approved by the Ethics Committee of Animal Experimentation and Welfare of the University of Valencia (Valencia, Spain). Mice were euthanized under anesthesia with isoflurane 3–5% and once they were unconscious they were exsanguinated. Death was confirmed by cervical dislocation.

2.2. Determination of GSH and GSSG

Frozen liver samples were homogenized in phosphate saline buffer (PBS) with 10 mM N-ethyl maleimide (NEM). Perchloric acid (PCA) was then added to obtain a concentration of 4% and centrifuged at 15,000 g for 15 min at 4 °C. The concentrations of GSH and GSSG were determined in the supernatants by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). The chromatographic system consisted of a Micromass QuattroTM triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Zspray electrospray ionization source operating in the positive ion mode with a LC-10A Shimadzu (Shimadzu, Kyoto, Japan) coupled to the MassLynx software 4.1 for data acquisition and processing. Samples were analyzed by reverse-phase HPLC with a C18 Mediterranean SEA column (Teknokroma, Barcelona, Spain). The mobile phase consisted of the following gradient system (min/%A/%B) (A,

Table 1
Transitions and retention times for analytes determined by LC-MS/MS.

Analyte	Cone (V)	Collision (eV)	Transition (m/z)	Retention time (min)
GS-NEM	30	15	433 > 304	4.32
GSSG	30	25	613 > 355	1.46

0.5% formic acid; B, isopropanol/acetonitrile 50/50; 0.5% formic acid): 5/100/0, 10/0/100, 15/0/100, 15.10/100/0, and 60/100/0. The flow rate was set at 0.2 ml/min. Positive ion electrospray tandem mass spectra were recorded using the following conditions: capillary voltage 3.5 kV, source temperature 120 °C, nebulization and cone gases were set at 500 and 30 L/h, respectively. Argon at 1.5610–3 mbar was used as the collision gas for collision-induced dissociation. An assay based on LC-MS/MS with multiple reaction monitoring was developed using the transitions m/z, cone energy (V), collision energy (eV) and retention time (min) for each compound that represents favorable fragmentation pathways for these protonated molecules (Table 1). Calibration curves were obtained using twelve-point (0.01–100 mmol/l) standards (purchased from Sigma-Aldrich, St Louis, USA) for each compound. The concentrations of metabolites were expressed as nmol/mg of protein.

2.3. Determination of malondialdehyde

Lipid peroxidation was assessed by the measurement of malondialdehyde levels in liver tissue according to Wong et al. [36] by HPLC. This method is based in the reaction of malondialdehyde with thiobarbituric acid (TBA) to yield the adduct MDA-TBA, which is determined specifically by HPLC.

Liver tissue was homogenized in phosphate buffer. Derivatization was performed mixing homogenized sample with 2 M sodium acetonitrile buffer pH 3.5 with TBA 0.2%. The mixture was heated in a boiling-water bath for 60 min at 95 °C. Then, 50 mM potassium phosphate pH 6.8 was added. The samples were centrifuged at 13000 rpm for 5 min at 4 °C and the supernatant was mixed 1:1 with 50 mM potassium phosphate pH 3.5.

Kromasil® 100–5C8, 15 × 0.46 cm column (Teknokroma, Barcelona, España) and 50 mM potassium phosphate pH 6.8 with acetonitrile (83/17) were used as stationary and mobile phase, respectively. The flow rate was 1 ml/minute and 532 nm was fixed for the peak detection for 4.5 min.

2.4. Western-blotting

Liver tissues were frozen at – 80 °C until homogenization in Hepes lysis buffer (100 mg/ml) on ice. The Hepes lysis buffer contained 75 mM NaCl, 750 μ M magnesium chloride, 25 mM Hepes (pH 7.4), 500 μ M EGTA, 5% glycerol, 0.5% Igepal, 1 mM dithiothreitol, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, and 1 mM sodium orthovanadate. A protease inhibitor cocktail (Sigma) containing AEBSF, aprotinin, bestatin, leupeptin, pepstatin A, and E-64 was added just before its use at a concentration of 5 μ l/ml. The homogenates were sonicated with a Branson Sonicator SLPe for 30 sec (2 sec each pulse) at 30% of amplitude and centrifuged at 15,000 g for 15 min at 4 °C. In case of nuclei isolation, a slight modification of the nuclei isolation method described by [32] was used. Chemiluminescence was detected with a charge-coupled device camera (Biorad ChemiDoc XRS + Molecular Imager and LAS-3000, Fujifilm) using the Luminata Classico Western HRP Substrate (Millipore, Billerica, USA).

The following antibodies were used: antibody against Nrf2 (1/1000) (Abcam, Cambridge, UK), antibody against PGC-1 α (Cell Signaling Technology, Danvers, USA), antibody against AP-1 (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against p-p65 Ser536 (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against

p65 (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against p-p38 α Thr180/Tyr182 (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against p38 α (1/1000) (Santa Cruz Biotechnology, Santa Cruz, CA), antibody against p-IKK α / β Ser173/180 (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against IKK α (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against IKK β (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against p-RSK1 Ser3880 (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against RSK1 (1/500) (Cell Signaling Technology, Danvers, USA), antibody against p-Akt Ser473(1/1000) (Cell Signaling Technology, Danvers, USA), antibody against Akt (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against Hsp27 (1/1000) (Santa Cruz Biotechnology, Santa Cruz, CA), antibody against phospho-Hsp27 (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against p53 (1/500) (Cell Signaling Technology, Danvers, USA), antibody against TBP (1/1000) (Abcam, Cambridge, UK) and antibody against α -tubulin (1/1000) (Sigma-Aldrich, St. Louis, MO).

2.5. RT-PCR

A small piece (approximately 30 mg) of liver was excised and immediately immersed in RNA-later solution (Ambion, Foster City, CA, USA) to stabilize the RNA. Total RNA was isolated using Trizol (Sigma-Aldrich, St Louis, USA). The cDNA for amplification in the PCR assay was constructed by reversion transcription reaction using Revertaid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA). Real-time PCR was performed using an iQTM5 multi-color real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The threshold cycle (CT) was determined and the relative gene expression was expressed as follows: fold change = $2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT_{\text{target}} - CT_{\text{housekeeping}}$, and $\Delta(\Delta CT) = \Delta CT_{\text{treated}} - \Delta CT_{\text{control}}$. The specific primers used are shown in Table 2 with the exception of TaqMan probes. TaqMan probes (Thermo Fisher Scientific, Massachusetts, USA) of *Cxcl1* (Mm04207460_m1), *Il-1 β* (Mm00434228_m1), *Tnf- α* (Mm00443260_g1) and *Rplp0* (Mm00725448_s1) (housekeeping) were employed for mRNA cytokine determination.

2.6. ChIP

Chromatin immunoprecipitation (ChIP). EZ-Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit (Millipore, California, USA) was used to DNA immunoprecipitation from frozen liver tissue as described by the manufacturer. Briefly, 37% formaldehyde was used to crosslink 100 mg of liver tissue for 10 min. Chromatin was isolated and sonicated for 35 min. Magnetic beads provided by the kit and antibodies against NF-kappa-B p65 subunit and H3K4me3 (Millipore, California, USA)

Table 2
Oligonucleotides used for RT-PCR.

Gene	Direct/Reverse oligonucleotide
<i>Catalase</i>	5'- GGAGCAGGTGCTTTTGGATA - 3' 5'-GAGGGTCACGAACGTGTGCA - 3'
<i>Sod1</i>	5'- TTTTGGCGCGTCTTTTC - 3' 5'-CCATACTGATGGACGTGGAA - 3'
<i>Sod2</i>	5'- GGCCAAGGGAGATGTTACAA - 3' 5'- GAACCTTGGACTCCCACAGA - 3'
<i>Gclc</i>	5'- CCATCACTTCATTCCCAGAG - 3' 5'- GATGCCGGATGTTCTTGTT - 3'
<i>Gankyrin</i>	5'- ACTAGAAGTATCAGGACAGCAGA - 3' 5'- AGCGGAGGCAGCAATATG - 3'
<i>Il-1α</i>	5'- TGACCTGCAACAGGAAGTAAAA - 3' 5'- TTTCTGGCAACTCCTTCAGC - 3'
<i>G6pdh</i>	5'- ATGGGTGCATCGGGTGAC - 3' 5'- GAAGGGCTCACTCTGCTTTC - 3'
<i>Tbp</i>	5'- CAGCCTTCCACCTTATGCTC - 3' 5'- CCGTAAGGCATCATTGGACT - 3'

Table 3
Oligonucleotides used for ChIP.

Gene	Direct/Reverse oligonucleotide
<i>Catalase</i>	5'- TGCACCCTGTATTATGTGAT - 3' 5'- AAAGTGGCCTGGATTGACTG - 3'
<i>Sod1</i>	5'- TAACCCAGCAACTGAGAGG - 3' 5'- GGGACTCCCAGATAGCATT - 3'
<i>Sod2</i>	5'- GAAGGTCACTCCGGGATAA - 3' 5'- CTCCTGCTTCTGGAAACCC - 3'
<i>Gclc</i>	5'- AACTCCAGCTGTGCCAAGTT - 3' 5'- GGGTGATGAATGACCTGTT - 3'
<i>Cxcl1</i>	5'- ACTTTGGGGCAAAAAGCAA - 3' 5'- GCCCTTTTATGCTCGAAAC - 3'
<i>Tnf-α</i>	5'- CTCCCAGAGACATGGTGGAT - 3' 5'- CACCCTCCACTCTAAACA - 3'

were used for immunoprecipitation. Immunoprecipitated DNA was analyzed using real-time PCR. Primers were designed according to the consensus binding sequence for NF-kappa-B GGGACTTTCC [37] to evaluate the recruitment of p65 to the promoter region of *Gclc*, *Sod1*, *Sod2*, *Cxcl1*, *Tnf- α* and catalase genes (Table 3). Immunoprecipitation without antibody was performed as a negative control.

2.7. Statistical analysis

Results are expressed as mean \pm standard deviation (S.D.). Statistical analysis was performed in two steps. One-way analysis of variance (ANOVA) was performed first. When the overall comparison of groups was significant, differences between individual groups were investigated by the Scheffé test. Statistical differences are indicated in the figure legends.

3. Results

3.1. p38 α -deficiency triggers glutathione depletion and lipid peroxidation in the liver of young mice but not in old mice

The liver of young p38 α knockout mice exhibited oxidative stress and lipid peroxidation when compared with young wild-type mice as evidenced by the decrease in GSH levels together with the increase in GSSG/GSH ratio and malondialdehyde levels (Fig. 1). Old wild-type mice exhibited diminished GSH levels and a remarkable increase in malondialdehyde levels in the liver when compared with young wild-type mice. However, unexpectedly old mice deficient in p38 α had higher hepatic GSH levels and lower GSSG/GSH ratio than young p38 α knock-out mice, and also lower hepatic malondialdehyde levels than old wild-type (Fig. 1).

3.2. p38 α -deficiency causes a dramatic down-regulation of antioxidant enzymes in the liver of young mice but their up-regulation in old mice

In order to explain the changes in glutathione redox status observed upon p38 α deficiency and aging, the expression levels of the mRNAs encoding the catalytic subunit of glutamate cysteine ligase (*Gclc*) as well as the antioxidant enzymes superoxide dismutase 1 (*Sod1*), *Sod2*, and catalase were measured in the liver of young and old wild-type and liver specific p38 α knockout mice. A dramatic decrease in the mRNA expression levels of *Gclc*, *Sod1*, *Sod2*, and catalase was found in the liver of young p38 α deficient mice when compared with young wild-type animals (Fig. 2). However, in old wild-type mice the expression of the four enzymes markedly diminished when compared with the young wild-type group (Fig. 2). Strikingly, old mice deficient in p38 α exhibited up-regulation of *Gclc*, *Sod1*, *Sod2*, and catalase in comparison with old wild-type mice (Fig. 2).

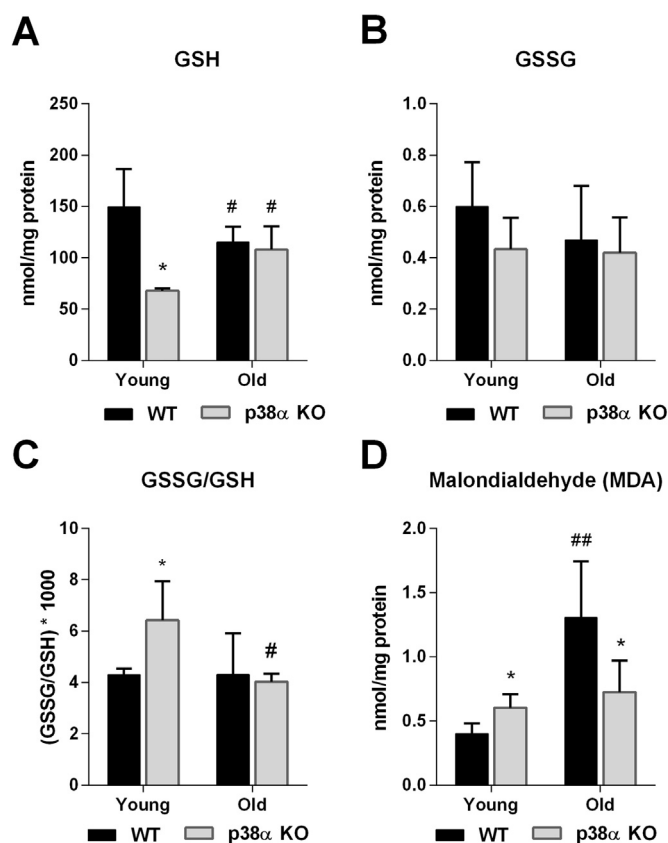


Fig. 1. Levels of GSH (A) and GSSG (B), GSSG/GSH ratio (C) and malondialdehyde levels (D) in the liver of young WT and p38 α KO mice and in old WT and p38 α KO mice. The number of samples per group was 4–6. Statistical significance is indicated as * $p < 0.05$ WT vs. KO; # $p < 0.05$ and ## $p < 0.01$ old vs. young.

3.3. p38 α -deficiency reduces nuclear translocation of phospho-p65 in the liver of young mice but induces its nuclear translocation in old mice

To assess whether Nrf-2, PGC-1 α , and NF- κ B were involved in the down-regulation of antioxidant enzymes observed upon p38 α deficiency in young mice as well as in the age-related up-regulation, nuclear levels of Nrf-2, PGC-1 α , and phospho-p65 were measured as an index of their nuclear translocation and activation. Nrf-2 nuclear levels did not change significantly in the liver of young mice upon p38 α deficiency, but nuclear levels of PGC-1 α and phospho-p65 decreased in the knockout mice (Fig. 3A and S1A). Nrf-2 levels clearly diminished upon p38 α deficiency in the liver of old mice (Fig. 3B and S1B). As in young animals, nuclear PGC-1 α significantly diminished in the liver of old mice upon p38 α deficiency (Fig. 3B and S1B). However, it is noteworthy that nuclear levels of phospho-p65 increased in the liver of old knock-out mice when compared with old wild-type mice (Fig. 3B and S1B).

Moreover, immunohistochemistry analysis confirmed that nuclear translocation of p65 was higher in the liver of young wild-type mice as well as in old p38 α knockout mice, showing these latter animals remarkably the highest levels of p65 in the nuclei of hepatocytes (Fig. 3C).

As p38 α deficiency has been previously related with down-regulation of Hsp25 [33] that could be responsible for glutathione depletion, we also measured the protein levels of HSP25 and the mRNA levels of its target gene *glucose 6-phosphate dehydrogenase*, but no significant change was found in any of these parameters upon p38 α deficiency in the liver of young mice (see Supplementary Fig. S3).

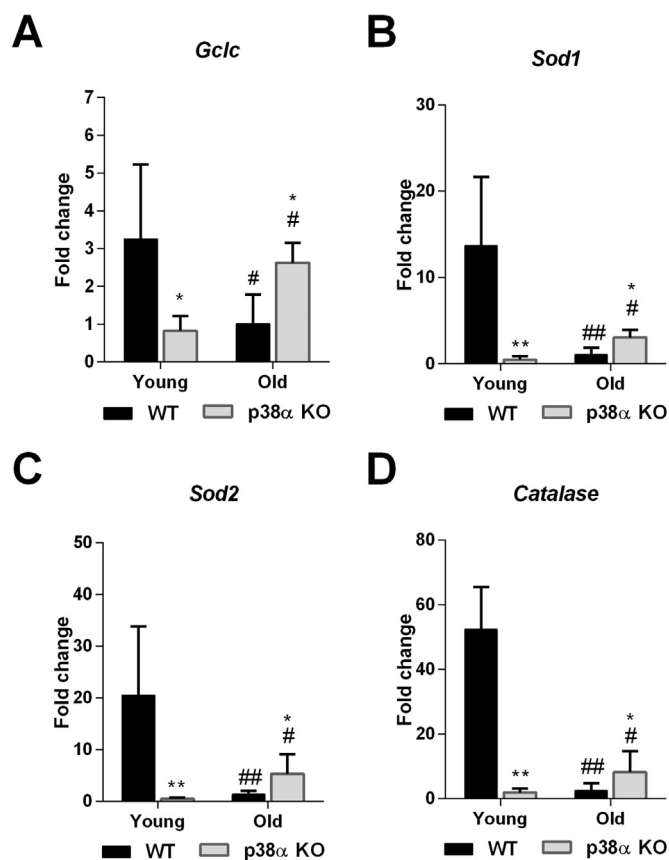


Fig. 2. mRNA relative expression of *Gclc* (A), *Sod1* (B), *Sod2* (C) and *catalase* (D) vs *Tbp* in the liver of young WT and KO mice and in the liver of old WT and p38 α KO mice. The number of samples per group was 4–6. Statistical significance is indicated as * $p < 0.05$ and ** $p < 0.01$ WT vs. KO; # $p < 0.05$ and ## $p < 0.01$ old vs. young.

3.4. p38 α -deficiency abrogates phosphorylation of IKK, RSK1 and AKT in the liver of young mice but promotes IKK and AKT phosphorylation in old mice

As phospho-p65 levels were higher in the nuclei of livers from young wild-type and old p38 α knockout mice, we investigated the status of the canonical pathway of NF- κ B activation via phospho-IKK. Indeed, phospho-IKK α / β levels were lower in livers of young p38 α knockout mice than in young wild-type mice, whereas in contrast phospho-IKK α / β levels were higher in livers of old p38 α knockout mice than in old wild-type mice (Fig. 4A; Supplementary Fig. S2A).

The alternative pathway responsible for NF- κ B activation via RSK1 and AKT [38–41] was also investigated. Interestingly, phospho-RSK1 and phospho-AKT levels were higher in the liver of young wild-type mice than in young p38 α knockout mice (Fig. 4B and C; see densitometries in Supplementary Figs. S2B and C). However, no increase in phospho-RSK1 levels was found in the liver of old p38 α knockout mice (Fig. 4B and S3B), whereas phospho-AKT levels were higher in the liver of old knockout mice compared with old wild-type mice (Fig. 4C; see densitometries in Supplementary Fig. S3C).

3.5. p38 α -deficiency abrogates recruitment of p65 and H3K4me3 to promoters of antioxidant genes in the liver of young mice but induces their recruitment in old mice

In order to confirm the implication of NF- κ B in the regulation of antioxidant genes upon p38 α deficiency and aging, ChIP assays were performed to assess p65 recruitment to the promoters of *Gclc*, *Sod1*, *Sod2*, and *catalase*. As it has been previously reported the regulation of the expression of antioxidant genes by PGC1- α in mouse liver, the ChIP

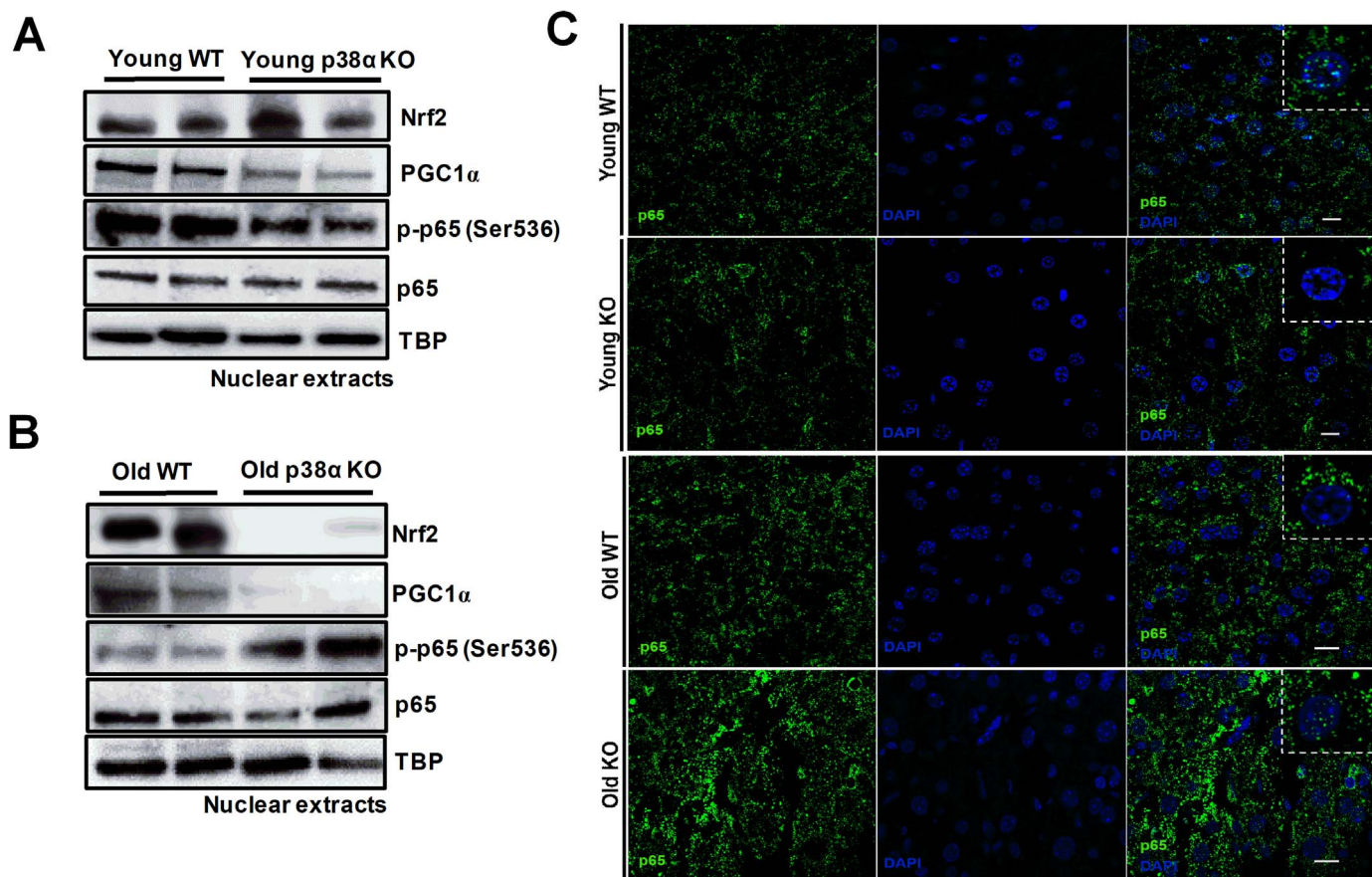


Fig. 3. Representative image of western blotting of nuclear levels of Nrf-2, PGC-1α, p-p65 (Ser536), p65, and TBP (TATA-binding protein) as loading control in the liver of young WT and p38α KO mice (A) and in the liver of old WT and p38α KO mice (B). The number of samples per group was 4. Representative images of immunohistochemistry of p65 in liver histological sections (C). Scale bar = 30 μm; selected squares with magnification 200%.

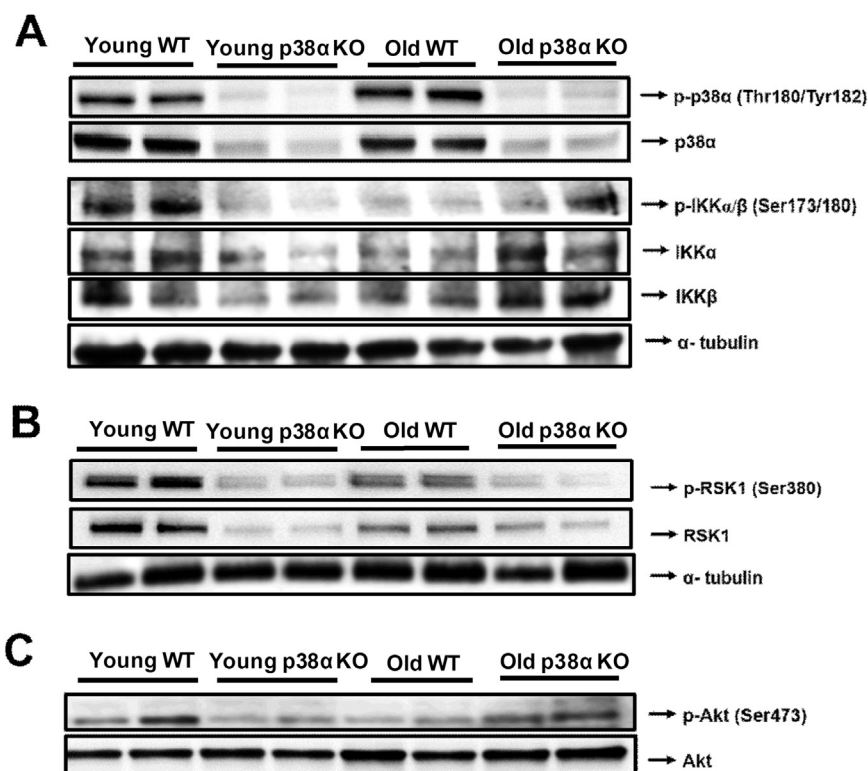


Fig. 4. Representative image of western blotting of p-p38α (Thr180/Tyr182), p38α, p-IKKα/β (Ser173/180), IKKα and IKKβ in the liver of young WT and p38α KO mice and in the liver of old WT and p38α KO mice. α-tubulin was used as a loading control (A). Representative image of western blotting of p-Rsk1 (Ser380) and Rsk-1 in the liver of young WT and p38α KO mice and in the liver of old WT and p38α KO mice. α-tubulin was used as a loading control (B). Representative image of western blotting of p-Akt (Ser473) and Akt in the liver of young WT and p38α KO mice and in the liver of old WT and p38α KO mice (C). The number of samples per group was 4.

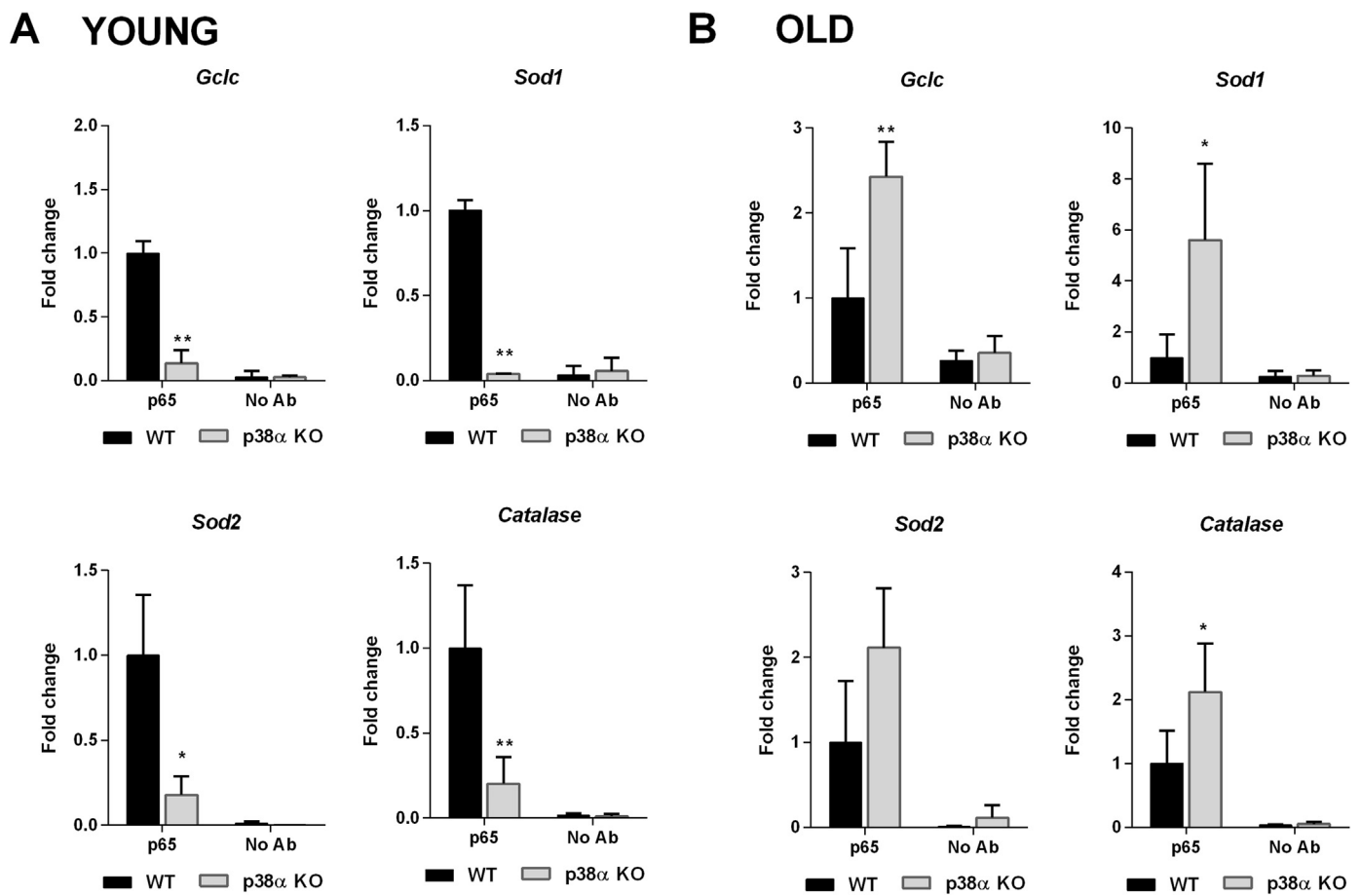


Fig. 5. Histograms showing the recruitment of p65 in the promoter regions of *Gclc*, *Sod1*, *Sod2* and *catalase* in the liver of young WT and p38α KO mice (A) and in the liver of old WT and p38α KO mice (B), measured by chromatin immunoprecipitation (ChIP) assay. The number of samples per group was 4. Statistical significance is indicated as * $p < 0.01$ WT vs. KO.

assay was not performed for PGC1-α [42,43]. p38α deficiency markedly reduced the recruitment of p65 to the promoters of the four genes in the liver of young mice (Fig. 5A). In contrast, long term p38α deficiency induced p65 recruitment to the promoters of *Gclc*, *Sod1*, and *catalase* when compared with old wild-type mice (Fig. 5B).

The recruitment of p65 to the promoters of antioxidant genes in livers of young wild-type mice and old p38α knockout mice was paralleled by detection of the euchromatin marker H3K4me3. Indeed, Fig. 6A shows that H3K4me3 was found in the promoters of *Gclc*, *Sod1*, *Sod2*, and *catalase* in the liver of young wild-type mice, but clearly diminished in young p38α knockout mice. However, similarly to p65, long-term p38α deficiency resulted in strong H3K4me3 signal in the promoters of *Gclc*, *Sod1*, *Sod2*, and *catalase* (Fig. 6B).

3.6. p38α-deficiency did not affect pro-inflammatory gene expression in the liver of young mice

As NF-κB generally controls the expression of pro-inflammatory genes, we assessed whether NF-κB activation associated with p38α deficiency resulted in pro-inflammatory gene up-regulation. Thus, the mRNA expression levels of *Tnf-α*, *Cxcl-1* (mouse analogue of interleukin 8) and interleukin 1β (*Il1-β*) were measured. Strikingly, the enhanced phospho-p65 levels found in the nuclei of liver cells from young wild-type mice was not associated with up-regulation of *Tnf-α*, *Cxcl-1* or *Il1-β* mRNAs (Fig. 7A and Supplementary Fig. S3). Old mice exhibited higher expression levels of *Tnf-α* mRNA than young mice in the liver, and this increase was higher in p38α knockout mice (Fig. 7). No changes with age or with p38α deficiency were found in the recruitment of p65 to the promoter of *Cxcl-1*, whereas in the case of *Tnf-α*, p65 recruitment

increased with age but without differences between wild-type and p38α knockout livers (Fig. 7B and C).

Additionally, protein levels of p53 and mRNA levels of *Il1-α* and *gankyrin* were measured as they have been previously related with p38α signaling and cell fate, particularly in oncogenesis. p53 protein levels as well as *Il1-α* and *gankyrin* mRNA levels increased with age, but no significant changes were found between old wild-type mice and old p38α-knock out mice (see Supplementary Fig. S3).

4. Discussion

p38α MAPK is essential for development in mammals. In fact, p38α deficiency results in mouse embryo lethality due to a placental defect [44], but when p38α is deleted only in the embryos, these complete development and die soon after birth likely due to pulmonary insufficiency caused by insufficient vascularization [6]. Nevertheless, mice with tissue-specific deficiency of p38α are viable and, liver-specific p38α knockout mice in particular exhibit neither liver dysfunction nor damage under basal conditions [33]. However, we previously showed that p38α is required for cytokinesis completion in hepatocytes and accordingly liver-specific p38α deficiency enhances hepatocyte binucleation [35]. Furthermore, long-term p38α deficiency leads to severe impairment of the actin cytoskeleton in hepatocytes of old mice, which seems to be mediated by lack of Hsp25 phosphorylation [32].

Here we show that liver-specific p38α deficiency triggers a dramatic down-regulation of the mRNAs encoding key antioxidant enzymes *Gclc*, *Sod1*, *Sod2*, and *catalase* in young mice, which is probably mediated by the lack of p65 recruitment to their promoters and by down-regulation of PGC1α, with no change in the nuclear translocation of Nrf-2. The

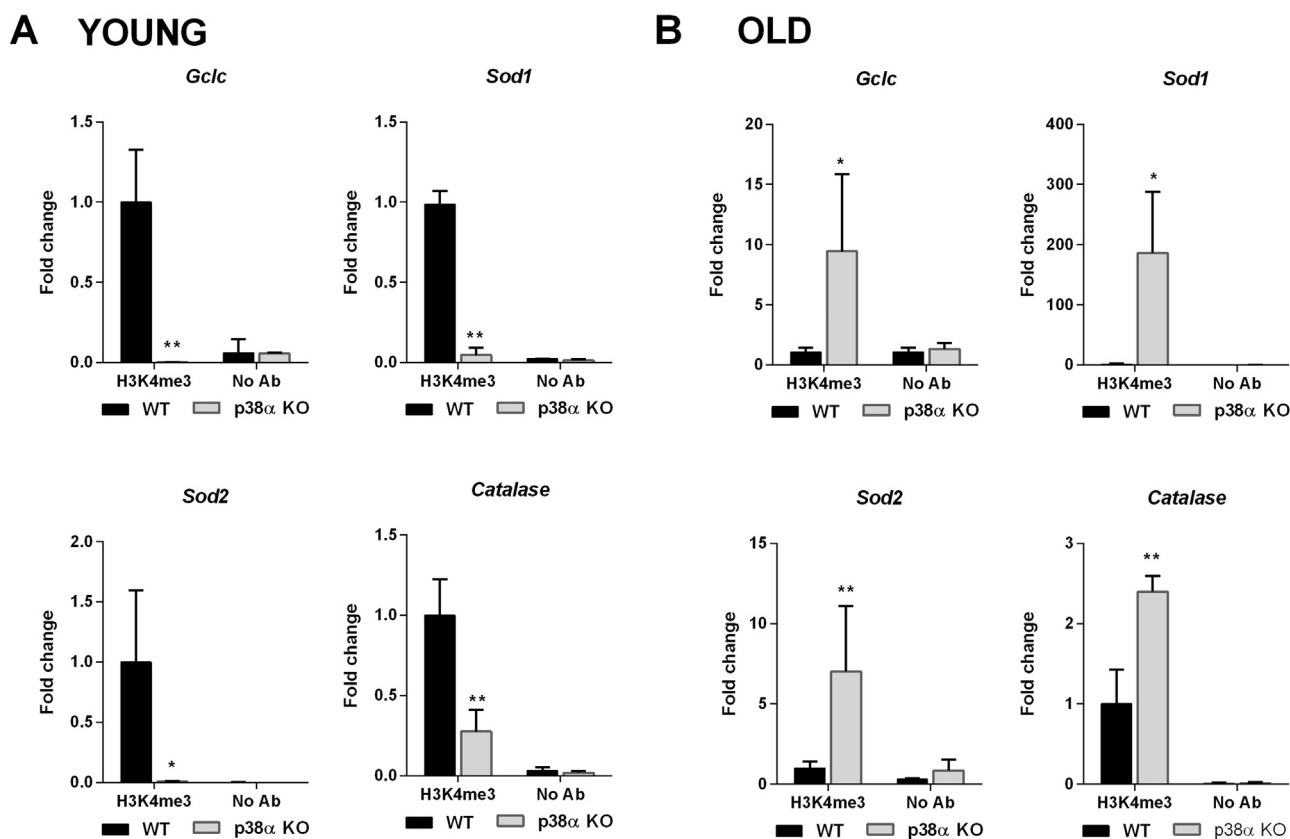


Fig. 6. Histograms showing chromatin immunoprecipitation (ChIP) assay of the histone modification H3K4me3 in the promoter regions of *Gclc*, *Sod1*, *Sod2* and *catalase* in the liver of young WT and p38 α KO (A) and in the liver of old WT and p38 α KO mice (B). The number of mice per group was 4. Statistical significance is indicated as ** $p < 0.01$ WT vs. KO.

marked decrease in the expression of these antioxidant enzymes leads to oxidative stress evidenced by GSH depletion together with increases in the GSSG/GSH ratio and malondialdehyde levels. The deficiency in key antioxidant enzymes and the associated oxidative stress might contribute to the diminished lifespan that we found in mice with biliary cirrhosis and liver-specific p38 α deficiency [35]. As a hypothesis for further studies, we propose that the p38 α /NF- κ B and p38 α /PGC-1 α pathways maintain the normal antioxidant defense in the liver under basal physiological conditions, whereas the adaptive up-regulation of the hepatic antioxidant response upon exposure to electrophilic stimuli or oxidative stress would rely mainly on the Nrf-2 pathway [45,46].

AKT is known to activate NF- κ B via IKK [38], and our data is consistent with the p38 α -dependent activation of NF- κ B occurring in the liver of young mice through both the classical IKK pathway and also via RSK1 and AKT. As total IKK α / β protein levels were also increased in the liver of young mice in comparison with p38 α knock out mice, those changes in the phosphorylation might be due to changes in the expression of IKK α / β subunits. We previously reported that p38 α is required for the development of hepatomegaly upon chronic cholestasis and its deficiency leads to shorter lifespan likely due to down-regulation of the AKT/mTor pathway [35]. The canonical pathway of RSK1 activation is through ERK1/2 [39], and it has been reported that RSK1-dependent NF- κ B activation may be induced by p53 or IL-1 β [40,41]. However, neither p53 protein levels nor IL-1 β mRNA were affected by p38 α deficiency in the liver of young animals (see Supplementary Fig. S3). Hence, RSK1 phosphorylation may be triggered by p38 α in the liver, as previously reported in dendritic cells [39,47], which seems to be a pathway independent of p53 and IL-1 β . Nevertheless, as RSK1 protein levels also increased in the liver of young mice in comparison with p38 α knock out mice, those changes in RSK1 phosphorylation might be due to changes in RSK1 expression. Unexpectedly, the activation of NF- κ B regulated by p38 α leads to up-regulation of antioxidant

genes, but not of pro-inflammatory cytokines. This differential up-regulation of NF- κ B target genes is intriguing and deserves further investigation.

Unexpectedly, the long-term deficiency of p38 α in liver cells triggers a compensatory up-regulation of antioxidant enzymes, which correlates with recruitment of p65 to their promoters, and results in increased GSH levels and lower GSSG/GSH ratio and malondialdehyde levels. The compensatory NF- κ B activation observed upon long-term p38 α deficiency probably involves both classical IKK and also AKT. Accordingly, phospho-IKK and phospho-AKT levels were higher in the liver of old p38 α knockout mice than in old wild-type mice. Strikingly, Nrf-2 nuclear levels were much higher in the liver of wild-type mice than in old p38 α knockout mice, but antioxidant gene levels were lower in wild-type mice. The explanation for this age-related loss of efficient transcription driven by Nrf-2 in the liver remains to be elucidated.

Interestingly, the combined deficiency in p38 α and IKK- β sensitized hepatocytes to cytokine-induced apoptosis, and accordingly LPS triggered liver failure in mice deficient in both p38 α and IKK- β , but not in mice lacking only p38 α [25]. Hence, NF- κ B compensates p38 α deficiency to protect hepatocytes against cytokine-induced cell death [25]. Our results suggest that NF- κ B-mediated up-regulation of antioxidant genes might contribute to the protection exerted by NF- κ B.

Liver-specific p38 α deficiency led to ROS accumulation, which is likely to be due to decreased expression of Hsp25 in DEN-treated mice [33]. Hsp25 is known to increase GSH levels and glutathione peroxidase activity in skeletal myoblasts, leading to lower accumulation of hydrogen peroxide [48]. Hsp25 may also up-regulate glutathione reductase and glucose 6-phosphate dehydrogenase favoring the increase in GSH levels [49]. However, we did not find any significant change upon p38 α deficiency in the protein levels of Hsp25, nor in the mRNA expression of its target gene *glucose 6-phosphate dehydrogenase* (see Supplementary Fig. S3). Consequently, Hsp25 does not seem to be

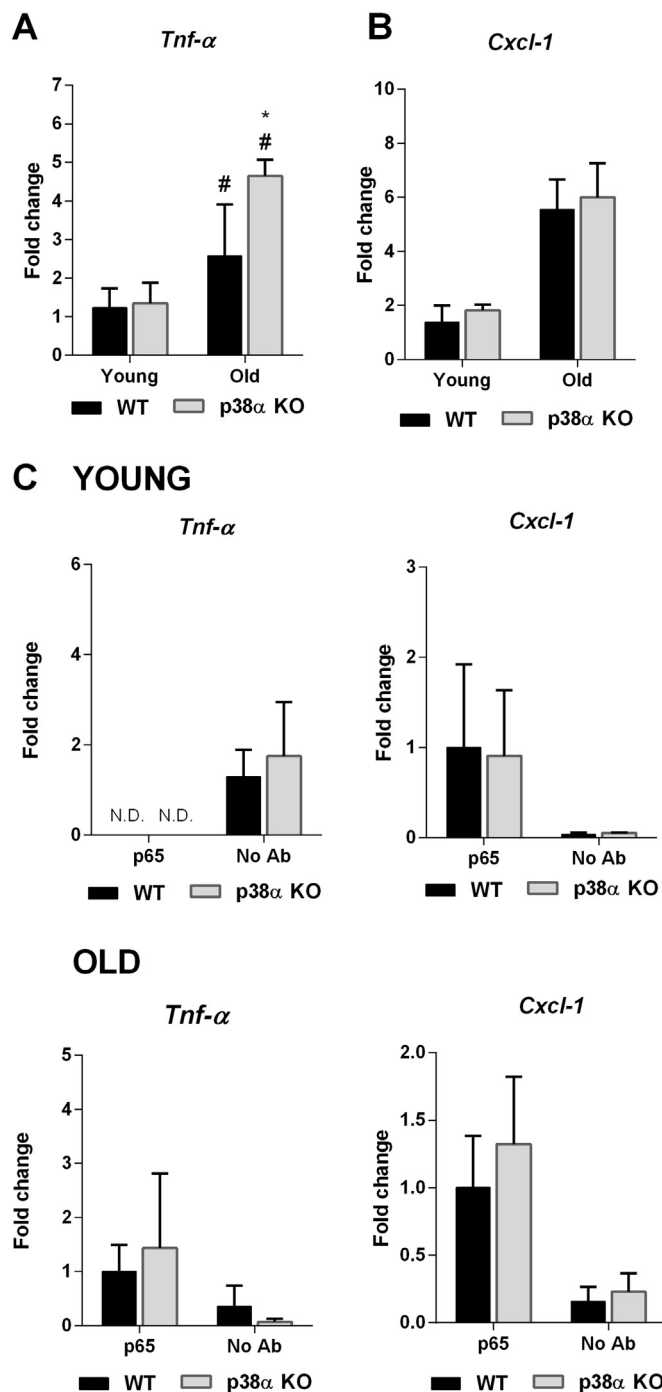


Fig. 7. mRNA relative expression of *Tnf-α* and *Cxcl-1* vs *Rplp0* in the liver of young WT and p38α KO mice and in the liver of old WT and p38α KO mice (A). Histograms showing the recruitment of p65 in the promoter regions of *Tnf-α* and *Cxcl-1* in the liver of young WT and p38α KO mice (B) and in the liver of old WT and p38α KO mice (C), measured by chromatin immunoprecipitation (ChIP) assay. The number of samples per group was 4–6 for RT-PCR and 4 for ChIP assay. Statistical significance is indicated as * $p < 0.05$ WT vs. KO; # $p < 0.05$ old vs. young.

involved in the down-regulation of antioxidant genes triggered by p38α deficiency in the liver of young animals.

p38α inhibits the G1/S and the G2/M checkpoints of the cell cycle, and down-regulates the JNK and EGFR pathways; hence it has been generally considered as a tumor suppressor in normal epithelial cells [2,41,50]. The role of p38α as tumor suppressor has been also supported by its ability to mediate oncogene-induced apoptosis or senescence (OIS) *in vitro* and by the enhanced tumorigenesis induced in mice

by p38α deficiency [51–58]. Accordingly, liver-specific p38α deficiency consistently leads to enhanced hepatocyte proliferation and tumor development in DEN-induced hepatocarcinogenesis in mice [6,33]. The pro-tumorigenic effect of p38α deficiency in the liver is mainly ascribed to the up-regulation of the JNK pathway [6], accumulation of superoxide and hydrogen peroxide, and release of IL-1α [33]. We found neither changes in the expression of *Il-1α* or *Gankyrin* mRNAs nor tumor development in the mice with p38α deficient livers (see Supplementary Fig. S3), suggesting that p38α deficiency alone is not sufficient to induce tumorigenesis.

p38α is activated by oncogene-induced ROS and inhibits tumor initiation by triggering apoptosis [52]. Strikingly, highly tumorigenic cancer cell lines tend to up-regulate Gstm1 and/or 2, which in turn bypass p38α activation and allow the accumulation of high ROS levels [52]. Interestingly, H-RasV12 transformed MEFs deficient in p38α exhibit in the long-term a dramatic increase in ROS levels [52]. Although H-Ras-induced p38α activation seemed to be downstream of ROS. Our findings point to a key role of p38 in the regulation of the expression of genes encoding antioxidant enzymes, which would contribute to modulate ROS levels during tumorigenesis.

5. Conclusions

In conclusion, p38α MAPK maintains the expression of antioxidant genes in the liver of young animals via NF-κB and PGC-1α under basal physiological conditions. However, its long-term deficiency triggers compensatory up-regulation of antioxidant enzymes through NF-κB. It is also noteworthy that the p38α-dependent NF-κB activation observed in young animals does not result in the up-regulation of pro-inflammatory genes, which deserves further investigation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.redox.2018.02.017>.

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