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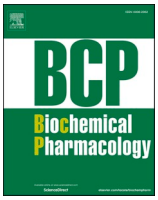

# Human 5-lipoxygenase regulates transcription by association to euchromatin

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ABSTRACT

Human 5-lipoxygenase (5-LO) is the key enzyme of leukotriene biosynthesis, mostly expressed in leukocytes and thus a crucial component of the innate immune system.

In this study, we show that 5-LO, besides its canonical function as an arachidonic acid metabolizing enzyme, is a regulator of gene expression associated with euchromatin. By Crispr-Cas9-mediated 5-LO knockout (KO) in MonoMac6 (MM6) cells and subsequent RNA-Seq analysis, we identified 5-LO regulated genes which could be clustered to immune/defense response, cell adhesion, transcription and growth/developmental processes. Analysis of differentially expressed genes identified cyclooxygenase-2 (COX2, *PTGS2*) and kynureninase (*KYNU*) as strongly regulated 5-LO target genes. 5-LO knockout affected MM6 cell adhesion and tryptophan metabolism via inhibition of the degradation of the immunoregulator kynurenine. By subsequent FAIRE-Seq and 5-LO ChIP-Seq analyses, we found an association of 5-LO with euchromatin, with prominent 5-LO binding to promoter regions in actively transcribed genes. By enrichment analysis of the ChIP-Seq results, we identified potential 5-LO interaction partners. Furthermore, 5-LO ChIP-Seq peaks resemble patterns of H3K27ac histone marks, suggesting that 5-LO recruitment mainly takes place at acetylated histones.

In summary, we demonstrate a noncanonical function of 5-LO as transcriptional regulator in monocytic cells.

## 1. Introduction

Leukotrienes are potent pro-inflammatory lipid mediators that play a role in the pathogenesis of inflammatory diseases such as asthma, rheumatoid arthritis and inflammatory bowel diseases [1–3]. 5-Lipoxygenase (5-LO, gene name *ALOX5*) catalyzes the first two oxygenation steps in the biosynthesis of leukotrienes from arachidonic acid and is therefore generally considered as key enzyme of the leukotriene pathway [4]. Consequently, 5-LO is a promising target for therapeutic intervention in inflammatory diseases [5,6].

Beside its function as a fatty acid oxygenase, there is emerging evidence that 5-LO can act as regulator and interacting partner of proteins

*Abbreviations:* 1,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; 5-LO, 5-Lipoxygenase; CDS, coding sequence; ChIP, chromatin immunoprecipitation; CML, chronic myeloid leukemia; FAIRE, formaldehyde assisted isolation of regulatory elements; FCS, fetal calf serum; FDR, false discovery rate; GO, gene ontology; HUVEC, human umbilical vein endothelial cells; LC-MS, liquid chromatography-mass spectrometry; LLOQ, lower limit of quantification; LPS, lipopolysaccharide; NGS, next generation sequencing; MΦ, macrophages; MM6, MonoMac 6; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; STRaND, shuttling transcriptional regulator and non-DNA binding; SMaRT, sensing, acting as a messenger and regulating transcription; TGFβ, transforming growth factor-β; TNFα, tumor necrosis factor-α; TSS, transcription start site; WT, wild type.

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directly or indirectly involved in the regulation of gene expression. In fact, 5-LO binds to Dicer and modifies its miRNA processing activity, it interacts with tumor promoting β-catenin/Wnt signaling, as well as with p53 nuclear trafficking [7–11]. Furthermore, the pathogenesis of certain forms of cancer is associated with the dysregulation of 5-LO [12–14]. Several reports have shown that 5-LO overexpression correlates with tumor progression and poor patient survival but it is unclear whether 5-LO plays a causative role [15–17].

Physiologically, expression of human 5-LO is mostly restricted to leukocytes. In monocytic cells, 5-LO is strongly upregulated by transforming growth factor-β (TGFβ) and 1α,25-dihydroxyvitamin $D_3$ (1,25 $(OH)_2D_3$) [18–20]. Lately, a synergistic activation of the *ALOX5* promoter by SMAD signaling involving the MLL protein and the vitamin D receptor in the monocytic cell line MM6 was found [21,22]. Furthermore, 5-LO expression was detected in ischemic tissue after cerebral ischemia [23].

Animal studies in mice also showed that a loss of 5-LO protein prevents development of chronic myeloid leukemia (CML) in the BCR/ABL model of CML [24]. The restricted stem cell renewal in 5-LO KO mice resulted in better survival by the reduction of β-catenin expression suggesting a link between BCR/ABL activity, 5-LO and Wnt signaling. This relationship was confirmed in another study where a screening for Wnt inhibitors led to the identification of lipoxygenin which turned out to be a non-redox 5-LO inhibitor that modulates the intracellular translocation of a β-catenin/5-LO complex [25]. Mechanistically, it appears that 5-LO serves as a chaperone for β-catenin and regulates its translocation into the nucleus because β-catenin itself lacks both, functional nuclear import and export sequences [26]. Of note, 5-LO is a highly mobile enzyme, which contains nuclear localization and export sequences. Thereby, the cellular localization of 5-LO depends on the cell type and is regulated by phosphorylation at Ser271 and Ser523 [27–29]. For example, in monocytes during cell differentiation and in neutrophils after surface adherence, 5-LO shuttles from the cytosol to the nucleus [30]. Furthermore, protein/protein interactions of 5-LO with several transcription factors were observed. Besides p53 and β-catenin, the NF-κB proteins c-Rel and p65 were found to interact with 5-LO suggesting that 5-LO might regulate the expression of some NF-κB target genes [31–33].

In this study we show that 5-LO has a significant impact on gene expression in the human monocytic AML cell line MM6. Using RNA-Seq we identified a network of 5-LO regulated genes, including genes of the integrin pathway that are important for adhesion processes as well as *KYNU*, a gene of the tryptophan-kynurenine pathway which metabolizes the immunosuppressant kynurenine. *KYNU* is upregulated in inflammatory diseases such as psoriasis and was recently suggested as biomarker for Crohn's disease [34–37]. Additionally, genes involved in biological processes such as immune response, cell proliferation and adhesion were regulated.

Mechanistically, the protein–protein interactions with transcription factors suggest that 5-LO may have a function as a shuttling regulator of transcription (STRaND) [38]. Using ChIP-Seq experiments, we now present an association of 5-LO with promoter regions of the human genome, suggesting that 5-LO is modulating gene expression and thereby exerting an additional noncanonical function beside its well established role in leukotriene biosynthesis.

## 2. Materials and methods

### 2.1. Cell culture

MonoMac 6 cells (DSMZ, ACC 124, Braunschweig, Germany) and their genetically modified derivatives were maintained at passages 2–20 at 37 °C and 5% $CO_2$ (V/V) in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% (V/V) FCS (Capricorn Scientific, Ebsdorfergrund, Germany), 1% (V/V) GlutaMAX (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 10 μg/mL human insulin (Sigma-Aldrich, St. Louis, Missouri, USA), 1% (V/V) MEM nonessential amino acids (Sigma-Aldrich, St. Louis, Missouri, USA), 1 mM sodium pyruvate (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1 mM oxaloacetate (Merck, Darmstadt, Germany), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cells were differentiated for 72 h at 37 °C and 6% $CO_2$ (V/V) using 1 ng/mL transforming growth factor-β (TGFβ) (PeproTech, Cranbury, New Jersey, USA) and 50 nM 1,25 $(OH)_2D_3$ (Cayman Chemical Company, Ann Arbor, Michigan, USA). For stimulation, cells were incubated with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, Missouri, USA from *E. coli 0111:B4*) for 2 to 24 h depending on the experiment.

Monocytes from human peripheral blood, taken from informed volunteers, were isolated from leukocyte concentrates (DRK-Blutspendedienst, Frankfurt, Germany) and purified by adherence as reported recently [39]. Macrophages (MΦ) were obtained by treatment with 10 ng/mL CSF-2 (M1) or CSF-1 (M2) (PeproTech, Cranbury, New Jersey, USA) for 7 days together with 10 ng/mL IFNγ (M1) or IL-4 (M2) for the final 24 h. 0.1 μg/mL LPS was also added 24 h before harvest.

### 2.2. Plasmid construction

For the re-expression of 5-LO, MM6 KO cells were lentivirally transduced with a codon-optimized *ALOX5* coding sequence (CDS) (Geneart, Regensburg, Germany) with an altered sgRNA binding site of Cas9. Using pT3 as a cloning vector, point mutations of the *ALOX5* CDS were introduced by site directed mutagenesis. Subsequently, the generated *ALOX5* CDS variants were transferred into the expression vector LeGO-iG2 (backbone restricted with NotI + BamHI (both New England Biolabs, Ipswich, Massachusetts, USA)) (generous gift from Boris Fehse, Addgene plasmid #27341 [40]) in which GFP was also replaced by a blasticidin S resistance gene (backbone restricted with BsrGI + MscI (both New England Biolabs, Ipswich, Massachusetts, USA)) using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, Massachusetts, USA). All newly generated plasmids are available at Addgene (#182741-182745).

### 2.3. 5-LO knockout and knockin generation

The generation and validation including off-target analysis of MM6 5-LO KO cells was reported recently [8]. The re-expression of 5-LO variants in single cell knockout clones 1 and 2 (KO1 and KO2) was performed using lentiviral transduction. Production of lentiviral particles was perfomed in LentiX-293 T cells (Takara Bio, Kusatsu, Japan) after 5 h preincubation with 25 μM chloroquine (Sigma-Aldrich, St. Louis, Missouri, USA) using polyethylenimine-based transfection of a modified lentiviral expression vector containing a *ALOX5* variant and blasticidin S resistance gene (LeGO-BSD-5LO Addgene #182741-182745), a lentiviral packaging vector (psPAX2, Addgene #12260) and an envelope plasmid encoding the glycoprotein of vesicular stomatitis virus (VSV-G) (pCMV-VSV-G, Addgene #8454). The resulting supernatant was sterile filtered (PVDF, 0.45 μm; Carl Roth, Karlsruhe, Germany) and concentrated (20-fold) with LentiX-Concentrator (Takara Bio, Kusatsu, Japan) according to the manufacturer's protocol. Subsequently, $0.5 \times 10^6$ MM6 5-LO KO cells were infected in presence of 8 μg/mL polybrene (Sigma-Aldrich, St. Louis, Missouri, USA) with 250 μL of concentrated virus. The used titer of $1.0–1.6 \times 10^6$ TU/mL was estimated based on the survival rate (50–80%) under subsequent selection with 10 μg/mL blasticidin S (InvivoGen, San Diego, California, USA) for 2 weeks.

### 2.4. Total RNA-sequencing

RNA was extracted using TRIzol (Invitrogen, Waltham, Massachusetts, USA) and treated with DNase I (Invitrogen, Waltham, Massachusetts, USA) both according to manufacturer's specifications. Afterwards,

1 μg of total RNA was depleted from ribosomal RNA using RiboCop rRNA Depletion Kit (Lexogen, Vienna, Austria) and libraries, containing unique dual indices, if applicable, were created with the SENSE or CORALL total RNA-Seq Library Prep Kit (Lexogen, Vienna, Austria). RNA and cDNA quality, purity and average length were verified using the Agilent 2100 bioanalyzer System (Agilent Technologies, Santa Clara, California, USA). The libraries were paired-end sequenced on NextSeq 500 or NextSeq 2000 instruments with a read length of 2 × 75 bp or 2 × 50 bp respectively (Illumina, San Diego, California, USA). Subsequent data processing was performed using Cutadapt 3.4 followed by RSEM 1.3.0 with STAR 2.6.0c for alignment to the human genome (GRCh38 release 92) [41–44]. Differential expression analysis (DEA) was performed using EBSeq 1.2.0, supplied by RSEM with a false discovery rate (FDR) of 0.05, for final DEA only genes with a fold change ≥2 were included [45]. Afterwards, differentially expressed genes were analyzed with the Gene Set Enrichment Analysis (GSEA)/Molecular Signature Database (MSigDB) online available at gsea-msigdb.org using hallmark and GO biological process gene sets [46,47].

### 2.5. ChIP-sequencing

For ChIP-Seq analysis, differentiated MM6 cells were treated with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, Missouri, USA) for 2 h. ChIP assays were performed as described recently with the anti-5-LO antibody ab169755 (Abcam, Cambridge, UK) [48,49]. Purified DNA ChIP templates and input controls were sequenced with 50 bp read length at the Gene Core Facility at the EMBL (Heidelberg, Germany). Alignment was performed with Bowtie2 v. 2.3.4.3 to the reference genome GRCh38 and MACS2 v. 2.1.1 was used for peak calling with input control files and a q-value cutoff for peak detection of 0.1 [50,43,51]. Further analyses were carried out with deeptools v. 3.3.2.0.1 (arguments computeMatrix & plotProfiles) and pyGenomeTracks v. 3.5 for visualization [52,53].

### 2.6. FAIRE-sequencing

Differentiated MM6 cells were stimulated with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, Missouri, USA) for 2 h and cross-linked with a final concentration of 1% formaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA) for 10 min and then stopped by glycine (Sigma-Aldrich, St. Louis, Missouri, USA) addition. FAIRE sample preparation was conducted as reported recently with the modified protocol by Giresi et al. [54,55]. Library preparation of purified DNA samples was conducted with the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, Ipswich, Massachusetts, USA) and single-end sequencing was performed with a read length of 75 bp on a NextSeq 500 (Illumina, San Diego, California, USA). Subsequent bioinformatic analysis was performed in the same way as for ChIP-Seq samples.

### 2.7. mRNA analysis

RNA was extracted from 5 × $10^6$ cells using TRIzol (Invitrogen, Waltham, Massachusetts, USA) and treated with DNase I (Invitrogen, Waltham, Massachusetts, USA) both according to the manufacturer's protocol. RNA integrity, purity and concentration were verified by agarose gel electrophoresis and UV/Vis spectroscopy (Nanodrop 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Subsequent reverse transcription of 2 μg purified RNA was performed using the high-capacity RNA-to-cDNA Kit (Applied Biosystems, Waltham, Massachusetts, USA) according to the manufacturer's protocol. Relative quantification of mRNA levels was performed by qPCR using 10 ng cDNA (RNA equivalent) with the PowerUp SYBR Mastermix (Applied Biosystems) on a StepOnePlus System (Applied Biosystems) in a final volume of 10 μL. Ubiquitin C (UBC) was used as internal standard for quantifications. The primer sequences are shown in Table 1. Specificity of all primer pairs was experimentally verified by agarose gel electrophoresis and melting curve. All runs included no template controls for all targets and measurements were performed in triplicates.

**Table 1**
**qPCR primer sequences (5′ → 3′)** (All primers were supplied by Eurofins Genomics, Ebersberg, Germany).

| Gene symbol/ target | Forward | Reverse |
|---|---|---|
| *ALOX5* | G AAT TAC TCC AAA GCG ATG G | AT GAC CCG CTC AGA AAT AGT G |
| *ALOX5*-KI | AAC TAC AGC AAG GCC ATG GAA AA | T CAC TCT CTC GCT GAT GGT GT |
| *KYNU* | GGC TCT CCA CCT AGA TGA GGA | GCT GCT ATT TTG GCC CAC TTA T |
| *ITGAM* | GCC TTG ACC TTA TGT CAT GGG | CCT GTG CTG TAG TCG CAC T |
| *PTGS2* | CTG GCG CTC AGC CAT ACA G | CGC ACT TAT ACT GGT CAA ATC CC |
| *UBC* | ATT TGG GTC GCG GTT CTT G | TGC CTT GAC ATT CTC GAT GGT |

### 2.8. Cell adhesion assay

Confluent human umbilical vein endothelial cells (HUVEC) in 24-well plates were either activated with 1 ng/mL TNFα (PeproTech, Cranbury, New Jersey, USA) for 24 h or remained untreated as indicated. MM6 cells were differentiated for 3 days and treated with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, Missouri, USA) for the last 24 h of cell differentiation. Afterwards, MM6 cells (1 × $10^4$ cells) were stained with CellTracker Green (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and added onto the HUVEC monolayer. After 5 min, non-adherent MM6 cells were washed off. Adhesion of fluorescent MM6 cells was finally measured using fluorescence emission at 535 nm with an excitation at 485 nm on a Tecan SPECTRAFlour Plus microplate reader (Tecan Group, Maennedorf, Switzerland).

### 2.9. Kynureninase activity assay

Differentiated MM6 cells were stimulated for 24 h with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, Missouri, USA). After stimulation, cells were washed twice with PBS pH 7.4 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and counted using trypan blue solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA). 2.5 × $10^6$ MM6 cells or 1.0 × $10^6$ detached primary Macrophages/Monocytes (MΦ) were centrifuged (300 × g, 5 min) and then resuspended in 250 μL PBS pH 7.4 with or without 3 μM of the kynureninase substrates kynurenine or 3-hydroxy-D/L-kynurenine (Cayman Chemical Company, Ann Arbor, Michigan, USA) and incubated for 1 h at 37 °C. Finally, cells were centrifuged at 15,000 × g for 5 min at 4 °C and the supernatant was snap-frozen in liquid nitrogen. For sample extraction, 100 μL cell supernatant were spiked with 20 μL methanol/water 1:1 (V/V) and 20 μL of internal standard ($^{13}$C(6)-3-hydroxyanthranilic acid; Alsachim, Illkirch, France). After addition of 300 μL ice cold methanol (LC-MS grade Carl Roth, Karlsruhe, Germany), the mixture was incubated at −20 °C for 30 min to allow complete protein precipitation. Afterwards, samples were centrifuged at 2000 × g, the supernatant was transferred to an amber glass vial and evaporated at 45 °C under a slight stream of nitrogen. For LC-MS/MS measurement, samples were dissolved in 50 μL 0.1% acetic acid (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The LC-MS/MS system consisted of a Shimadzu Nexera X2 UHPLC system (Shimadzu, Duisburg, Germany) coupled to a mass spectrometer 5500 QTRAP (Sciex, Darmstadt, Germany) equipped with a Turbo V ion source operated in positive electrospray ionization mode. Chromatographic separation was performed using a Hypersil Gold aQ Ultra High Performance Liquid Chromatography (UHPLC) column (100 mm × 2.1 mm ID, 1.9 μm; Thermo Fisher Scientific, Waltham, Massachusetts, USA) under gradient conditions. Mobile phase A was 0.1% formic acid (VWR International, Radnor, Pennsylvania, USA) and mobile phase B was

methanol with 0.1% formic acid. The gradient program was as follows: 0–1 min 100% A, 2.5 min 70% A, 6–9 min 0% A, 9, 5–12 min 100% A. The flow rate was 0.35 mL/min, column temperature was 30 °C and injection volume was 5 μL.

The mass spectrometric analysis was performed in multiple reaction monitoring mode, recording at least two precursor-to-product ion transitions per analyte. Data acquisition and evaluation were performed using Analyst Software v 1.6.3 (Sciex, Darmstadt, Germany) and MultiQuant Software v 3.0.2 (Sciex, Darmstadt, Germany).

### 2.10. Western blot analysis

For the analysis of protein expression, PBS washed cells were lysed in lysis buffer (20 mM Tris·HCl, pH 8.0, 1% Triton X-100, 2 mM EDTA, 2 mM PMSF, 100 mM NaCl, 1 mM $Na_3VO_4$, 0.1% SDS, and cOmplete protease and PhosSTOP phosphatase inhibitor mixture (Roche, Basel, Switzerland)), sonicated 2 × 10 sec and centrifuged (15,000 × g, 10 min, 4 °C). Then, cell lysates containing 40 μg protein were separated via SDS-PAGE by 10% gels and transferred onto nitrocellulose membranes (Li-Cor Biosciences, Bad-Homburg, Germany). Proteins were finally identified by specific primary antibodies (5-LO, (ab169755, abcam, Cambridge, UK), β-actin (ab8227, abcam, Cambridge, UK), kynureninase (ab225916, abcam, Cambridge, UK), prostaglandin synthase 2 (ab23672, abcam, Cambridge, UK)) and detected using the matching secondary antibodies (Li-Cor Biosciences, Bad-Homburg, Germany). Imaging was performed using a two channel Odyssey infrared imaging system (Li-Cor Biosciences, Bad-Homburg, Germany).

### 2.11. Determination of 5-LO product formation

$5 \times 10^6$ MM6 cells were resuspended in 1 mL PBS pH 7.4 containing 1 mg/mL glucose. 1 mM $CaCl_2$ was added and preincubated for 30 sec at 37 °C. The reaction was started by addition of 20 μM arachidonic acid (Cayman Chemical Company, Ann Arbor, Michigan, USA) and 2.5 μM calcium ionophore A23187 (Sigma Aldrich, St. Louis, Missouri, USA) followed by another incubation period of 10 min at 37 °C. Finally, the reaction was terminated with 1 mL ice-cold methanol (LC-MS grade, Carl Roth, Karlsruhe, Germany). Sample preparation was performed as reported before [56].

5-LO products were analyzed using an Acquity UPLC H-class coupled to TUV and QDa detectors (Waters, Milford, Massachusetts, USA), the QDa was operated in negative electrospray ionization mode. Chromatographic separation was performed using an ACQUITY UPLC HSS T3 1.8 μM, 2.1 × 100 mm column (Waters, Milford, Massachusetts, USA). The mobile phase consisted of (A) ultra-pure water, (B) acetonitrile, (C) methanol and (D) 1% acetic acid. The gradient program was as follows: 0–5.2 min A 45% /B 34% /C 16% /D 5% linear gradient to A 39%/ B 38.1%/ C 17.9%/ D 5%, 5.2–7.4 min A 20%/ B 51%/ C 24%/ D 5% linear gradient to A 12.7%/ B 56%/ C 26.3%/ D 5%. The flow rate was 0.5 mL/min, column temperature was 40 °C and injection volume was 10 μL.

$PGB_1$, 6-*trans*-$LTB_4$, 6-*trans*-12-epi-$LTB_4$, and $LTB_4$ were detected at 280 nm and 335.2 Da, 5-HETE at 235 nm and 319.2 Da, respectively. Data acquisition and processing was performed using Empower 3 software (Waters, Milford, Massachusetts, USA). All data obtained were normalized to $1 \times 10^6$ cells and corrected for non-enzymatic oxidation products present in control samples without cells.

### 2.12. Statistical analyses

Data are presented as mean + standard deviation (SD) of at least three independent experiments. Statistical significance was analyzed by one-way-Welsh's or ordinary two-way-ANOVA dependent on the experimental design using GraphPad Prism 8 (GraphPad Software, San Diego, California, USA). Multiple comparisons were corrected by controlling the FDR using the two-stage step-up method of Benjamini, Krieger and Yekutieli [57].

## 3. Results

### 3.1. Identification of 5-LO regulated target genes

In order to study the role of 5-LO on gene expression, 5-LO expression in MM6 cells was induced by TGFβ and $1,25(OH)_2D_3$ and stimulated by LPS to mimic inflammatory conditions (Fig. 1A). To identify genes regulated by 5-LO, RNA-sequencing was performed with MM6 WT, 5-LO KO and 5-LO KI cells (GEO: GSE200336) with or without LPS stimulation and with or without TGFβ/$1,25(OH)_2D_3$ differentiation. To eliminate possible cloning effects, two 5-LO KO single cell clones (5-LO KO1 & 2) were selected which were generated by CRISPR gene editing showing a frameshift mutation on both alleles. Starting from these KO single cell clones, we additionally generated 5-LO KI cells (5-LO KI1 & 2) where a codon-optimized *ALOX5* coding sequence lacking the Cas9 sgRNA binding site under the control of the SFFV promoter (Fig. 1A, B) was lentivirally integrated in each KO clone. In 5-LO KO cells, *ALOX5* mRNA can still be detected (Fig. 1B) whereas the frame shift in the 5-LO coding sequence prevents translation of the 5-LO mRNA so that no 5-LO protein was detectable in the KO cells (Fig. 1A).

In order to determine the regulated genes with the highest possible certainty, two differential expression analyses were performed. Analysis 1) MM6 5-LO KO clone 1 and 2 vs. MM6 WT respectively and analysis 2) MM6 5-LO KO clone 1 and 2 vs. the corresponding MM6 5-LO KI cells. When merging these data, only those genes were considered which were regulated in both clones to avoid possible clonal effects (analysis 1) and which showed a significant opposite regulation in the corresponding 5-LO KI (rescue effect) (analysis 2). Finally, genes were filtered for an FDR $< 0.05$ and a fold change $\geq 2$.

After these strict selection criteria, only a few regulated genes remain and the distribution of those regulated genes in regard of the treatments is shown in a Venn diagram (Fig. 2A). In undifferentiated MM6 cells, four genes were down and 15 genes were upregulated by 5-LO KO. In LPS treated undifferentiated MM6, 12 genes were down and 31 genes were upregulated. In differentiated MM6 cells, 4 genes were down and 26 genes were upregulated by 5-LO KO (Fig. 2B). Thus, for all treatments the expression of differentially expressed genes was mostly enhanced in the absence of 5-LO. Interestingly, the spectrum of differentially expressed genes varied between the different treatments, only two genes were regulated in all four conditions (*ISL2* upregulated by 5-LO KO and *NAV3* downregulated by 5-LO KO) and four genes were regulated in three treatments (*MAP3K9*, *NPDC1*, *P2RY6* and *YAP1P1*). Nevertheless, in untreated MM6 cells, the majority (10 out of 19) of the differentially expressed genes were regulated in at least two treatments (highlighted in yellow).

In differentiated and LPS treated MM6 cells, 44 genes were down and 110 genes were upregulated by 5-LO KO (Fig. 3A). Genes belonging to the 10 most prominent clusters are arranged in the figure according to their Gene Ontology (GO) biological function using the Geneset Enrichment Analysis and the Molecular Signatures Database (GSEA-MSigDB). The assignment of the individual genes to the 10 clusters is shown in Fig. 3A. As expected, a large percentage of the genes is involved in immune/defense response. More surprisingly, some genes regulate (leukocyte) cell adhesion and migration including prominent genes of the integrin signaling pathway. Furthermore, genes could be assigned to the following clusters: regulation of population proliferation, positive regulation of multicellular organismal processes and regulation of intracellular signal transduction including well-known members of the epithelial–mesenchymal transition and the JAK-STAT pathway.

Next, we performed a pathway analysis using MSigDB Hallmark revealing the pathway TNFα signaling via NF-κB to be highly enriched, containing amongst others, *LIF*, *PTGS2*, *SOCS3*, *KYNU* and *LAMB3* (Fig. 3B).

Overall, the gene *KYNU* showed the strongest regulation by 5-LO KO

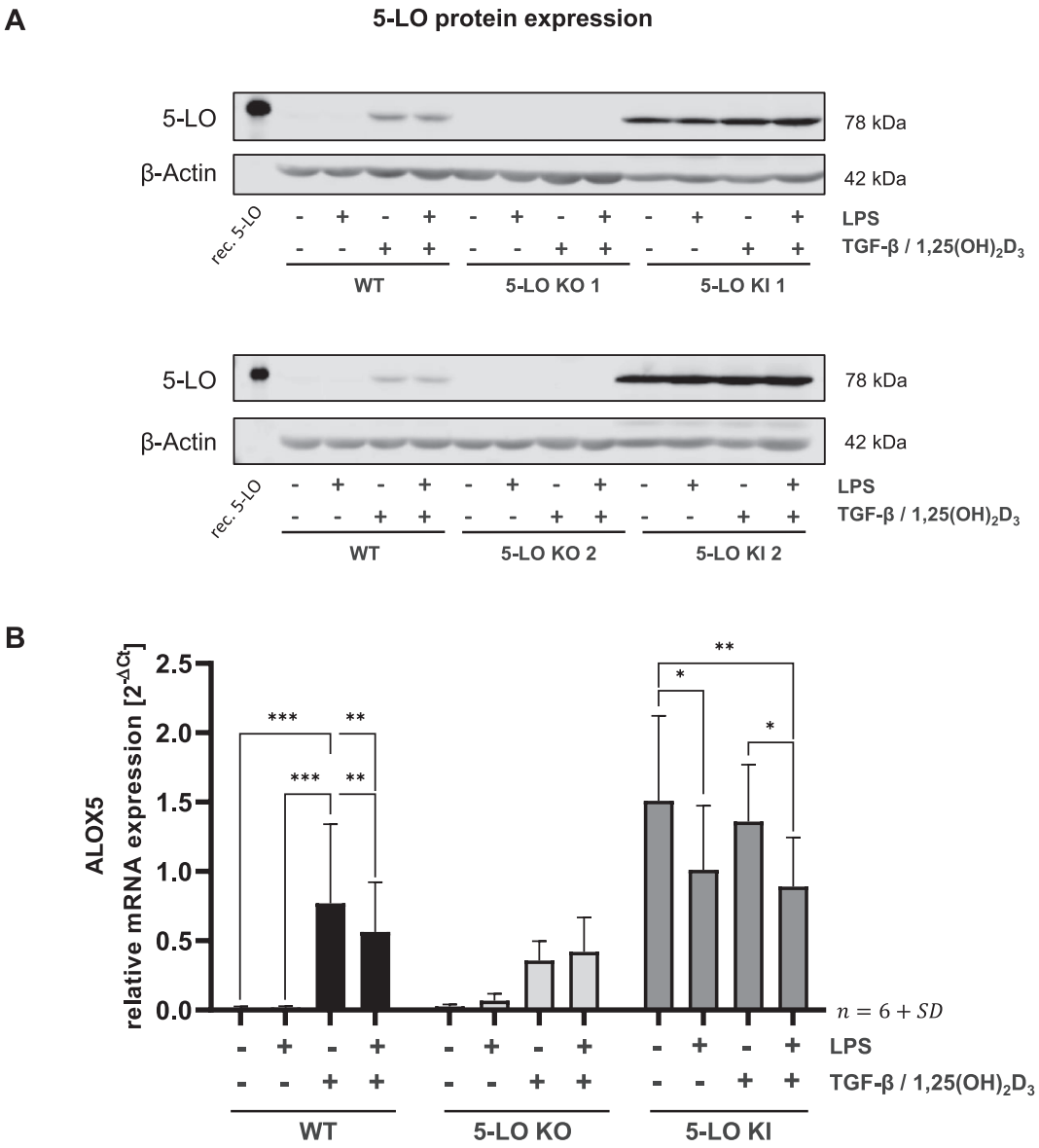


**Fig. 1. 5-LO protein and mRNA expression.** MM6 cells (WT, 5-LO KO and 5-LO KI) with or without differentiation by 1 ng/mL TGFβ/50 nM $1,25(OH)_2D_3$ for 3 days and with or without stimulation with 1 μg/mL LPS for the last 6 h were used. **(A)** 5-LO protein expression was analyzed by Western blot with recombinant 5-LO as positive control. The housekeeping protein β-actin was used as loading control. **(B)** Relative *ALOX5* mRNA expression of MM6 WT, 5-LO KO/KI1 and 2 cells was analyzed by RT-qPCR. Data are normalized to the reference gene UBC and presented as $2^{-\Delta Ct}$ (mean + SD) (n = 6). Since the coding sequence of the *ALOX5* CDS in MM6 KI cells does not correspond to the exact sequence of *ALOX5* gene in MM6 WT, adapted primers had to be used with the same binding location, for primer sequences see Table 1.

among the protein coding genes. *KYNU* encodes for kynureninase, the key enzyme in the tryptophan-kynurenine pathway. Most impressively, this gene is tightly regulated in an on/off manner by the presence or absence of 5-LO protein upon LPS-stimulation, especially in differentiated cells. Whereas most other genes are upregulated by 5-LO KO and subsequently downregulated by reexpression of 5-LO, *KYNU* expression is positively linked to the presence of the 5-LO protein.

### 3.2. 5-LO strongly induces expression of kynureninase in MM6 cells

Next, we verified the RNA-Seq results by RT-qPCR. For this, we selected a gene that was strongly regulated (kynureninase, *KYNU*) and representative genes belonging to the adhesion/migration (integrin alpha-M, *ITGAM*) and immune/defense response (COX2, *PTGS2*). From this selection, *ITGAM* and COX2 are upregulated whereas *KYNU* is downregulated by 5-LO KO.

Integrin α-M (CD11b, *ITGAM*) and integrin β2 (CD18, *ITGB2*) form the heterodimer macrophage antigen 1 (Mac-1). Mac-1 is involved in various adhesion processes of monocytes, macrophages and granulocytes as well as in the uptake of complement-coated particles and pathogens [58–60].

The *PTGS2* gene encodes COX2, a key enzyme in prostaglandin biosynthesis [61]. It is a prominent NF-κB target gene and is therefore particularly strongly upregulated in differentiated MM6 cells after LPS stimulation (Fig. 4A) [62–64].

In 5-LO KO cells, *ITGAM* and COX2 show an increased upregulation by LPS compared to MM6 WT cells. When 5-LO is reintroduced into MM6 (5-LO KI cells), this effect is reverted and for *ITGAM* and COX2, mRNA levels were even lower than in MM6 WT cells (Fig. 4A). In summary, the presence of 5-LO in MM6 cells inhibits the LPS-stimulated expression of *ITGAM* and COX2.

For *KYNU* there is a strong LPS triggered upregulation in MM6 WT

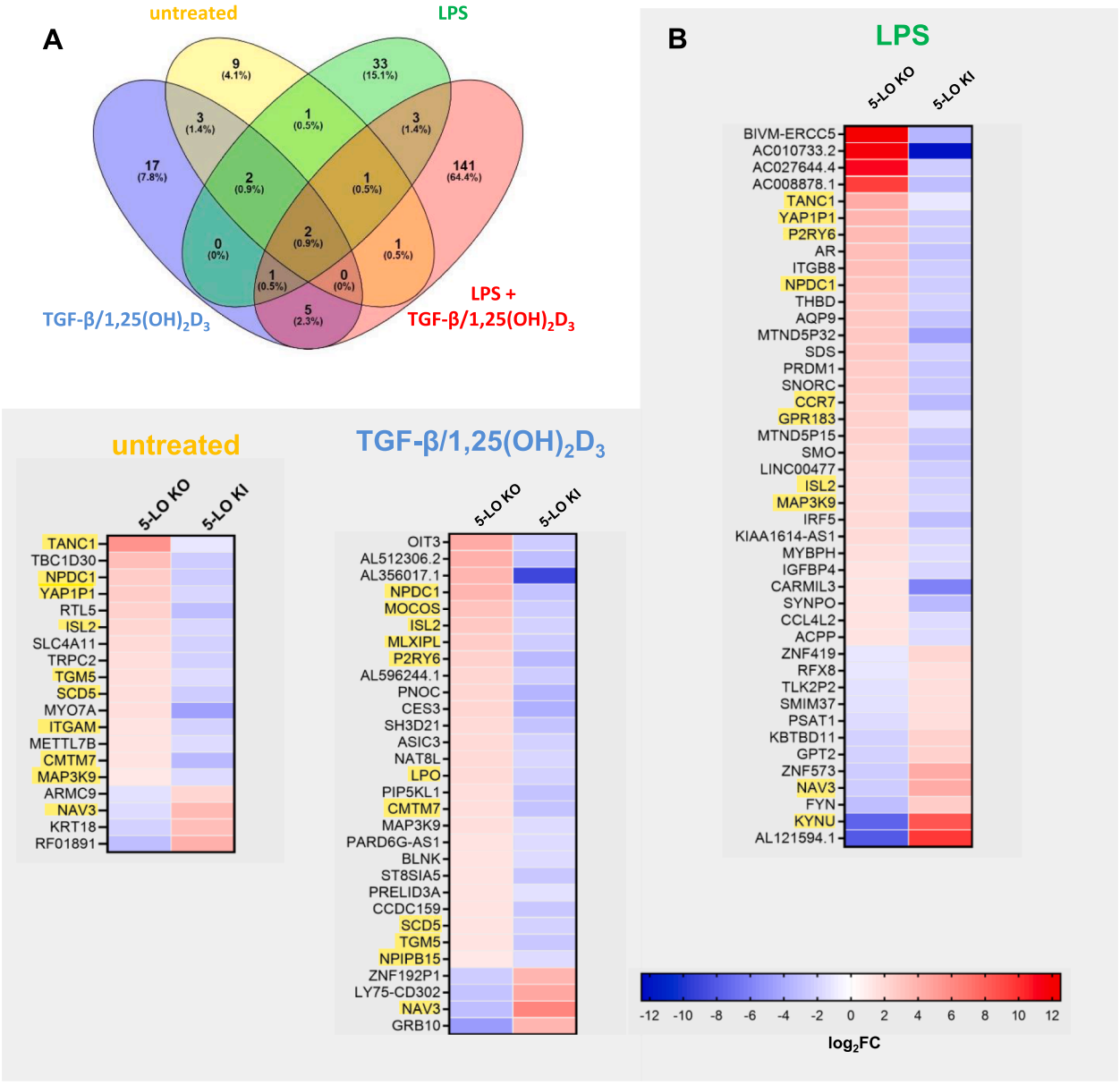


**Fig. 2. Venn diagram and heat map of differentially expressed genes obtained from RNA-Seq analyses.** For differential expression analysis, MM6 WT cells were compared with 5-LO KO1 and 5-LO KO2 separately. In a second differential expression analysis, 5-LO KO1 and KO2 cell lines were compared with their corresponding 5-LO KI cell line. **(A)** Venn diagram showing all differentially expressed genes and overlap of differentially expressed genes in multiple treatments. **(B)** MM6 cells (WT, 5-LO KO and 5-LO KI) were cultured without additions or differentiated by 1 ng/mL TGFβ/50 nM $1,25(OH)_2D_3$ for three days or stimulated with 1 μg/mL LPS for the last 6 h of cell differentiation. Only genes with a fold change ≥ 2 and FDR < 0.05 were considered. The heat maps include all genes that were up- and downregulated by 5-LO KO and show a rescue effect by 5-LO KI. Values presented are $log_2$(FC) 5-LO KO vs. WT and 5-LO KI vs. corresponding 5-LO KO. Differentially expressed genes that were regulated in more than one treatment are shown in yellow.

cells, which is independent of the cellular differentiation state. In 5-LO KO cells, the LPS triggered upregulation is completely hampered but can be restored to WT level after 5-LO re-expression (Fig. 4A). Thus, 5-LO appears to be critical to enhance the expression of *KYNU* after LPS stimulation.

Since mRNA expression does not always correlate with the protein level, kynureninase, COX2 and 5-LO expression was analyzed by Western blot using cell lysates of LPS stimulated M1 and M2 macrophages as positive controls (Fig. 4B). Western blot analysis confirmed the results from RNA-Seq as LPS clearly induces kynureninase protein expression in MM6 WT and MM6 5-LO KI cells, whereas kynureninase expression is strongly reduced in 5-LO KO cells (Fig. 4B). The opposite effect could be observed for COX2. Upregulation of COX2 occured in 5-LO KO cells, whereas COX2 expression in 5-LO KI cells was suppressed (Fig. 4B).

Kynureninase which is encoded by *KYNU* catalyzes two reactions in tryptophan metabolism, the conversion of kynurenine to anthranilic acid and the corresponding 3-hydroxy derivative to 3-hydroxyanthranilic acid (Fig. 4C) [65,66]. Next to the downregulation of kynureninase protein, we determined whether we could also detect a decrease in kynureninase activity. For this purpose, $2.5 \times 10^6$ intact MM6 WT, 5-LO KO or 5-LO KI cells as well as $1.0 \times 10^6$ M1-, M2- and M0-MΦ were incubated with the kynureninase substrates kynurenine or 3-hydroxykynurenine and the products (anthranilic acid and 3-hydroxyanthranilic acid) were subsequently determined by LC-MS/MS. Interestingly, we could mainly detect 3-hydroxyanthranilic acid whereas the concentration of anthranilic acid was below the lower limit of quantification

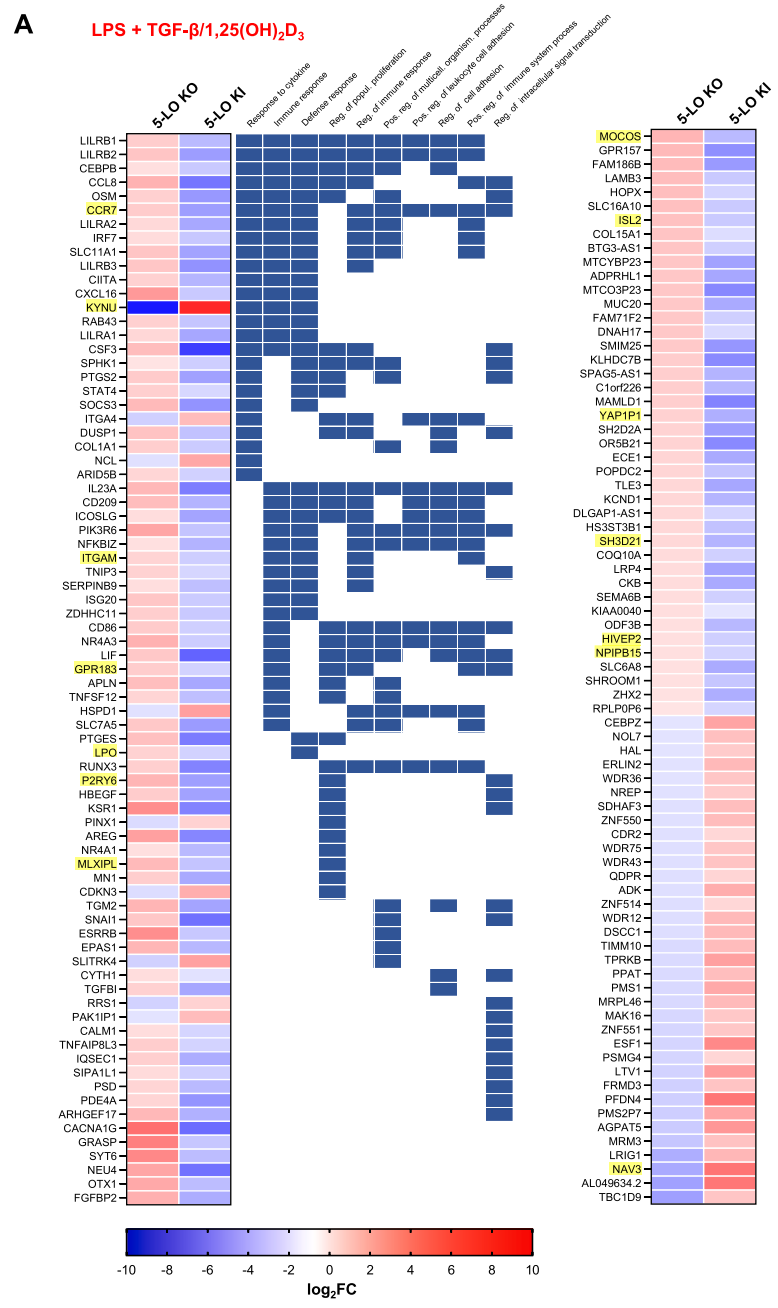


**Fig. 3. RNA-Seq analysis with functionally clustered heat map and pathway analysis of LPS treated, differentiated MM6 cells.** For differential expression analysis, MM6 WT cells were compared with 5-LO KO1 and 5-LO KO2 separately. In a second differential expression analysis, both 5-LO KO cell lines were compared with their corresponding 5-LO KI cell line. MM6 cells (WT, 5-LO KO and 5-LO KI) were differentiated by 1 ng/mL TGFβ/50 nM 1,25 $(OH)_2D_3$ for three days and stimulated with 1 μg/mL LPS for the last 6 h of cell differentiation. Only genes with a fold change $\geq 2$ and FDR $< 0.05$ were considered. **(A)** The heat map shows functionally clustered genes using GSEA GO Biological Process regulated by 5-LO KO and KI. Values presented are $\log_2$(FC) 5-LO KO vs. WT as well as 5-LO KI vs. the corresponding 5-LO KO. Differentially expressed genes that are regulated in more than one treatment are shown in yellow. **(B)** Pathway analysis of those differentially expressed genes using GSEA hallmark gene sets. Data are sorted and shaded by significance (log 1/p-value).

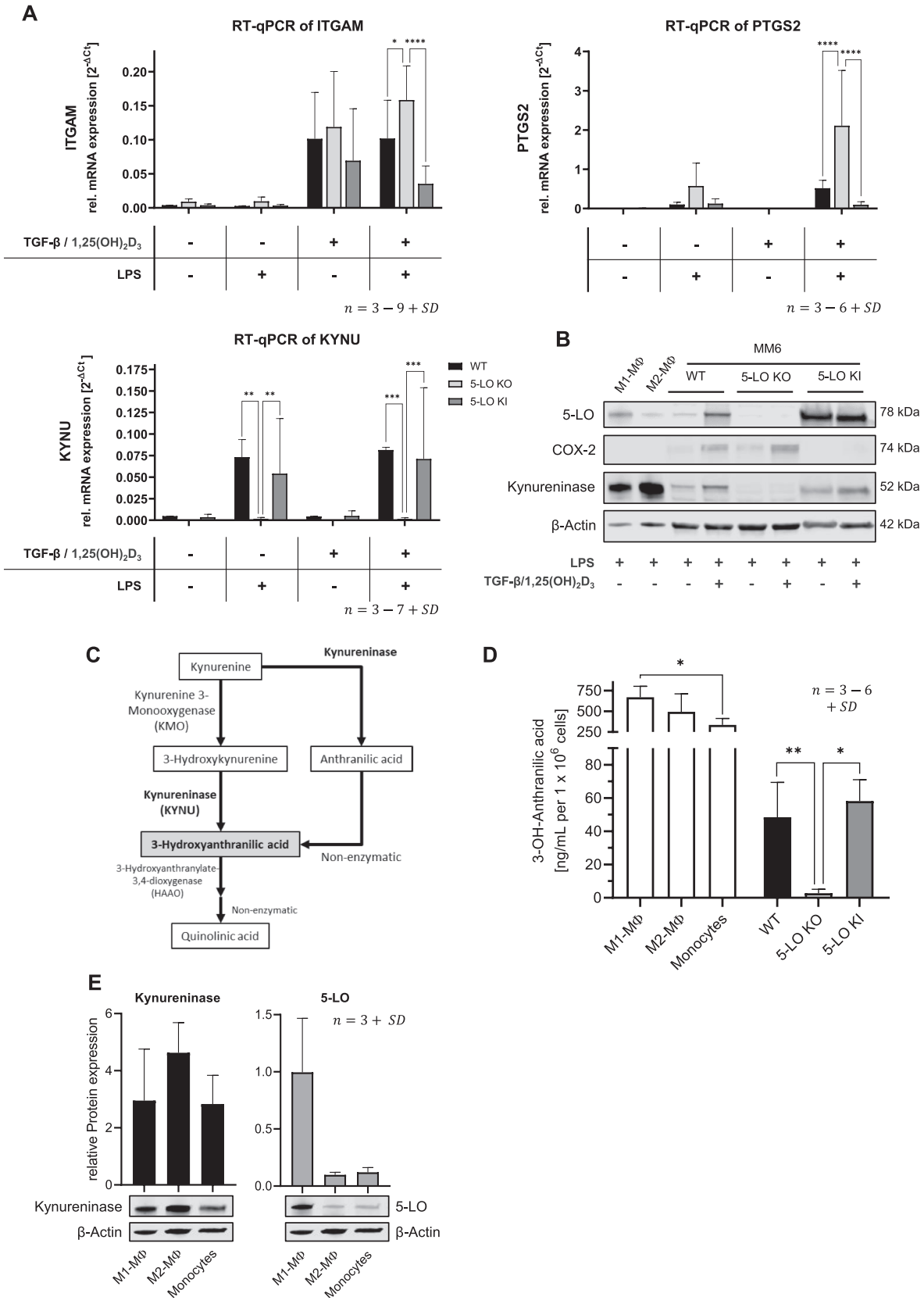


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**Fig. 4. Effect of 5-LO KO on integrin alpha M (*ITGAM*), cyclooxygenase 2 (*PTGS2* / COX2) and kynureninase (*KYNU*) expression as well as cellular kynureninase activity.** MM6 cells (WT, 5-LO KO and 5-LO KI) were cultured with or without 1 ng/mL TGFβ/50 nM $1,25(OH)_2D_3$ for 3 days and with or without 1 μg/mL LPS for the last 6 h of cell differentiation. **(A)** mRNA expression was analyzed by RT-qPCR and is presented as $2^{-\Delta Ct}$ (mean + SD) normalized to UBC expression. Statistical analysis was obtained with a two-way ANOVA based on n = 3–9 individual experiments. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) **(B)** Protein expression of kynureninase and COX2 was analyzed by Western blot using β-actin as loading control. Macrophages (MΦ) derived from peripheral blood were chosen as positive controls for kynureninase (control sample loading: 4 μg protein). For MM6 cells the 5-LO KO1 clone with the corresponding KI is shown. For measurement of kynureninase product formation, differentiated MM6 cells (WT, MM6 5-LO KO1 and MM6 5-LO KI1) were treated with 1 μg/mL LPS for the last 24 h of cell differentiation. **(C)** The kynurenine pathway. **(D)** $2.5 \times 10^6$ MM6 cells or $1.0 \times 10^6$ primary MΦ/monocytes were incubated with 3-hydroxy-kynurenine (3 μM) for 1 h and 3-hydroxy-anthranilic acid was measured in the supernatant using LC-MS/MS. **(E)** Kynureninase and 5-LO expression in M1-, M2-MΦ and monocytes was analyzed by quantitative Western blot analysis using β-actin as loading control. Statistical analysis was obtained with a one-way ANOVA with Welch correction based on n = 3–6 individual experiments. (+SD, *p < 0.05, **p < 0.01).

(LLOQ). Taken together, kynureninase activity correlated with the detected amount of kynureninase protein. In MM6 cells, *KYNU* expression strongly depended on the presence or absence of 5-LO protein (Fig. 4D). However, regarding monocytes, M1- and M2-MΦ, there was a much stronger 5-LO expression in M1-MΦ but all three cell types showed similar kynureninase expression and activity (Fig. 4D,E).

### 3.3. Transcriptional regulation is partially independent of 5-LO enzymatic activity

As 5-LO is highly active in differentiated MM6 cells leading to prominent leukotriene formation, we wanted to rule out that leukotrienes and other 5-LO-derived oxylipins are responsible for the differential gene expression induced by 5-LO KO. Therefore, we created another MM6 5-LO KI cell line expressing the enzymatically inactive 5-LO mutant E376Q (MM6 E376Q KI). By exchanging Glu to Gln, this variant loses its enzymatic activity due to the loss of a ligand to the non-heme iron [67,68]. For MM6 WT and MM6 5-LO KI, 5-LO activity correlates well with the amount of 5-LO protein quantified via Western blotting. As expected, we could not detect 5-LO products in MM6 E376Q KI cells (Fig. 5A, B). Despite the lack of 5-LO products and the reduced amount of 5-LO protein compared to MM6 WT or MM6 5-LO KI cells, *KYNU* mRNA expression was completely restored in MM6 E376Q KI cells after LPS treatment (Fig. 5C). For COX2 and *ITGAM*, the E376Q mutant restored the gene expression to levels of MM6 WT cells whereas in MM6 5-LO KI cells, mRNA levels of *ITGAM* and COX2 were even lower compared to the WT cells (Fig. 5D, E). This could be due to a concentration effect, as 5-LO KI cells contain more 5-LO protein compared to MM6 WT and E376Q KI cells (Fig. 5B) and therefore may show a stronger rescue effect.

Furthermore, we tested known inhibitors and receptor antagonists of the 5-LO pathway in MM6 WT cells. Thus, differentiated MM6 WT cells, which were treated with LPS (for the final 6 h of the 72 h differentiation period) were incubated with different inhibitors for 24 h (Fig. 5F–H). To cover different steps in the 5-LO pathway, the 5-LO inhibitors zileuton (4 μM, iron-ligand type inhibitor) and CJ-13610 (1 μM, non-redox type inhibitor), MK-886 (1 μM, 5-LO activating protein (FLAP) inhibitor), CP-10569 (1 μM, leukotriene $B_4$ receptor 1 (BLT1) antagonist) and montelukast (10 μM, cysteinyl leukotriene receptor (CysLT1) antagonist) were used. As shown in the Fig. 5F–H, none of the inhibitors or antagonists were able to mimic the effect of 5-LO KO on *KYNU* and COX2 gene expression in MM6 WT cells, implying that the regulatory mechanism is not linked to 5-LO product formation. In contrast, *ITGAM* mRNA expression was enhanced in all treatments except for the CysLT1 antagonist montelukast, suggesting that *ITGAM* regulation by 5-LO is product-dependent and seems to be mediated via $LTB_4$ signaling.

### 3.4. 5-LO KO in MM6 cells leads to increased MM6 cell adhesion

Besides *KYNU*, the differential expression analysis revealed several genes regulated by 5-LO KO that are involved in cell adhesion of leukocytes (Fig. 3A). To prove that these observations have a functional impact on the behavior of MM6 cells, we performed a cell adhesion assay. Thus, confluent human umbilical vein endothelial cells (HUVEC) were activated by stimulation with TNFα for 24 h. Subsequently, LPS stimulated differentiated MM6 WT, 5-LO KO and 5-LO KI cells were fluorescent labeled and added to the HUVEC monolayer. After 5 min adhesion time, non-adhering cells were washed off and adhering MM6 cells were determined using fluorescence. Interestingly, adhesion was significantly enhanced in MM6 5-LO KO cells, while this effect was completely abrogated by the re-expression of 5-LO in MM6 5-LO KI cells (Fig. 6). This result is in line with the previously observed upregulation of several integrins in MM6 5-LO KO cells.

### 3.5. FAIRE- and ChIP-Seq analysis reveal 5-LO association with euchromatin

As the experiments with the 5-LO pathway inhibitors/antagonists suggest a mechanism independent of the canonical enzymatic function of 5-LO, we performed two additional genome-wide analyses. On the one hand, formaldehyde-assisted isolation of regulatory elements (FAIRE) experiments were performed to examine whether the overall amount of active regulatory elements of genes influenced by 5-LO KO changes and on the other hand, a ChIP-Seq analysis was done to prove a localization of 5-LO at genomic DNA and to additionally determine the genomic loci (GEO: GSE200338). FAIRE-Seq identifies the accessible regions of chromatin and thereby loci of active enhancers and promoters [51]. To demonstrate the effect of 5-LO on active enhancers and promoters, FAIRE-Seq tracks of differentiated MM6 WT with and without LPS stimulation were compared to MM6 5-LO KO cells with the same treatment. For MM6 WT cells without LPS treatment, 184,240 peaks were identified and for MM6 5-LO KO cells 142,486 peaks, respectively. Thereof, 116,954 peaks are shared, representing 63.5% (MM6 WT) and 82.1% (MM6 5-LO KO) of the total peaks. For differentiated cells with LPS treatment, 102,417 peaks were identified for MM6 WT and 128,048 for MM6 5-LO KO with 71,806 peaks in common, representing 70.1% (MM6 WT) and 56.1% (MM6 5-LO KO), respectively (Fig. 7A).

For 5-LO ChIP-Seq analysis, differentiated MM6 WT cells with and without LPS stimulation were fixed with formaldehyde and chromatin immunoprecipitation was performed using a 5-LO antibody (ab6755, abcam, Cambridge, UK). Peak identification against input control by MACS2 revealed 4,544 significant peaks for MM6 WT cells without LPS treatment and 10,318 significant peaks for MM6 WT cells after LPS treatment. Hence, LPS treatment increased the number of the identified peaks by more than 2-fold. Analysis of the 5-LO ChIP-Seq data sets revealed that approximately 35% of all 5-LO peaks are bound to promoters within 3 kb upstream of transcription start sites (TSS), no matter whether the cells had been treated with LPS or not (Fig. 7B, blue).

Fig. 8A shows the chromatin accessibility in the *KYNU* gene locus along with the corresponding FAIRE-Seq and ChIP-Seq peaks determined by MACS2. Since chromatin-modeling effects occur prior to mRNA expression, LPS stimulation was shortened to 2 h compared to 6 h used in the mRNA and protein expression experiments. Interestingly, the accessibility of the *KYNU* promoter region is higher in MM6 WT (red panel) than in MM6 5-LO KO cells (green panel) (Fig. 8A) shown by a decreased FAIRE peak signal strength at the transcription start site (TSS) in MM6 5-LO KO cells (Fig. 8, red panel). This indicates that 5-LO increases the activity of the *KYNU* promoter, which is in-line with the

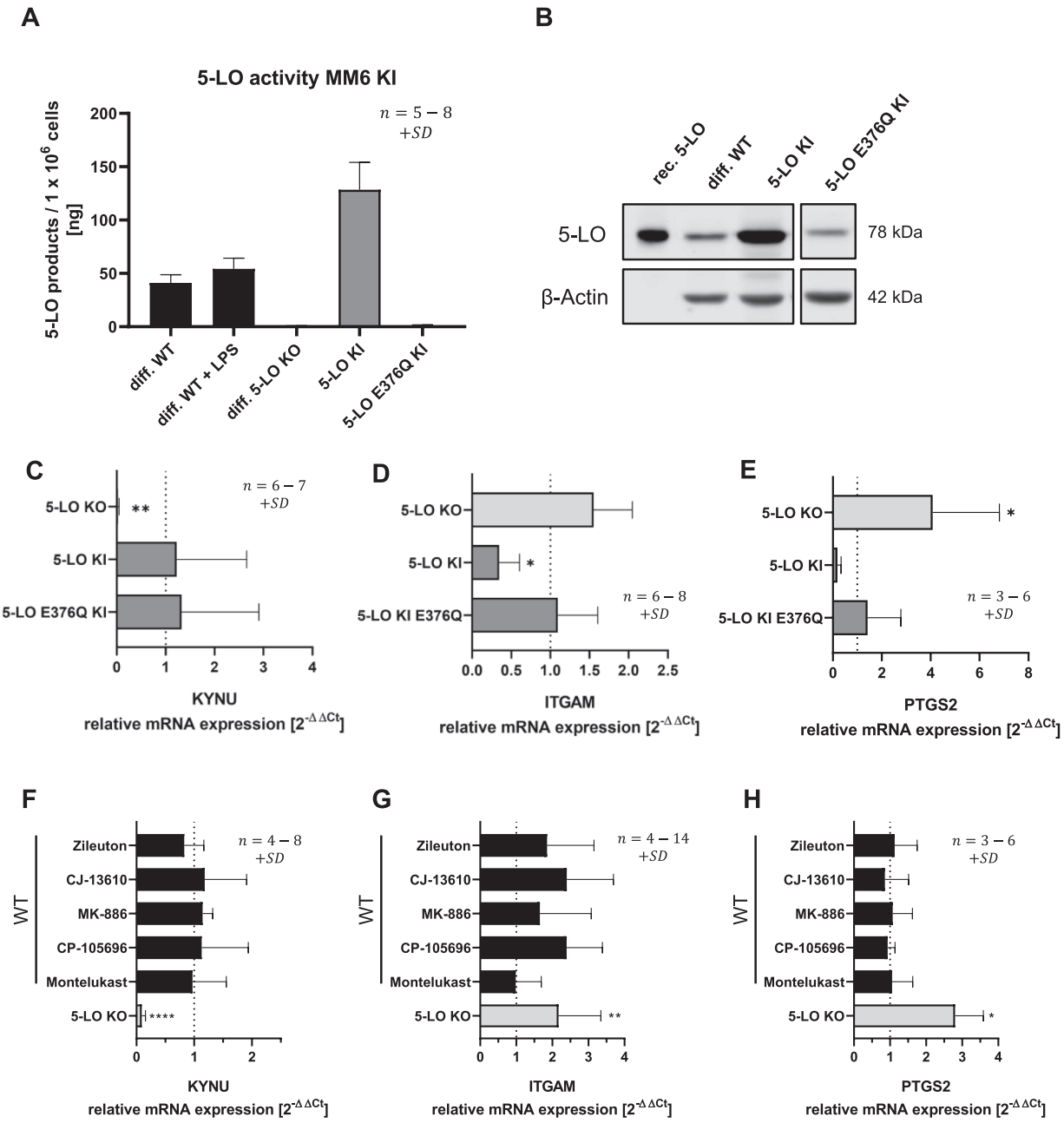


**Fig. 5. Effect of an enzymatically inactive 5-LO mutant (E376Q) and 5-LO pathway inhibitors/antagonists on *KYNU*, *ITGAM* and *PTGS2* (COX2) mRNA expression.** MM6 cells were differentiated by 1 ng/mL TGFβ/50 nM $1,25(OH)_2D_3$ for 3 days and stimulated with 1 μg/mL LPS for the last 6 h of cell differentiation. **(A)** Determination of 5-LO activity in whole cells. 5-LO products (5-H(p)ETE, $LTB_4$, and the respective all-trans isomers) were measured by UPLC and 5-LO activity was normalized to $10^6$ cells (mean + SD). **(B)** 5-LO protein expression in MM6 WT, MM6 5-LO KI and MM6 5-LO E376Q KI was analyzed by Western blot using β-actin as loading control. A representative blot of 5-LO KI1 is shown. **(C-E)** mRNA expression of *KYNU* (C), *ITGAM* (D) and *PTGS2* (COX2) (E) was analyzed by RT-qPCR, data are shown as $2^{-\Delta\Delta Ct}$ (mean + SD) normalized to UBC expression and WT control. Statistical analysis was obtained with a one-way ANOVA with Welch correction against WT control based on n = 3–8 individual experiments. (*$p < 0.05$, **$p < 0.01$). **(F-H)** The indicated 5-LO pathway inhibitors were added to MM6 WT and KO cells during the last 24 h of cell differentiation and the cells were finally stimulated for 6 h with 1 μg/mL LPS before harvest. The 5-LO inhibitors zileuton and CJ-13610 were used at 4 μM and 1 μM, respectively. The FLAP inhibitor MK-886 and the LTB4 receptor antagonist CP-105696 were used at 1 μM, while the CysLT1 inhibitor montelukast was used at 10 μM. Then, mRNA expression of *KYNU* **(F)**, *ITGAM* **(G)** and *PTGS2* (COX2) **(H)** was analyzed by RT-qPCR, data are shown as $2^{-\Delta\Delta Ct}$ (mean + SD) normalized to UBC and WT control. Statistical analysis was obtained with a one-way ANOVA with Welch correction against WT control based on n = 3–14 individual experiments. (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

effects of the 5-LO KO seen on *KYNU* mRNA expression. Next, we retrieved available data on the histone modification H3K27ac from primary $CD14^+$ monocytes (GEO: GSM1003559). This histone modification is a well-known marker of enhancers/promoters. Noticeably, the H3K27ac signals matched well with our FAIRE-Seq peaks at the promoter region of *KYNU*, fortifying the validity of our results.

Opposite effects of 5-LO KO are found at the *PTGS2* (COX2) locus. Fig. 8B shows the chromatin accessibility and FAIRE-/ChIP-Seq peaks in the *PTGS2* gene locus, where 5-LO KO clearly leads to an enhanced FAIRE peak signal strength, which is in-line with the results of the RNA-Seq where we detected enhanced COX2 mRNA levels after 5-LO KO. Thus, our data show an association of 5-LO protein to active chromatin

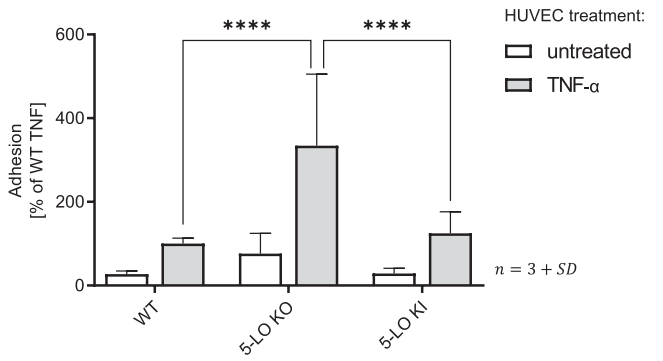


**Fig. 6. Impact of 5-LO knockout (KO) and knockin (KI) on MM6 cell adhesion.** MM6 cells (WT, MM6 5-LO KO1 and MM6 5-LO KI1) were differentiated by 1 ng/mL TGFβ / 50 nM 1,25(OH)$_2$D$_3$ for 3 days and stimulated with 1 μg/mL LPS for the last 24 h of cell differentiation. MM6 cells were fluorescence labeled and then transferred to a HUVEC monolayer which had been either activated with 1 ng/mL TNFα for 24 h or remained untreated as indicated. After 5 min adhesion time, non-adhering cells were washed off and adhering MM6 cells were determined by fluorescence measurement using a microplate reader. Statistical analysis was obtained with a two-way ANOVA based on n = 3 individual experiments. (mean + SD, ****p < 0.0001).

regions, demonstrating a novel noncanonical function of 5-LO as a direct regulator of transcription.

Subsequently, a binding pattern analysis ± 2 kb around the TSS of all genes was performed with the 5-LO ChIP-Seq data sets followed by comparison to the FAIRE-Seq results. Deeptools was used to calculate the mean binding profile around the TSS of all annotated genes from bigWig score files (GEO: GSE200338) revealing that 5-LO binds predominantly upstream and downstream in close proximity to the TSS, while the FAIRE-Seq peak locates exactly at the TSS (Fig. 9). Thus, it appears that 5-LO binds preferentially around active promoters, possibly stabilizing euchromatin or acting as a chromatin remodeler. Nevertheless, 5-LO does not seem to associate to all promoters of differentially expressed genes in MM6 cells.

In the next step, we aimed to identify transcription factors, which may co-localize with 5-LO on DNA, because the complex nature of gene regulation suggests the involvement of one or more transcription factors, which might interact with 5-LO. Using ChIP-Atlas, we compared the complete 5-LO ChIP-Seq datasets with a database of public data stored in sequence read archives of other ChIP-Seq experiments with transcription factors or histone modifications. Fig. 10 presents the results sorted and shaded by significance (p-value), where only the most significant data set for each ChIP antigen is shown. Additionally, only data sets derived from myeloid cell lines were considered to ensure maximum cell-type specificity. The transcription factor RBFOX2 intersected with around 15% of the 5-LO peaks in K562 cells followed by the chromatin reader proteins BRD4 and BRD2 and the histone methyl transferase MLL1 (*KMT2A*).

As the identified transcription factors are known chromatin binders [69–75], we also included histone marks in the analysis. Interestingly, activating histone acetylations at H3K27, H3K18 and H3K9 share a high rate of overlaps with 5-LO peaks, independent of LPS stimulation

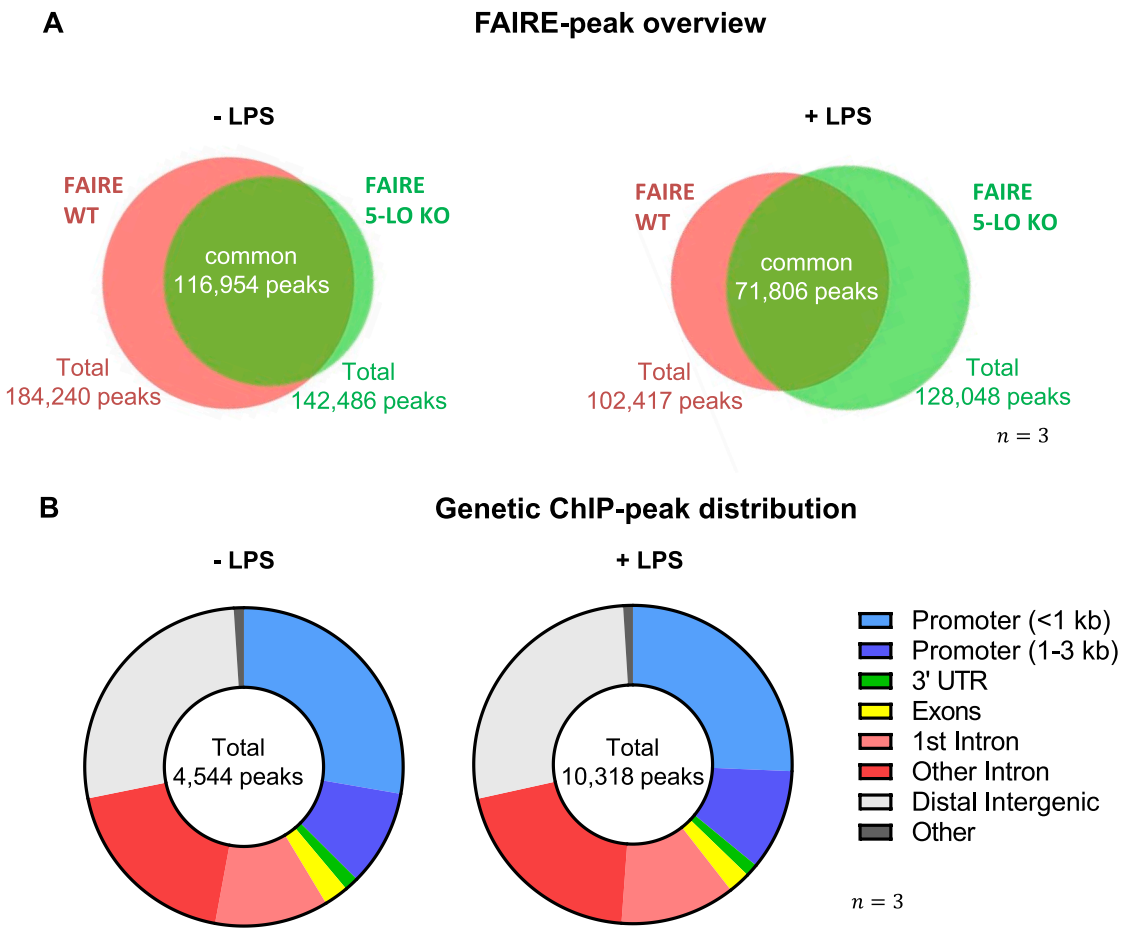


**Fig. 7. FAIRE- and 5-LO ChIP-sequencing of differentiated MM6 cells.** MM6 cells (WT and 5-LO KO) cells were differentiated by 1 ng/mL TGFβ / 50 nM 1,25 (OH)$_2$D$_3$ for 3 days and stimulated with or without 1 μg/mL LPS for the last 2 h of cell differentiation. **(A)** Venn diagram of FAIRE-Seq comparing peaks of MM6 WT and 5-LO KO cells. **(B)** The distribution of the number of significant ChIP peaks across different genomic loci (using ChIP-Seeker) in MM6 WT cells.

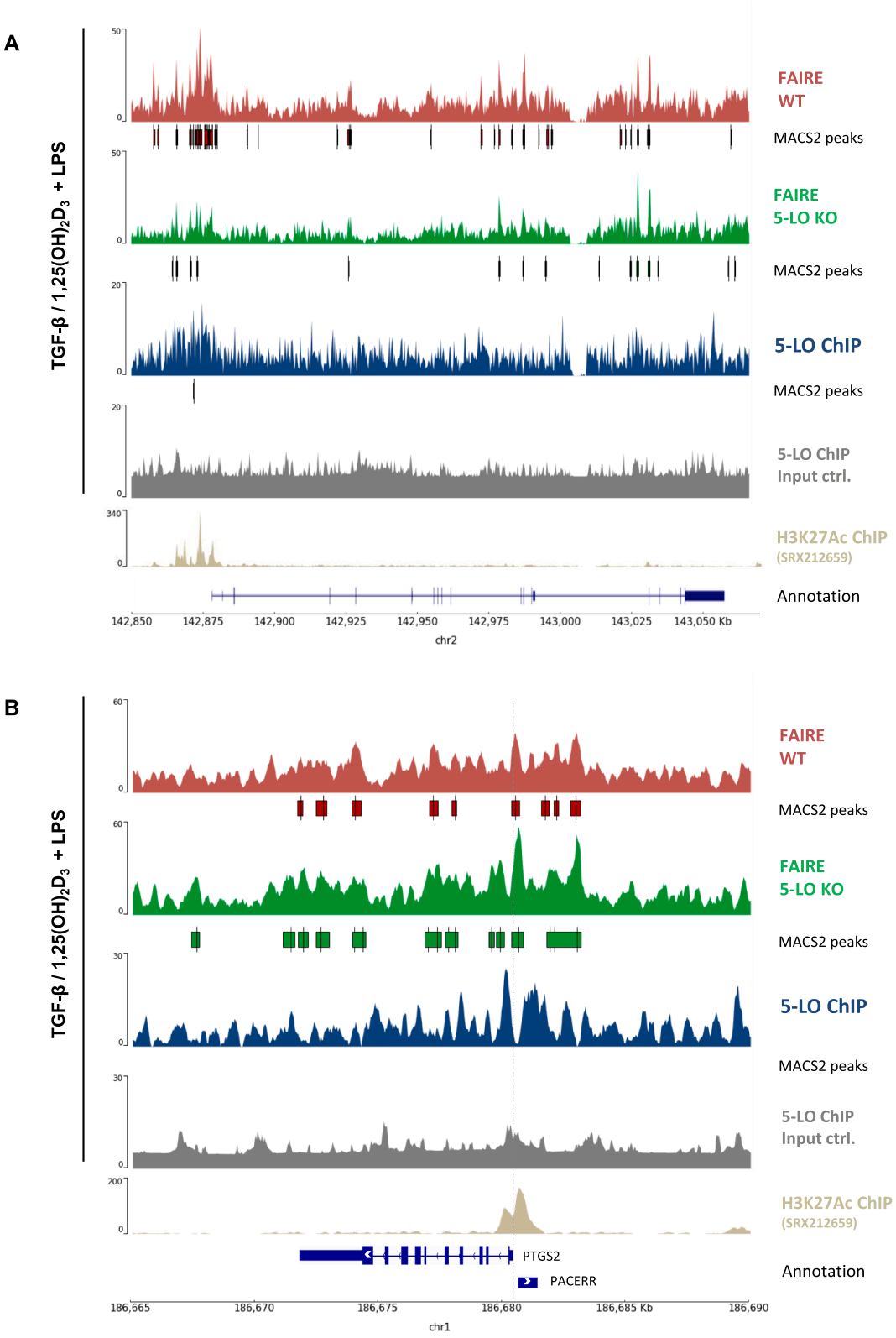


**Fig. 8. Genome browser tracks of the gene *KYNU* (A) and *PTGS2* (COX2) (B) with FAIRE- and 5-LO ChIP-Seq data in combination with the histone marker H3K27Ac.** MM6 cells (WT and 5-LO KO1) were differentiated with 1 ng/mL TGFβ / 50 nM $1,25(OH)_2D_3$ for 3 days and stimulated with 1 μg/mL LPS for the last 2 h of cell differentiation. Peaks recognized as significant by MACS2 are indicated below the tracks. Histone modification track H3K27Ac was obtained from CD14 + monocytes by the ENCODE project (ChIP-Atlas: SRX212659).

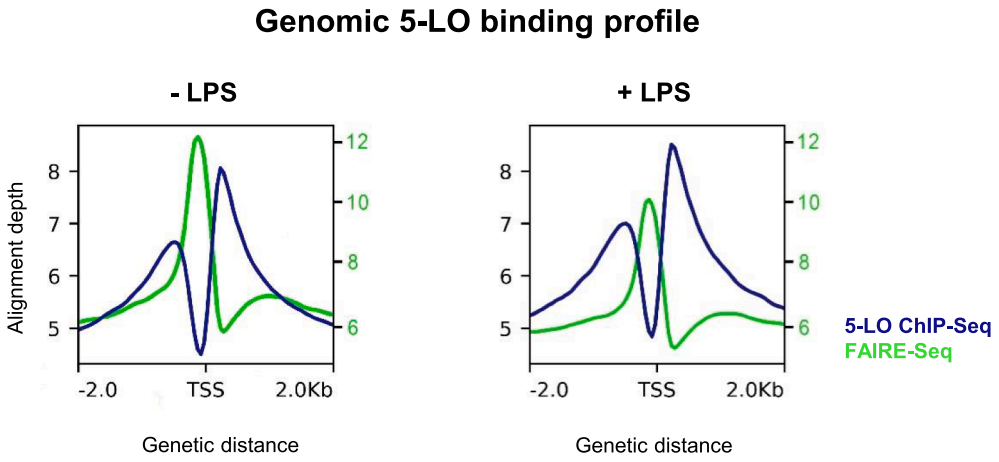


**Fig. 9. FAIRE-Seq signal and 5-LO ChIP-Seq binding profile around TSSs.** MM6 cells (WT and 5-LO KO) were differentiated by 1 ng/mL TGFβ / 50 nM 1,25 $(OH)_2D_3$ for 3 days and stimulated with or without 1 μg/mL LPS for the last 2 h of cell differentiation. The 5-LO binding pattern ± 2 kb around transcription start sites of the whole genome (using deepTools) in MM6 WT cells is shown in blue in comparison to the FAIRE-Seq profile in green.

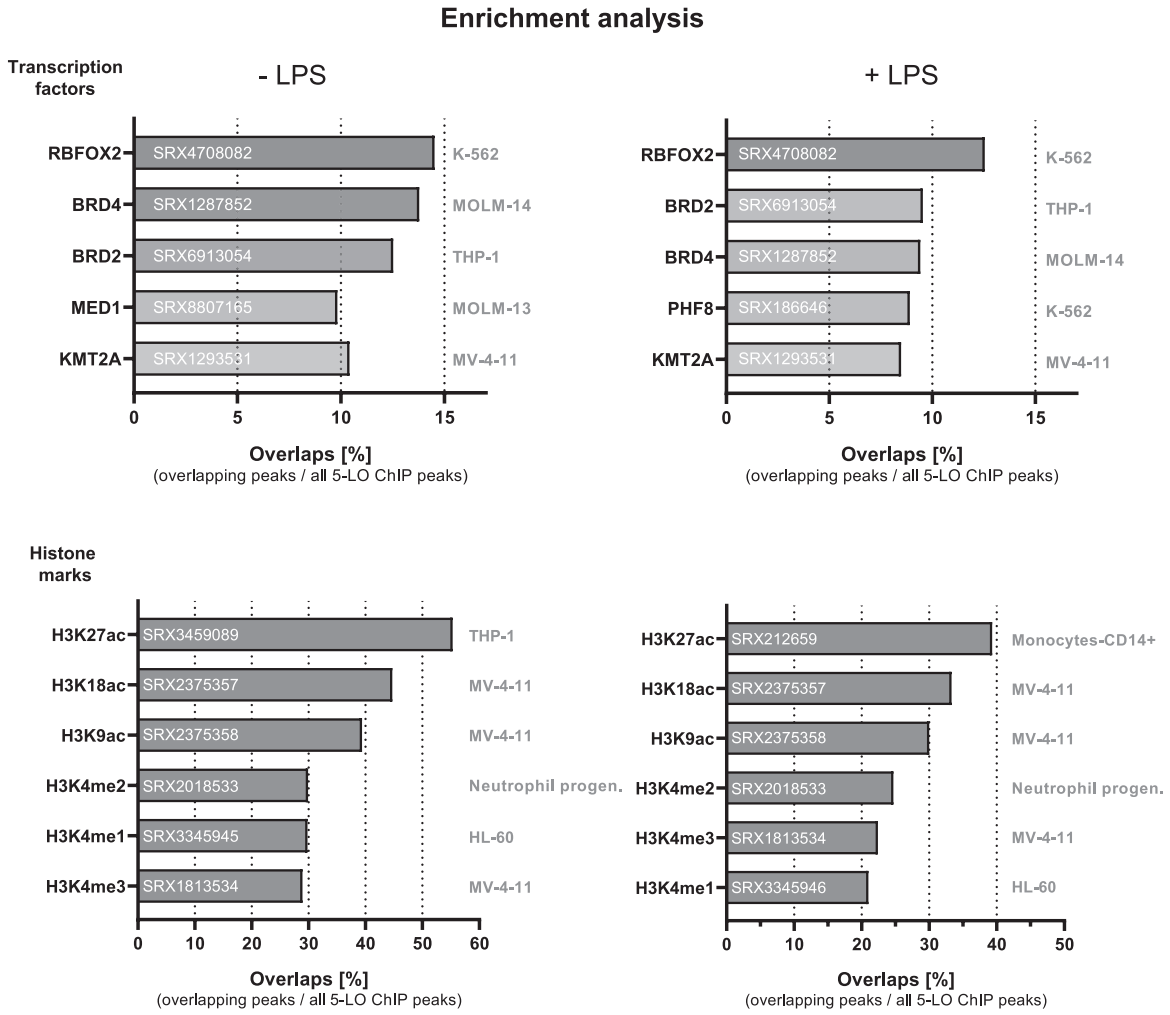


**Fig. 10. Enrichment analysis for identification of transcription factors that co-localize with 5-LO.** Significant 5-LO ChIP-Seq peaks of differentiated MM6 WT cells (+/- LPS) were compared to other ChIP-Seq datasets of blood cells using the ChIP-Atlas. The overlaps of those datasets were calculated by dividing the overlapping peaks by all 5-LO ChIP peaks and only the most significant data set of the respective transcription factor or histone is shown. Data are sorted and shaded by significance (log p-value) and in second order by number of overlaps both descending from top to bottom.

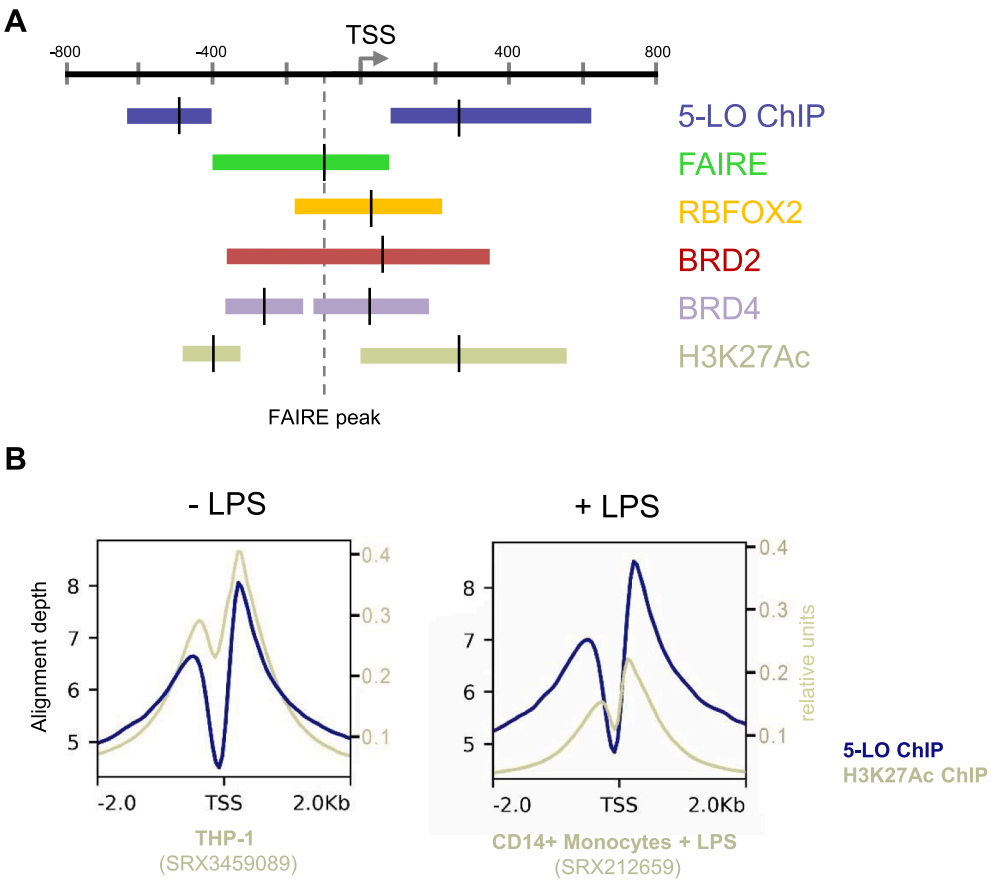


**Fig. 11. Comparison of DNA binding pattern around TSSs of 5-LO with the H3K27 acetylation pattern and other enriched transcription factors.** MM6 WT cells were differentiated by 1 ng/mL TGFβ / 50 nM $1,25(OH)_2D_3$ for 3 days and stimulated with 1 μg/mL LPS for the last 2 h of cell differentiation. **(A)** Comparison of the 5-LO binding pattern with the binding pattern of other transcription factors in combination with regulatory elements at the TSS. Data are taken from the ChIP-Atlas database (ChIP-Atlas: SRX4708082, ChIP-Atlas: SRX1287852, ChIP-Atlas: SRX1287852, ChIP-Atlas: SRX212659). **(B)** The 5-LO binding and H3K27Ac pattern ± 2 kb around the TSSs of the whole genome. THP-1 cells were differentiated by $1,25(OH)_2D_3$ for 24 h (ChIP-Atlas: SRX3459089) and CD14-positive monocytes from human leukapheresis production cultured with M-CSF (10 ng/mL) and stimulated with LPS (50 ng/mL) for 3 h (ChIP-Atlas: SRX212659).

(Fig. 10). Fig. 11A shows a comparison of the binding regions of 5-LO in ChIP-Seq, open chromatin by FAIRE-Seq, the first three hits of transcriptions factor analysis (RBFOX2, BRD2 and BRD4) and the histone modification H3K27ac. Interestingly, RBFOX2 and the transcription factors BRD2 and BRD4, unlike 5-LO, bind predominantly at the TSS of genes whereas the binding pattern of 5-LO around TSSs showed the highest similarity to the H3K27ac histone mark, independent of LPS treatment (Fig. 11). In Fig. 11B, the genome-wide binding profile of 5-LO and H3K27ac with and without LPS is shown. It is clearly visible that the distribution and distance around the TSS is similar. Typically, most activating histone marks show a dip at the position of bound transcription factors, as transcription factors can only bind at nucleosome-free regions [76]. Taken together, our data show that besides its canonical function, 5-LO is located in active promotors, colocalizes with the H3K27ac histone marker and can modulate gene transcription.

## 4. Discussion

In this study, we highlight the canonical and noncanonical functions of 5-LO on transcriptional regulation in MM6 cells. For the first time we provide a complete characterization of the so far unknown direct involvement of 5-LO in transcriptional regulation and prove that 5-LO protein has a strong effect on mRNA expression of a subset of genes. By RNA-Seq, we were able to identify many 5-LO regulated target genes in the myeloid cell line MM6, which varied upon the particular treatment (cell differentiation, LPS treatment) of the cells. This new noncanonical function of 5-LO as a DNA-associated regulator was demonstrated by ChIP-Seq, further analysis revealed a similarity of the 5-LO binding pattern with the histone modification H3K27ac. Furthermore, our FAIRE-Seq data from MM6 WT and 5-LO KO cells showed that the presence or absence of 5-LO in monocytes altered the accessibility of regulatory regions at the chromatin level.

Most of the differentially expressed genes were regulated within only one of the four treatments, (differentiated by TGFβ/$1,25(OH)_2D_3$ and stimulated with LPS), suggesting that the regulatory effect by 5-LO depends on the differentiation and activation state of the cells, even though several differentially expressed genes were found in more than one treatment.

Since transcription is a tightly regulated multi-step process, it can be affected on many levels. The classical canonical 5-LO pathway can modulate transcription via the enzymatic conversion of arachidonic acid to leukotriene $A_4$ ($LTA_4$), which, depending on the cellular environment, is converted into the biologically active $LTB_4$ or the highly potent CysLTs (Fig. 12). These leukotrienes act via G-protein coupled receptors, which, as in the case of $LTB_4$, upregulate MyD88 via SOCS1 and the JAK-Stat pathway and thus influence NF-κB mediated transcription [77]. To discriminate the effects of the canonical pathway from direct transcriptional regulation, we used inhibitors of the leukotriene cascade and reintroduced the enzymatically inactive 5-LO mutant E376Q into 5-LO KO cells. For *KYNU* and *PTGS2*, we were able to demonstrate an leukotriene-independent regulation of gene expression (Fig. 5) whereas regulation of *ITGAM* seems to be mediated via $LTB_4$ signaling, which is consistent with previous publications [24,78]. The observation that COX2 (*PTGS2*) is a 5-LO target gene which is inversely regulated by 5-LO independent of its catalytic activity is interesting, since both enzymes use arachidonic acid as substrate and produce oxylipins which are involved in the onset and resolution of inflammation. Thus, this regulatory mechanism could be involved in switching between leukotriene and prostaglandin formation during immune reactions.

The observed 5-LO KO effects on integrin expression and on cell adhesion of monocytes can be relevant for inflammatory processes. In LPS stimulated, differentiated MM6 cells, 5-LO inhibited the adhesion of the cells. This result is consistent with the increased expression of

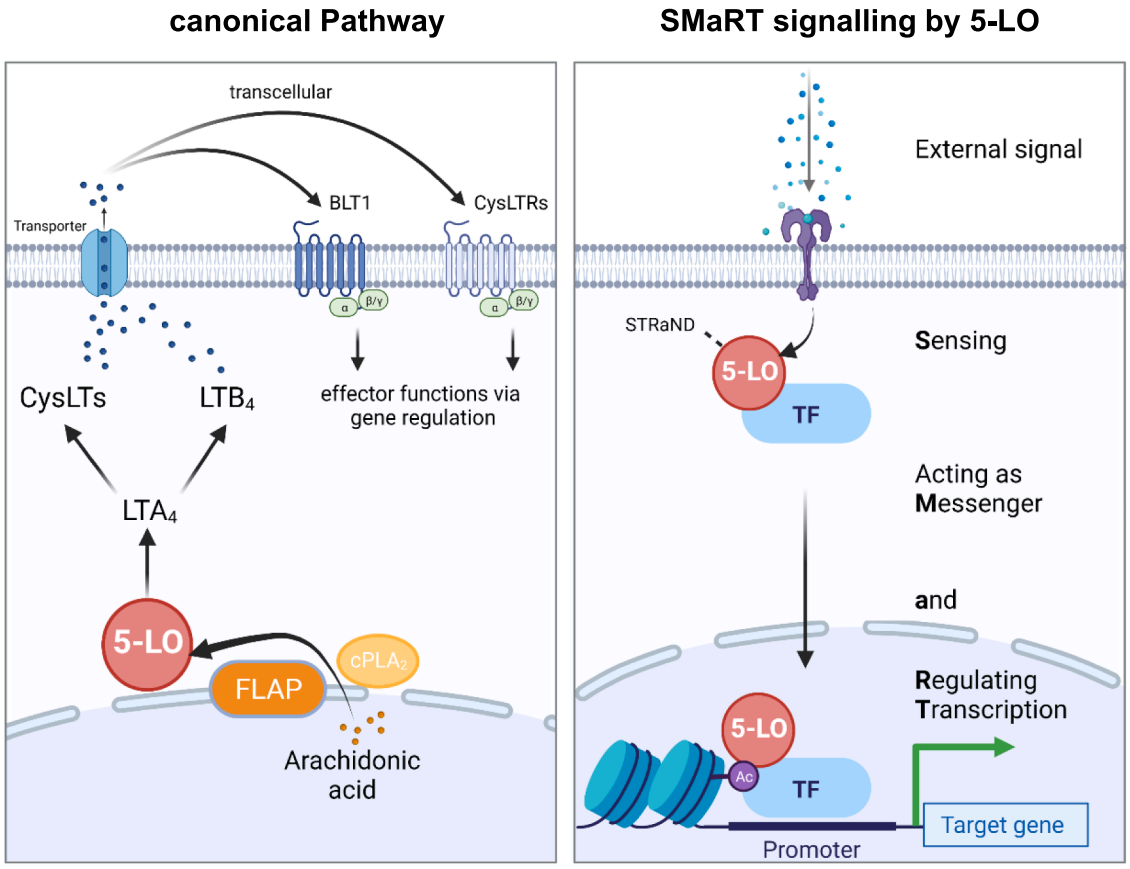


**Fig. 12.** Mechanisms of 5-LO regulated transcription.

*ITGAM* in MM6 5-LO KO cells, which, as a component of the Mac-1 heterodimer, has an important role in monocyte adhesion and migration [79,80]. This observation is in line with the previous data where a slightly increased Mac-1 expression in peripheral blood of 5-LO KO mice was found [24]. However, Lee et al. observed an opposite effect, showing an $LTB_4$-dependent upregulation of Mac-1 in LPS treated THP-1 cells and mouse macrophages. The increased expression of Mac-1 and enhanced monocyte adhesion was attenuated in 5-$LO^{(-/-)}$ mice [78]. Although all three studies show a $LTB_4$-dependent relationship between 5-LO and *ITGAM* expression, the outcome appears to be strongly dependent on the cellular environment. Besides inflammatory bowel disease and atherosclerosis, this 5-LO mediated regulation of integrins and cell adhesion may be relevant for other inflammatory diseases. Recently, the influence of 5-LO KO on the expression of inflammatory markers in mouse periodontitis was investigated and revealed that *ITGAM* was upregulated in 5-LO KO mice after 7 days compared to control mice [81]. Furthermore, Xia et al. demonstrated that 5-LO inhibitors or 5-LO KO in the mantle cell lymphoma (MCL) Jeko1 led to a hampered migration and adherence of the cells [82]. Taken together, the participation of 5-LO on adhesion processes of different cell types suggests a pivotal role of 5-LO in the inflammatory response and several inflammatory diseases.

Kynureninase is involved in tryptophan metabolism and is particularly expressed in innate immune cells. Recently, there is emerging evidence that the tryptophan-kynurenine metabolism is an important pro-inflammatory process in inflammatory bowel diseases. As a result of immune cell migration into inflammatory intestinal tissue [83,84], tryptophan metabolizing enzymes are expressed at the site of inflammation to maintain the intestinal barrier and prevent adaptive immunity [85,86]. This immunosuppressive effect is thought to be mediated by kynurenine, the substrate of kynureninase [65,66]. Interestingly, the results of our study show that the upregulation of *KYNU* mRNA and protein expression by LPS is tightly linked to the presence of 5-LO protein in MM6 cells. Furthermore, proinflammatory M1 macrophages exhibit significantly greater 5-LO expression than M2 macrophages [39], which does not appear to correlate with kynureninase expression levels. However, it has to be considered that intracellular localization of 5-LO seems to be different in M1 and M2 macrophages. Confocal microscopy experiments of 5-LO localization show that the 5-LO of M2 macrophages translocates completely to nuclear hotspots after LPS stimulation, whereas in M1 macrophages a large fraction remains cytosolic [87]. Furthermore, M1 macrophages show high 5-LO activity whereas activity in M2 macrophages is extremely low [39] which might be related to different 5-LO functions.

Recently, increased expression of kynureninase in inflammatory mucosa has been identified as a potential biomarker for ileal Crohn's disease, which suggests that 5-LO has a dual proinflammatory effect [37]. On the one hand, mediated through its canonical action and associated secretion of chemotactic $LTB_4$ and on the other hand, as a positive regulator of *KYNU* expression, leading to the degradation of the anti-inflammatory kynurenine [88,89].

Interestingly, for some cell types like lymphocytes or solid tumors like adenocarcinoma or glioblastoma it is known from literature that these cells or tissues express 5-LO protein but lack enzymatic activity. Furthermore, 5-LO expression in those tumor cells correlates with poor disease progression [15,16]. A noncanonical function of 5-LO as direct chromatin-associated regulator of transcription might explain the presence of enzymatically inactive 5-LO in these cells.

Former studies showed that 5-LO is localized in euchromatin, without providing a functional explanation [90]. To the best of our knowledge, we provide the first ChIP-Seq results for a lipoxygenase, mainly known for its enzymatic activity and prove its association to

chromatin. The local binding pattern of 5-LO around TSSs rather coincides with histone marks than with the binding pattern of transcription factors which predominantly occupy the TSSs. These observations are in line with a mechanism proposing that cytosolic proteins shuttling to the nucleus may serve as extracellular signal effectors. A so-called SMaRT signaling (sensing, acting as a messenger and regulating transcription) was suggested where these proteins sense extracellular signals, translocate to the nucleus and regulate gene transcription in association with transcription factors [38]. These STraNDs bind to DNA indirectly only via recruitment of transcription factors. This effect can possibly result in broad ChIP-Seq signals like the ones we observed here. Thereby, STRaNDs may regulate transcription on many levels. First, they may maintain or close a local open chromatin environment around transcription factor binding sites or second, they may act as regulator of transcription factors either by trapping the factors outside the nucleus or upon activation, guiding them inside the nucleus. By conducting 5-LO inhibitor experiments, Brand et al. already discovered that 5-LO is involved in the Wnt signaling pathway through an interaction with β-catenin and regulates the intracellular localization of β-catenin [25]. The data also suggested the involvement of 5-LO in other developmental pathways such as the Hedgehog pathway or TGFβ, BMP and activin-A signaling, further supporting the function of 5-LO as an effector in SMaRT signaling.

For the identification of potential 5-LO interacting proteins, an enrichment analysis of our ChIP-Seq peaks on the basis of myeloid cells was performed. Among the transcription factors, RBFOX2, BRD2 and BRD4 provided the strongest overlaps with the 5-LO ChIP-Seq peaks. RBFOX2 is an RNA binding protein with elucidated function as a splicing regulator but increasing evidence suggests that it is also involved in transcriptional control and chromatin interactions [69]. Its binding has a strong preference for hotspots in the genome, in particular gene promoters, where it is often localized in active chromatin regions [91]. Besides, BRD2 and BRD4 are also chromatin-reading proteins which bind to acetylated histones, playing a key role in the transmission of epigenetics and transcriptional regulations [72,92,93,71]. BRD4 recognizes the acetylated NF-κB component p65 in addition to acetylated histones, which ultimately leads to activation of NF-κB [70]. Interestingly, there is a previous report on a protein/protein interaction between 5-LO and p65 observed in HL-60 cells [32]. However, BRD4 following CK2 phosphorylation influences p53 controlled transcription [93] and 5-LO has been shown to regulate nuclear trafficking of p53 and to inhibit p53 transcriptional activity [10,94]. This presumed association of 5-LO with chromatin-altering proteins such as BRD2/4 and RBFOX2 is a possible explanation for the detected reduced chromatin accessibility at the *KYNU* promoter and strongly decreased mRNA expression in MM6 5-LO KO cells. MLL1 (*KMT2A*) is another transcription factor where a significant overlap in the ChIP-Seq signals exists. Interestingly, MLL1 and its oncogenic variant MLL-AF4 was previously identified as potent activator of the *ALOX5* promoter and it was subsequently found that the TGFβ-mediated induction of *ALOX5* promoter activity is MLL-dependent [95,22]. Thus, 5-LO itself is a MLL target gene which might then modulate MLL-dependent transcription at a certain set of genes. One might speculate at this point, that this crosstalk is part of a circuit that modulates MLL transcriptional activity in a gene-specific manner.

By analyzing ChIP-Seq data sets, we also identified RBFOX2, BRD2 and BRD4 as candidate association partners for 5-LO. All three displayed the typical binding pattern of transcription factors with preferential binding at histone-free core promoter regions. In contrast, 5-LO showed a binding profile typical for histone marks with strong binding only upstream and downstream of the core promoters. As we assume that 5-LO does not bind to DNA directly, this may indicate that 5-LO interacts with histones and stabilizes histone acetylation and/or maintains the nucleosome-free region to facilitate transcriptional activation or repression by RBFOX2, BRD2 and BRD4. Alternatively, 5-LO may be associated on the surface of transcription factor complexes bound at the TSS and act there as a STRaND (Fig. 12).

In conclusion, we provide evidence for association of 5-LO to chromatin and propose a noncanonical function as transcriptional regulator, in addition to its role in leukotriene signaling. The mechanism of this function may involve direct interaction with transcription factors or acetylated histones and results in the demonstrated genome-wide transcriptional effects of 5-LO. Importantly, this novel 5-LO action translates in functional outcomes as seen by effects on the kynurenine pathway which opens new therapeutic options for severe diseases.

## CRediT authorship contribution statement

**Marius Kreiß:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Project administration. **Julia Oberlis:** Investigation, Formal analysis. **Sabine Seuter:** Methodology, Validation, Investigation. **Iris Bischoff-Kont:** Investigation, Formal analysis. **Duran Sürün:** Methodology. **Dominique Thomas:** Methodology, Validation, Formal analysis, Investigation. **Tamara Göbel:** Methodology, Validation. **Tobias Schmid:** Methodology, Resources. **Olof Rådmark:** Resources. **Ralf P. Brandes:** Resources. **Robert Fürst:** Resources. **Ann-Kathrin Häfner:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Funding acquisition. **Dieter Steinhilber:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

## Data availability

Data will be made available on request.

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