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E74-like factor 3 and nuclear factor-κB regulate lipocalin-2 expression in chondrocytes

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Key points

- E74-like factor 3 (ELF3) is a transcription factor regulated by inflammation in different physio-pathological situations. Lipocalin-2 (LCN2) emerged as a relevant adipokine involved in the regulation of inflammation. In this study we showed for the first time the involvement of ELF3 in the control of LCN2 expression and its cooperation with nuclear factor-κB (NFκB).
- Our results will help to better understand of the role of ELF3, NFκB and LCN2 in the pathophysiology of articular cartilage.

Abstract E74-like factor 3 (ELF3) is a transcription factor induced by inflammatory cytokines in chondrocytes that increases gene expression of catabolic and inflammatory mediators. Lipocalin 2 (LCN2) is a novel adipokine that negatively impacts articular cartilage, triggering catabolic and inflammatory responses in chondrocytes. Here, we investigated the control of LCN2 gene expression by ELF3 in the context of interleukin 1 (IL-1)-driven inflammatory responses in chondrocytes. The interaction of ELF3 and nuclear factor-κB (NFκB) in modulating LCN2 levels was also explored. LCN2 mRNA and protein levels, as well those of several other ELF3 target genes, were determined by RT-qPCR and Western blotting. Human primary chondrocytes, primary chondrocytes from wild-type and *Elf3* knockout mice, and immortalized human T/C-28a2 and murine ATDC5 cell lines were used in *in vitro* assays. The activities of various gene reporter constructs were evaluated by luciferase assays. Gene overexpression and knockdown were performed using specific expression vectors and siRNA technology, respectively. ELF3 overexpression transactivated the LCN2 promoter and increased the IL-1-induced mRNA and protein levels of LCN2, as well as the mRNA expression of other pro-inflammatory mediators, in human and mouse chondrocytes. We also identified a collaborative loop between ELF3 and NFκB that amplifies the induction of LCN2. Our findings show a novel role for ELF3 and NFκB in the induction of the pro-inflammatory adipokine LCN2, providing additional evidence of the interaction between ELF3 and NFκB in modulating inflammatory responses, and a better understanding of the mechanisms of action of ELF3 in chondrocytes.

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Abbreviations AP-1, activating protein 1; C/EBPβ, CCAAT/enhancer binding protein β; COL2A1, type II collagen; COX2, cyclooxygenase 2; ECM, extracellular matrix; ELF3, E74-like factor 3; IL, interleukin; LCN2, lipocalin 2; LPS, lipopolysaccharide; MMP, matrix Metalloproteinase; NFκB, nuclear factor-κB; NO, nitric oxide; NOS2, nitric oxide synthase 2; OA, osteoarthritis; TNF-α, tumour necrosis factor α; VCAM-1, vascular cell adhesion molecule 1.

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Introduction

Articular cartilage is a highly specialized connective tissue that lines bone surfaces, providing low-friction and hydrodynamic load-bearing properties that protect articulating joints. In adult articular cartilage, chondrocytes are the unique cell component that is responsible for the turnover and maintenance of collagens, proteoglycans and other molecules of the extracellular matrix (ECM). In physiological conditions, chondrocytes remain as quiescent cells. However, during cartilage degenerative diseases, these cells exhibit an aberrant behaviour characterized by enhanced expression and activities of cartilage degrading enzymes and the production of inflammatory mediators, among other processes (Hunter & Felson, 2006; Goldring & Goldring, 2010). Several factors are involved in the pathogenesis of cartilage degenerative diseases, and stress- or inflammation-induced signalling pathways are known to contribute to destruction of articular cartilage in both osteoarthritis (OA) and rheumatoid arthritis (RA) (Felson, 2005; Otero *et al.* 2005; Berenbaum, 2013). Cytokines and adipokines play a major role in inflammatory responses (Goldring *et al.* 2011; Conde *et al.* 2013). These factors up-regulate the expression of a number of genes encoding catabolic and inflammatory regulators, including metalloproteinase (MMP) 13, cyclooxygenase 2 (COX2) or nitric oxide synthase 2 (NOS2), via the induction and activation of transcription factors such as nuclear factor-κB (NF-κB), CCAAT/enhancer binding protein β (C/EBPβ), activating protein 1 (AP-1) family members, and ETS factors (Grall *et al.* 2003; Hirata *et al.* 2012; Otero *et al.* 2012).

The ETS transcription factors constitute a family of at least 30 members involved in the regulation of differentiation, cell proliferation and ECM remodelling in both physiological and pathological conditions (Oettgen *et al.* 1997; Grall *et al.* 2003). The ETS family member E74-like factor 3 (ELF3), also known as ESE-1, ESX, ERT and JEN, is expressed exclusively in epithelial tissues in physiological conditions (Oettgen *et al.* 1997), but ELF3 can be found in different tissues and cell types under inflammatory conditions. For instance, ELF3 levels are increased in OA and RA synovial tissues (Grall *et al.* 2003), and it mediates inflammatory actions through transcriptional control of genes such as NOS2 and COX2 (Rudders *et al.* 2001; Grall *et al.* 2005). ELF3 displays a pro-catabolic/anti-anabolic role in chondrocytes, by acting as a repressor of type II collagen gene (*COL2A1*) promoter activity (Peng *et al.* 2008) and as a transactivator of the MMP13 promoter via a proximal evolutionarily conserved ETS binding site (EBS) (Otero *et al.* 2012).

Obesity is one of the major risk factors for degenerative joint diseases (Conde *et al.* 2013, 2014). It is characterized by excessive accumulation of dysfunctional white adipose tissue that synthesizes a plethora of factors, generally called adipokines. The relationship between adipokines and cartilage degeneration is now widely recognized (Otero *et al.* 2003; Lago *et al.* 2008; Conde *et al.* 2012), and a number of adipokines are known to exert direct pro-inflammatory and catabolic actions in articular chondrocytes via induction of interleukin (IL)-8, NOS2, vascular cell adhesion molecule 1 (VCAM-1), MMP3 and MMP13 (Gomez *et al.* 2011; Otero *et al.* 2003; Lago *et al.* 2008; Conde *et al.* 2012; Scotece *et al.* 2014). Lipocalin 2 (LCN2), also termed siderocalin, 24p3, uterocalin and neutrophil gelatinase-associated lipocalin, was described originally as a novel adipokine isolated from neutrophil granules, although it is believed that adipose tissue is the main source of this glycoprotein (Steppan *et al.* 2001). LCN2 protein has been isolated as a 25 kDa monomer, a 46 kDa homodimer, and in a covalent complex with MMP9 (Kjeldsen *et al.* 1994, 2000). This adipokine is involved in different biological processes such as apoptosis of haematopoietic cells (Devireddy *et al.* 2001), transport of fatty acids and iron (Chu *et al.* 1998), and modulation of inflammation (Cowland & Borregaard, 1997). More recently we reported that LCN2 is expressed by chondrocytes, where it induces the expression of NOS2 in combination with lipopolysaccharide (LPS; Conde *et al.* 2011), and we have recently described a novel inflammatory loop in which nitric oxide (NO) is able to regulate LCN2 expression (Gómez *et al.* 2013). Moreover, LCN2 levels are increased in synovial fluid of RA patients (Katano *et al.* 2009). All these data, together with the fact that LCN2 is able to participate in the stabilization of molecular complexes with MMP9 in articular cartilage (Gupta *et al.* 2007), clearly support the notion that LCN2 is a relevant factor in cartilage degradative processes.

Therefore, in the present study we focused on the mechanism(s) by which ELF3 regulates several inflammatory mediators in chondrocytes, with a special emphasis on IL-1-driven induction of LCN2.

Methods

Reagents

All culture reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) except Dulbecco´s modified Eagle's medium (DMEM)/Ham's F12 medium and trypsin-ethylenediaminetetraacetic acid (Lonza, Verviers, Belgium). For RT-qPCR analysis, First Strand reverse transcription kit, SYBR-green qPCR master mix, and primers were purchased from SABiosciences (Frederick, MD, USA). Nucleospin kits for RNA and protein isolation were from Macherey-Nagel (Düren, Germany). Human recombinant IL-1α, mouse recombinant IL-1α, PD098059, pyrrolidine dithiocarbamate (PDTC), and N^G-nitro-L-arginine methyl ester (L-NAME) were from Sigma-Aldrich. For transfection assays, Lipofectamine

Reagent, PLUS Reagent, and Opti-MEM I Reduced Serum Media were purchased from Invitrogen (Carlsbad, CA, USA), and the Luciferase Assay (Cat. no. E1500) and Renilla Luciferase Assay (Cat. no. E2810) systems were from Promega (Madison, WI, USA). For silencing experiments, the TriFECTa RNAi kit for human leptin receptor and human ELF3 were purchased from Integrated DNA Technologies (Coralville, IA, USA) and siLentFect from Bio-Rad Laboratories, Hercules, CA, USA.

Cell culture conditions and treatments

The human juvenile costal chondrocyte cell line T/C-28a2 was cultured as described previously (Goldring *et al.* 1994). For experiments, cells were seeded in six-well plates in DMEM/Ham's F12 supplemented with 10% fetal bovine serum (FBS) L-glutamine, and antibiotics (50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin). The murine chondrogenic cell line ATDC5 (purchased from RIKEN Cell Bank, Tsukuba, Japan) was cultured in DMEM/Ham's F12 supplemented with 5% FBS, 10 μg ml^{-1} human transferrin, 3×10^{-8} M sodium selenite, and antibiotics (50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin).

Human primary chondrocytes were isolated and cultured, as described (Otero *et al.* 2012), from articular cartilage samples obtained from knee joints of patients undergoing total joint replacement (with written patient consent and permission from the local ethics committee). Cells were treated with human or mouse IL-1 for the indicated times and concentrations.

Elf3 knockout mice (Ng *et al.* 2002) were obtained from Dr Melanie A. Pritchard, Monash University, Clayton, Victoria, Australia. All experiments were performed according to the guidelines of the American Veterinary Association, and were approved by the IACUC of the Hospital for Special Surgery. All mice used in this study were killed for tissue collection using methods that are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association, and animals were examined to confirm death before tissue was obtained. Primary mouse chondrocytes were isolated as described (Gosset *et al.* 2008; Otero *et al.* 2012) from 5- to 6-day-old wild-type (C57BL/6) or *Elf3* knockout mouse articular cartilage. Briefly, 5- to 6-day-old mice were killed using hypothermia-induced anaesthesia followed by decapitation. Immediately after killing, articular surfaces were dissected and digested in collagenase D for 16 h at 37°C, and filtered through a 40 μm cell strainer. Passage 0 chondrocytes were seeded in DMEM/F12 containing 10% FBS and allowed to reach confluence. Confluent cells were trypsinized and seeded in DMEM/F12/10% FBS for experimental purposes. For experimental purposes, passage 1 primary chondrocytes were seeded at a density of 2.5×10^4 cells cm^{-2} in DMEM/F-12 supplemented with 10% FBS. All experiments involving stimulation with inflammatory cytokines were performed in serum-free conditions.

Specific pharmacological inhibitors were added 1 h before stimulation at the indicated concentrations. All treatments were performed in at least three independent experiments.

Nitrite accumulation

Nitrite accumulation was measured in the culture medium by the Griess reaction, as previously described (Gómez *et al.* 2013; Santoro *et al.* 2015). Briefly, 100 μl of cell culture medium was incubated at room temperature for 10 min with 100 μl of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine HCl), and then the absorbance at 550 nm was measured in a microplate reader (Titertek Multiscan; Labsystems, Helsinki, Finland). Fresh culture medium was used as the blank in all experiments. The amount of nitrite (in μM) was calculated from a sodium nitrite standard curve in freshly prepared culture medium.

RNA extraction and real-time reverse transcription–polymerase chain reaction

mRNA levels were determined using SYBR-green based quantitative PCR (qPCR). Briefly, RNA was extracted using a NucleoSpin kit according to the manufacturer's instructions, and reverse-transcribed using an SABiosciences First Strand Kit. After the RT reaction, qPCR analysis was performed with an SABiosciences Master Mix and specific PCR primers for human *ELF3* (82 bp, PPH09786B, reference position 2868, GenBank accession no. NM_004433.4); mouse *Elf3* (105 bp, PPM04684E, reference position 420–440, GenBank accession no. NM_007921.2); human *GAPDH* (175 bp, PPH00150E, reference position 1287–1310, GenBank accession no. NM_002046.3); mouse *Lcn2* (81 bp, PPM03770A, reference position 364–383, GenBank accession no. NM_008491.1); human *LCN2* (87 bp, PPH00446E, reference position 626–644, GenBank accession no. NM_005564.3); mouse *Gapdh* (140 bp, PPM02946E, reference position 309, GenBank accession no. NM_008084.2); human *MMP13* (150 bp, PPH00121B, reference position 221–241, GenBank accession no. NM_002427.2); human *IL6* (160 bp, PPH00560B, reference position 755, GenBank accession no. NM_000600.3); human *TNFα* (110 bp, PPH00341F, reference position 749, GenBank accession no. NM_000594.3); human *COX2* (68 bp, PPH01136E, reference position 4078–4096, GenBank accession no. NM_000963.1); human *RELA* (65 bp,

PPH01812B, reference position 776, GenBank accession no. NM_021975.3). Amplification efficiencies were calculated for all primers utilizing serial dilutions of the pooled cDNA samples. The data were calculated, using the comparative ($\Delta\Delta C_t$) method and MxPro software (Stratagene, La Jolla, CA, USA), as the ratio of the expression of each gene to that of the housekeeping gene, as described previously (Otero *et al.* 2012). Data are shown as the mean ± SEM (error bars) of at least three independent experiments and represented as fold-change *vs.* control. Melting curves were generated to ensure a single gene-specific peak, and no-template controls were included for each run and each set of primers to control for unspecific amplifications.

Plasmid constructs and DNA transfection assays

The WT and NF-κB-mutant human ELF3 luciferase promoter sequences were described previously (Rudders *et al.* 2001; Grall *et al.* 2003). The luciferase vector containing the LCN2 promoter (p-LCN2 plasmid no. 28225) was purchased from Addgene (Cambridge, MA, USA). We also performed luciferase reporter assays using the pGL4.32 (luc2P/NF-κB-RE/Hygro) vector (a kind gift from Dr Pina Carbone, Institute of Oncology Research, Bellinzona, Switzerland).

Transfection experiments were carried out in the T/C-28a2 cells, basically as described (Otero *et al.* 2012). Briefly, cells were seeded at 2×10^5 cells per well in six-well plates and transfected with a total of 400 ng of DNA of each construct and 200 ng of *Renilla* luciferase reporter vector (Promega) as a transfection efficiency normalizer, using Lipofectamine PLUS reagents and Opti-MEM I reduced serum media. Luciferase activities were measured using the Luciferase Assay System and *Renilla* Luciferase Assay System, respectively, in a FLUO star BMG Labtech luminometer (Offenburg, Germany). We also transiently transfected chondrocytes with pCI-ELF3 (Peng *et al.* 2008), with p50 or p65 expression vectors (Addgene), or with the corresponding empty vector (pCI or pCMV4). Transfections were performed independently at least three times.

siRNA transfection

Chondrocytes were seeded at 2×10^5 cells per well in six-well plates and incubated overnight with DMEM/Ham's F12 with 10% FBS. The medium was then changed to serum and antibiotics-free medium. Transfections were performed following the manufacturer's instructions. For gene silencing we used 20 nM of ELF3 siRNA, and 20 nM of a non-targeting siRNA as control to verify the specificity of the ELF3 gene knockdown. Incubation was continued for 72 h after siRNA transfection, the ELF3 knockdown was verified at the mRNA and protein levels (data not shown), and the cells were subsequently stimulated with the appropriate treatments.

Western blotting analysis

Whole cell lysates were extracted using a lysis buffer for protein extraction (10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orhtovanadate, 0.5% Triton X-100, protease inhibitor cocktail), and analysed following immunoblotting procedures described previously (Lago *et al.* 2008). Immunoblots were incubated with specific antibody against mouse LCN2 (R&D Systems, Minneapolis, MN, USA) or human LCN2 (R&D Systems) and the bands were visualized using an Immobilon Western kit (Millipore, Billerica, MA, USA) and horseradish-peroxidase-labelled secondary antibody. To confirm equal loading for each sample, membranes were stripped in glycine buffer at pH 3 and reblotted with anti-actin antibody (Sigma-Aldrich). Autoradiographs were analysed with the EC3 imaging system (UVP, Upland, CA, USA).

Statistical analysis

Data are reported as means ± SEM (error bars) of at least three independent experiments. Statistical analyses were performed by ANOVA followed by Student's unpaired *t* test and Student–Newman–Keuls test, using Prism 4 software (GraphPad Software, La Jolla, CA, USA), with *P*-values < 0.05 considered significant.

Results

ELF3 potentiates the IL-1α-driven expression of pro-inflammatory and catabolic factors

In line with previously published articles (Grall *et al.* 2003, 2005), showing that ELF3 mediates inflammatory actions in chondrocytes, we determined that the over-expression of ELF3 was able to potentiate the effects of IL-1α on the induction of the pro-inflammatory mediators TNF-α, IL-6, and COX2 in human primary chondrocytes (Fig. 1*A*–*C*), as well as MMP13, one of the most relevant collagenases involved in articular cartilage degradation (Fig. 1*D*).

ELF3 regulates the expression of LCN2

Having demonstrated that in inflammatory conditions ELF3 is able to increase the expression of different factors that impair cartilage homeostasis, we focused our attention on the regulation of LCN2 by this transcription factor. The first evidence of a close

relationship between these two factors arose from the observation that IL-1α treatment induced rapid and sustained ELF3 gene expression in human primary chondrocytes (Fig. 1*E*), which was accompanied or followed by strong up-regulation of LCN2 mRNA (Fig. 1*F*). In mouse ATDC-5 chondrocytes, IL-1α-induced ELF3 mRNA expression peaked at 2 h, declining thereafter. However, the up-regulation of LCN2 followed the same pattern of human primary chondrocytes, being sustained even when ELF3 expression returned to basal levels (Fig. 1*G* and *H*).

Next, to gain further insights into the regulation of LCN2 by ELF3, we transiently transfected chondrocytes with the pCI-ELF3 expression vector (Peng *et al.* 2008) or with the pCI empty vector. While ELF3 over-expression alone did not alter the endogenous LCN2 levels, when pCI-ELF3-transfected cells were stimulated with IL-1α, both LCN2 mRNA and protein levels were increased significantly in comparison to IL-1α-treated cells transfected with the empty vector alone (Fig. 2*A* and *B*). To further confirm the contribution of ELF3 to the LCN2 induction, we knocked down ELF3 gene expression in human primary chondrocytes. As shown in Fig. 2*C*, the level of IL-1α-induced LCN2 mRNA was down-regulated significantly by ELF3 gene silencing. In line with this result, analysis of LCN2 mRNA in chondrocytes isolated from wild-type (WT) and *Elf3* knockout (KO) mice revealed that the IL-1α-driven LCN2 expression was significantly reduced in *Elf3* KO mouse primary chondrocytes compared to WT cells (Fig. 2*D*). Taken together, these results clearly indicate that ELF3 contributes to the IL-1α-induced LCN2 expression in

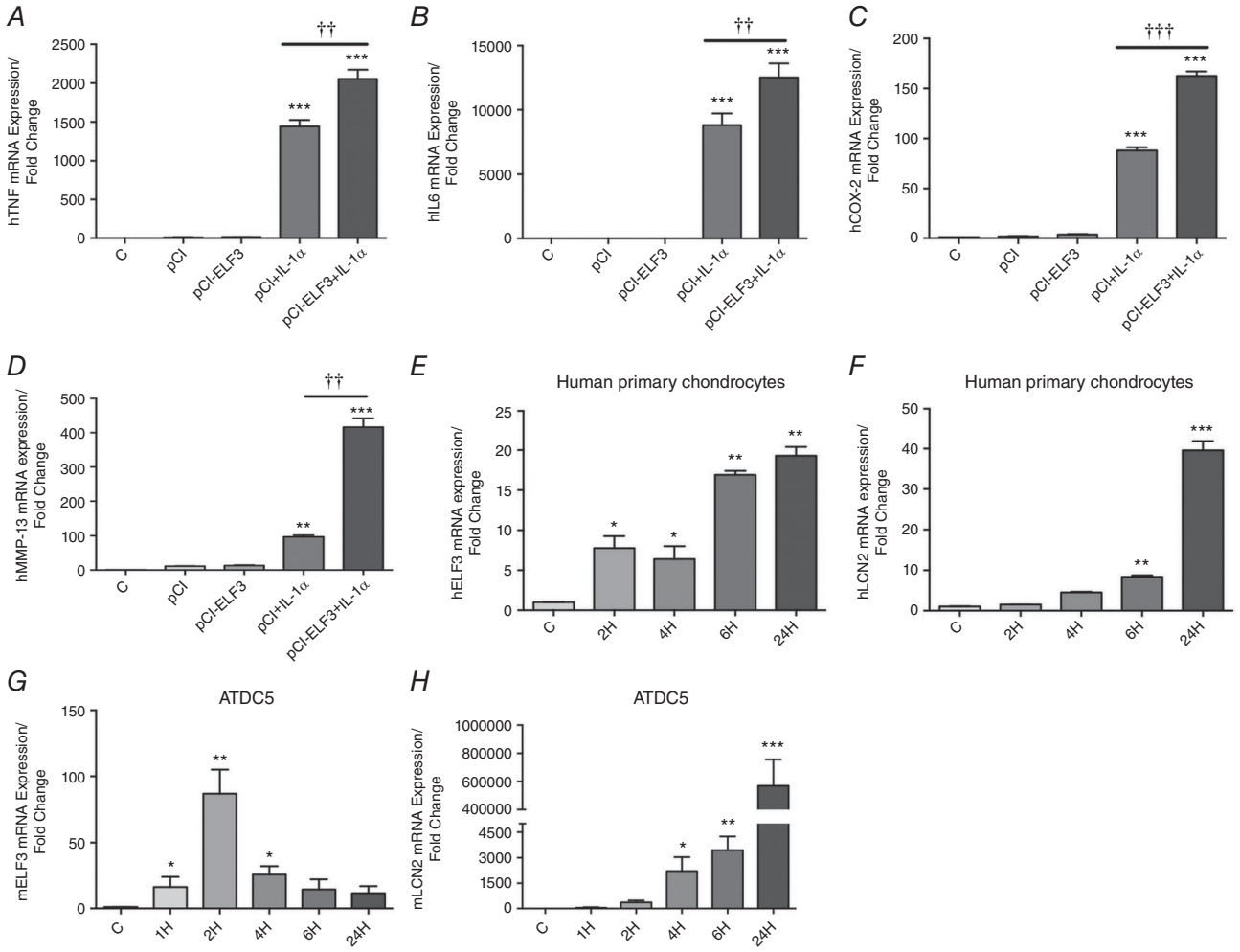

Figure 1. Expression of inflammatory factors upon ELF3 cell transfection and expression of ELF3 and LCN2 in IL-1 treated chondrocytes

A–D, TNF-α, IL-6, COX2 and MMP13 mRNA expression after transient transfection of human primary chondrocytes with the pCI-ELF3 (100 ng) expression vector or the empty vector (pCI), and IL-1α (1 ng ml^{-1}) treatment for 24 h. $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* unstimulated control; $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ *vs.* pCI+IL-1α. *E–H*, ELF3 and LCN2 mRNA expression in human primary chondrocytes and ATDC5 cells after IL-1α treatment (1 ng ml^{-1}) in a time-dependent manner (2, 4, 6 and 24 h). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* unstimulated control.

chondrocytes. However, the observation that, alone, ELF3 overexpression did not induce LCN2 expression, and that ELF3 knockdown is able to significantly, although not completely, abolish LCN2 expression, suggests that ELF3 may require activation of alternative or additional IL-1-driven mechanisms to modulate LCN2 levels in chondrocytes.

Having demonstrated that ELF3 participates in the induction of LCN2, we next analysed whether this ETS factor was able to control LCN2 gene transcription by regulating its promoter activation. To this end, we co-transfected T/C-28a2 human chondrocytes with a luciferase reporter construct containing the LCN2 promoter and different concentrations of pCI-ELF3 expression vector. As shown in Fig. 3*A*, ELF3 overexpression was able to transactivate the LCN2 promoter in a dose-dependent manner. Since ETS transcription factors are well-known MAPK effectors (Sharrocks, 2001; Tootle & Rebay, 2005), we next assessed whether the ELF3-driven LCN2 promoter transactivation was dependent upon the MAPK ERK1/2. As showed in Fig. 3*B*, inhibition of ERK1/2, by using the pharmacological inhibitor PD098059, significantly reduced the transactivation of the LCN2 promoter induced by ELF3 overexpression. For completeness, the participation of this kinase in the regulation of the endogenous levels of LCN2 was analysed

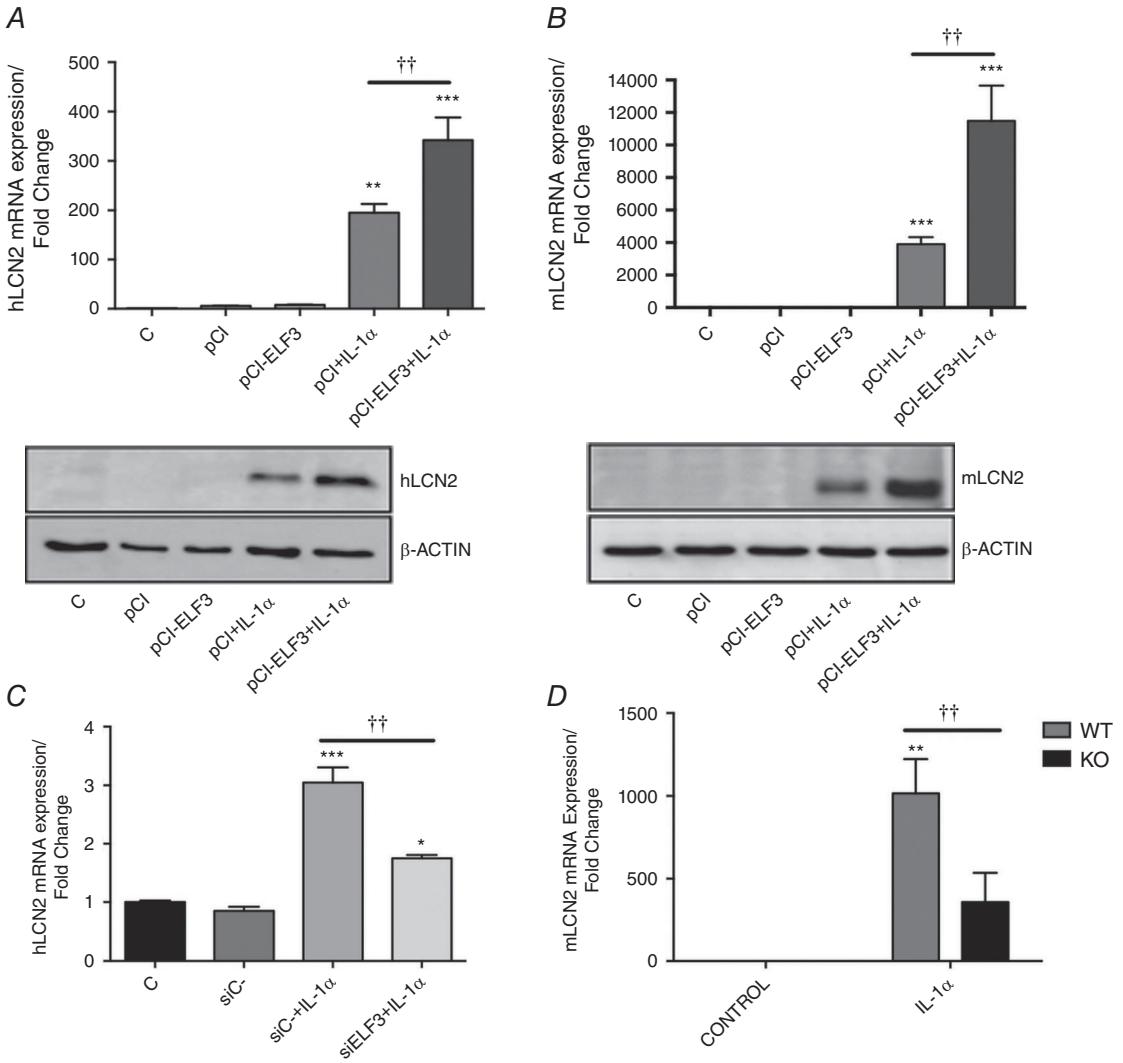

Figure 2. ELF3-induced LCN2 mRNA and protein in human primary chondrocytes and ATDC5 cells
A and *B*, hLCN2 mRNA and protein levels after transient transfection with the pCI-ELF3 (100 ng) expression vector or the empty vector (pCI) and IL-1α (1 ng ml^{-1}) treatment for 24 h. $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* unstimulated control; $^{\dagger\dagger}P < 0.01$ *vs.* pCI+IL-1α. *C*, hLCN2 mRNA levels after ELF3 gene knockdown with siELF3 or non-targeting siRNA (siC–) (20 nM) and IL-1α (1 ng ml^{-1}) treatment for 24 h. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* unstimulated control; $^{\dagger\dagger}P < 0.01$ *vs.* siC– + IL-1α. *D*, mLCN2 mRNA expression in chondrocytes isolated from wild-type (WT) and *Elf3* knockout (KO) mice after IL-1α (1 ng ml^{-1}) treatment for 24 h. $^{**}P < 0.01$ *vs.* unstimulated control; $^{\dagger\dagger}P < 0.01$ *vs.* IL-1α.

in cells transfected or not with ELF3 overexpressing vector and stimulated or not with IL-1α in the presence or absence of the pharmacological inhibitor of ERK1/2 kinase. As shown in Fig. 3*C*, ERK1/2 inhibition decreased the LCN2 protein expression induced by IL-1α stimulation even in ELF3-overexpressing cells, suggesting that this kinase can regulate the induction this adipokine by both IL-1α and ELF3.

NFκB also regulates LCN2 expression

As mentioned earlier, our results suggest that ELF3 is not the only transcription factor involved in the IL-1α-mediated LCN2 induction. Analysis of the human LCN2 promoter sequence using the ALGGEN Research software (http://alggen.lsi.upc.edu) revealed several NFκB binding sites (Glaros *et al.* 2012; Chang *et al.* 2013; Zhao *et al.* 2014; Xu *et al.* 2015), and NFκB has been shown to participate in the LCN2 expression in adipocytes (Zhang *et al.* 2014), suggesting that NFκB may account for the transcriptional control of LCN2 upon IL-1α stimulation in chondrocytes. We performed, therefore, co-transfection experiments in T/C-28a2 cells with the Luc-LCN2 reporter vector and the expression vectors encoding the NFκB subunits p50 and p65. As shown in Fig. 4*A*, co-transfection with pCMV4-p50 vector did not affect LCN2 promoter activity. In contrast, when cells were co-transfected with pCMV4-p65 or with a combination of p50 and p65 expression vectors, we observed clear transactivation of the LCN2 promoter.

To gain further insights into the potential involvement of NFκB subunit p65 in the IL-1α-mediated induction of LCN2 protein expression, we evaluated by Western blot analysis LCN2 protein in cells transfected with

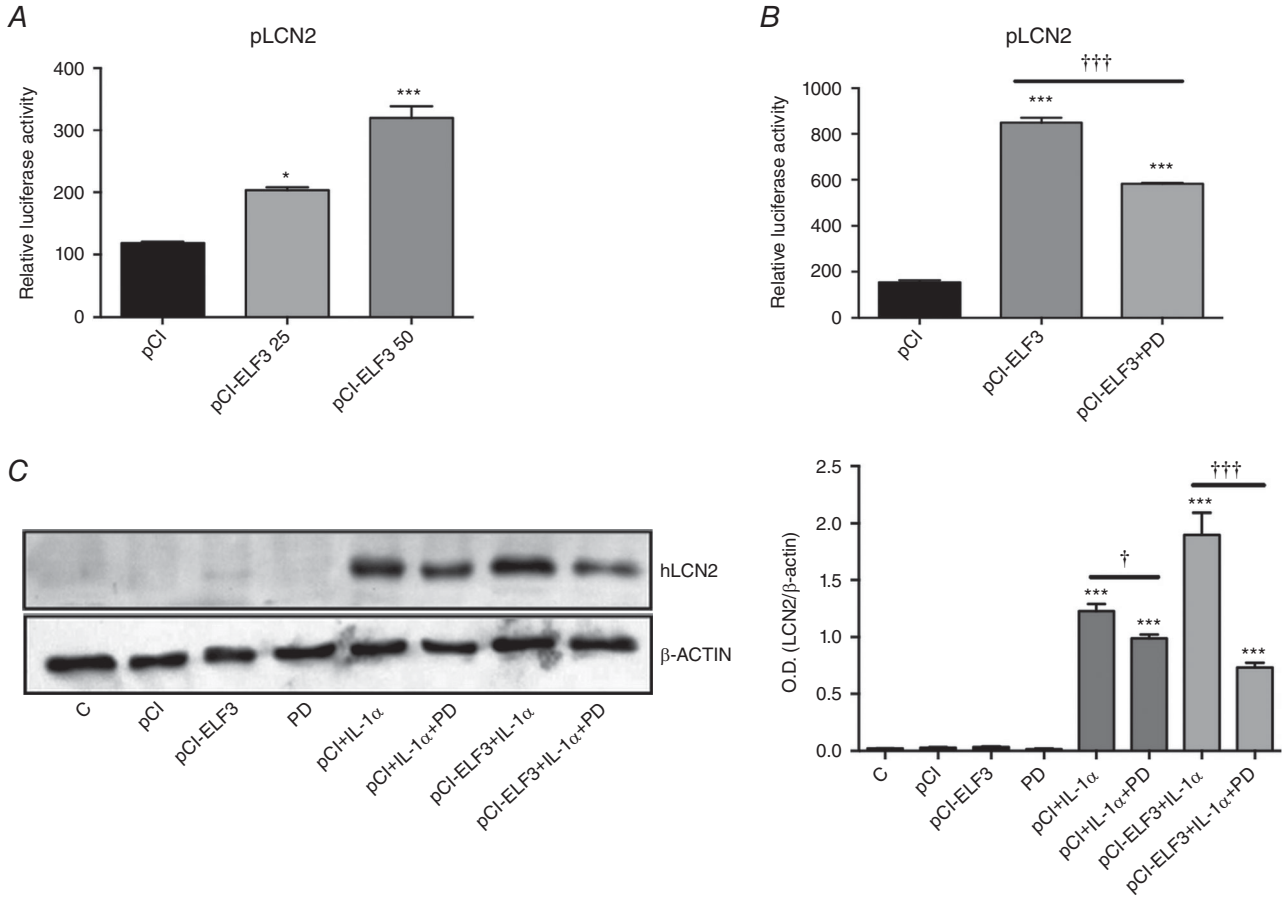

Figure 3. Effect of co-transfection with LCN2 promoter and ELF3
A, co-transfections with luciferase reporter constructs containing the LCN2 promoter (pLCN2) (400 ng) and the pCI-ELF3 expression vector (25 and 50 ng) or empty vector (pCI) for 48 h in T/C-28a2 human cells. *B*, co-transfections with luciferase reporter constructs containing the LCN2 promoter (pLCN2) (400 ng) and the pCI-ELF3 expression vector (50 ng) or the empty vector (pCI) and then treated with the inhibitor PD098059 (30 μM) for 48 h in T/C-28a2 human cells. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* pCI; $^{\dagger\dagger\dagger}P < 0.001$ *vs.* pCI-ELF3. *C*, left, hLCN2 protein expression after transient transfection with a pCI-ELF3 (100 ng) expression vector or an empty vector (pCI) and IL-1α (1 ng ml^{-1}) treatment during 24 h. PD098059 (30 μM) inhibitor was added 1 h before cytokine treatment. Right, data showing densitometric analysis of all performed Western blots.

pCMV4-p65 or empty vector with or without stimulation with IL-1α. As shown in Fig. 4*B*, overexpression of NFκB subunit p65 increased the induction of LCN2 elicited by IL-1α. To the contrary, inhibition of NFκB by using the selective pharmacological inhibitor PDTC significantly decreased the expression of LCN2 after IL-1α treatment (Fig. 4*C*).

ELF3 and NFκB act in a collaborative fashion in inducing LCN2

ETS transcription factors, like ELF3, can act as co-factors with other transcriptional regulators (Sharrocks, 2001; Tootle & Rebay, 2005; Otero *et al.* 2012), and ELF3 has been shown to interact with NFκB in different contexts (Grall *et al.* 2005; Longoni *et al.* 2013). We thus investigated

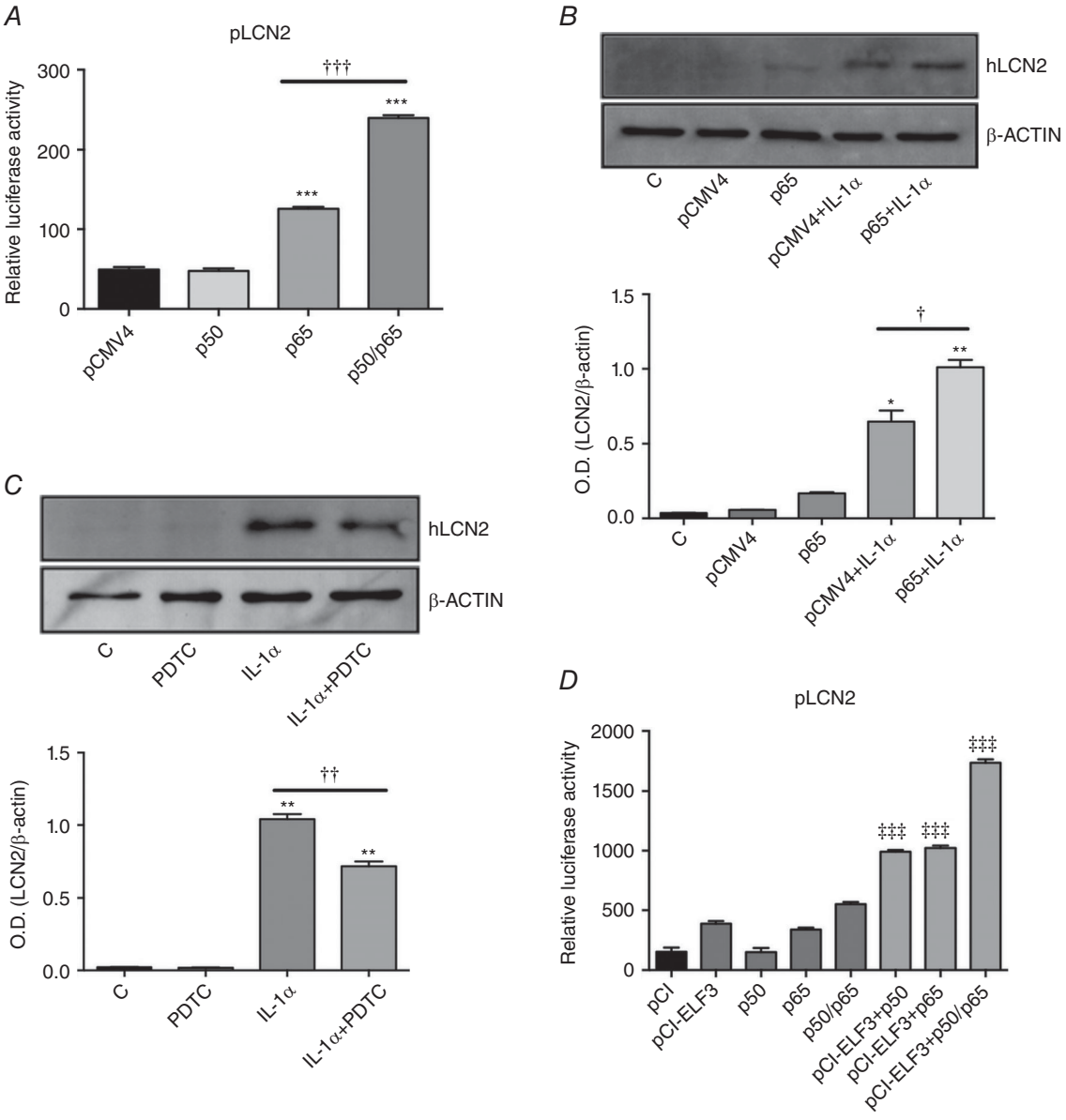

Figure 4. Effect of co-transfection with LCN2 and p50 and p65
A, co-transfections with luciferase reporter construct containing the LCN2 promoter (pLCN2) (400 ng) and the p50 (100 ng) and p65 (100 ng) expression vectors. ***$P < 0.001$ *vs.* pCMV4; †††$P < 0.001$ *vs.* p65. *B*, top, hLCN2 protein after transient transfection with the p65 (100 ng) expression vector or the empty vector (pCMV4) and IL-1α (1 ng ml^{-1}) treatment in T/C-28a2 human cells. Bottom, data showing densitometric analysis of all performed western blots. *C*, top, hLCN2 protein after IL-1α (1 ng ml^{-1}) treatment; PDTC inhibitor (10 μM) was added 1 h before cytokine treatment in T/C-28a2 human cells. Bottom, data showing densitometric analysis of all performed Western blots. *D*, co-transfections with luciferase reporter constructs containing the LCN2 promoter (pLCN2) (400 ng) and the ELF3 (100 ng), p50 (100 ng) or p65 (100 ng) expression vectors. ‡‡‡$P < 0.001$ *vs.* pCI-ELF3.

whether ELF3 was able to collaborate with the NFκB subunits p50 and p65 in the transactivation of the LCN2 promoter. As shown in Fig. 4*D*, co-transfection of T/C28a2 chondrocytes with pCI-ELF3 and plasmids encoding NFκB subunits, either alone or together, induced strong and significant increases in the transactivation of the LCN2 promoter. Note, LCN2 promoter transactivation was also significant in cells co-transfected with pCI-ELF3 or pCMV4-p65 alone, whereas overexpression of the p50 subunit alone did not transactivate the LCN2 promoter.

Since IL-1 can induce ELF3 and p65 mRNA expression (Fig. 5*A*), we wished to analyse the existence of a loop between these two transcription factors that finally leads to LCN2 induction in the presence of IL-1α. In agreement

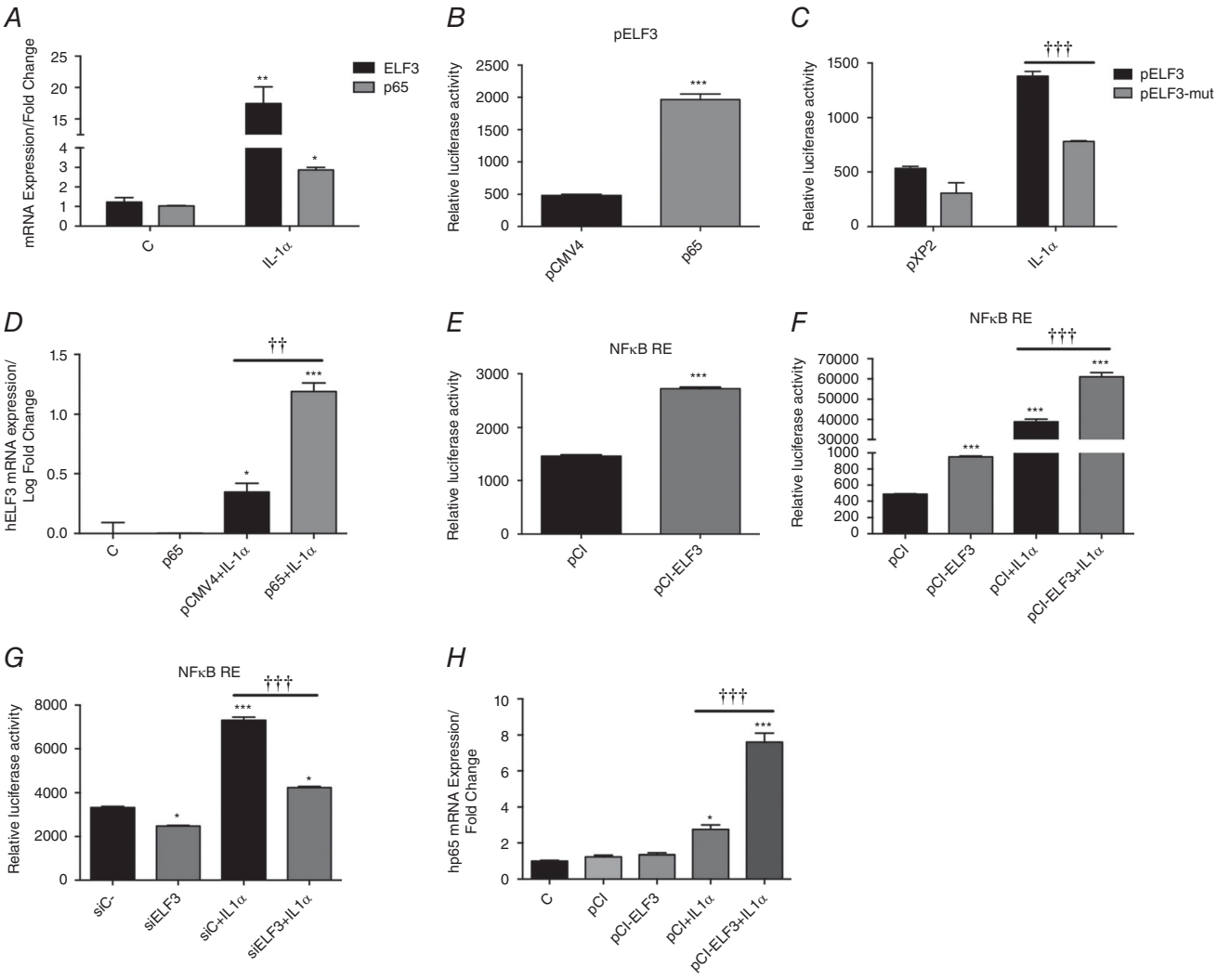

Figure 5. Expression of ELF3 and p65 in transfected human T/C-28a2 chondrocytes
A, ELF3 and p65 mRNA levels in T/C-28a2 human cells after IL-1α treatment (1 ng ml^{-1}) for 24 h. *B*, co-transfections with luciferase reporter construct containing the WT human ELF3 promoter (pELF3) (400 ng) and p65 expression vector (100 ng) or empty vector (pCMV4). *C*, co-transfections with luciferase reporter construct containing the WT human ELF3 promoter (pELF3) or the human ELF3 promoter with mutant NF-κB-binding site (pELF3-mut). *D*, hELF3 mRNA expression after transient transfection with the p65 (100 ng) expression vector or the empty vector (pCMV4) and IL-1α (1 ng ml^{-1}) treatment for 24 h. $^{*}P < 0.05$; $^{**}P < 0.01$ $^{***}P < 0.001$ *vs*. unstimulated control or pCMV4; $^{††}P < 0.01$; $^{†††}P < 0.001$ *vs*. IL-1α or p65+IL-1α. *E* and *F*, co-transfections with luciferase reporter construct containing the NFκB response element (NFκB) (400 ng) and pELF3 expression vector (100 ng) or the empty vector (pCI) and IL-1α treatment (1 ng ml^{-1}) for 24 h in T/C-28a2 cells. *G*, transfection with luciferase reporter construct containing the NFκB response element (NFκB) (400 ng), ELF3 knockdown with siELF3 or non-targeting siRNA (siC–) (20 nM), and IL-1α (1 ng ml^{-1}) treatment for 24 h in T/C-28a2 cells. *H*, p65 mRNA expression after transient transfection with the pCI-ELF3 (100 ng) expression vector or the empty vector (pCI) and IL-1α (1 ng ml^{-1}) treatment for 24 h in T/C-28a2 cells. $^{*}P < 0.05$; $^{***}P < 0.001$ *vs*. unstimulated control, pCI or siC–; $^{†††}P < 0.001$ *vs*. pCI + IL-1α or siC– + IL-1α.

with previous studies (Grall *et al.* 2003), we found that NFκB transactivates the ELF3 promoter (Fig. 5*B*), and that the IL-1α-driven ELF3 promoter activation depends upon a proximal NFκB binding site, as revealed by the reduced activation of the ELF3 promoter containing a mutation of the NFκB binding site (pCI-ELF3-mut) compared to the activation of the wild-type ELF3 promoter sequence (pELF3) (Fig. 5*C*). Further, p65 overexpression significantly up-regulated the IL-1α-induced ELF3 mRNA expression in human chondrocytes (Fig. 5*D*), which, together with the results obtained in luciferase reporter assays, confirmed that NFκB regulates transcription of the ELF3 gene. We next assessed the ELF3-dependent regulation of NFκB in chondrocytes. As shown in Fig. 5*E*, ELF3 overexpression increased the activity of a luciferase reporter construct containing five copies of the NFκB response element (NFκB RE). Moreover, while the activity of the NFκB RE was increased by IL-1α and this effect was enhanced by ELF3 overexpression (Fig. 5*F*), ELF3 knockdown decreased the activation of the NFκB RE exerted by IL-1α treatment (Fig. 5*G*). Finally, we observed that ELF3 overexpression increased the IL-1α-induced p65 mRNA expression in human chondrocytes (Fig. 5*H*).

Together, these results further demonstrate a mutual regulation between ELF3 and NFκB. We next investigated whether this interaction impacted on the induction of LCN2 by IL-1α in human chondrocytes. In agreement with the aforementioned results, siELF3 gene knockdown decreased LCN2 protein after IL-1α treatment (Fig. 6*A*). Notably, ELF3 silencing prevented the induction of LCN2 even when the cells were stimulated with IL-1α and CMV4-p65 (Fig. 6*A*). Similarly, pharmacological inhibition of NFκB using PDTC strongly reduced the IL-1α-induced LCN2 even when ELF3 was overexpressed, indicating that NFκB is also critical for the LCN2 induction (Fig. 6*B*).

Discussion

White adipose tissue is an active organ that sends out and replies to a wide spectrum of biochemical factors, including a substantial number of soluble

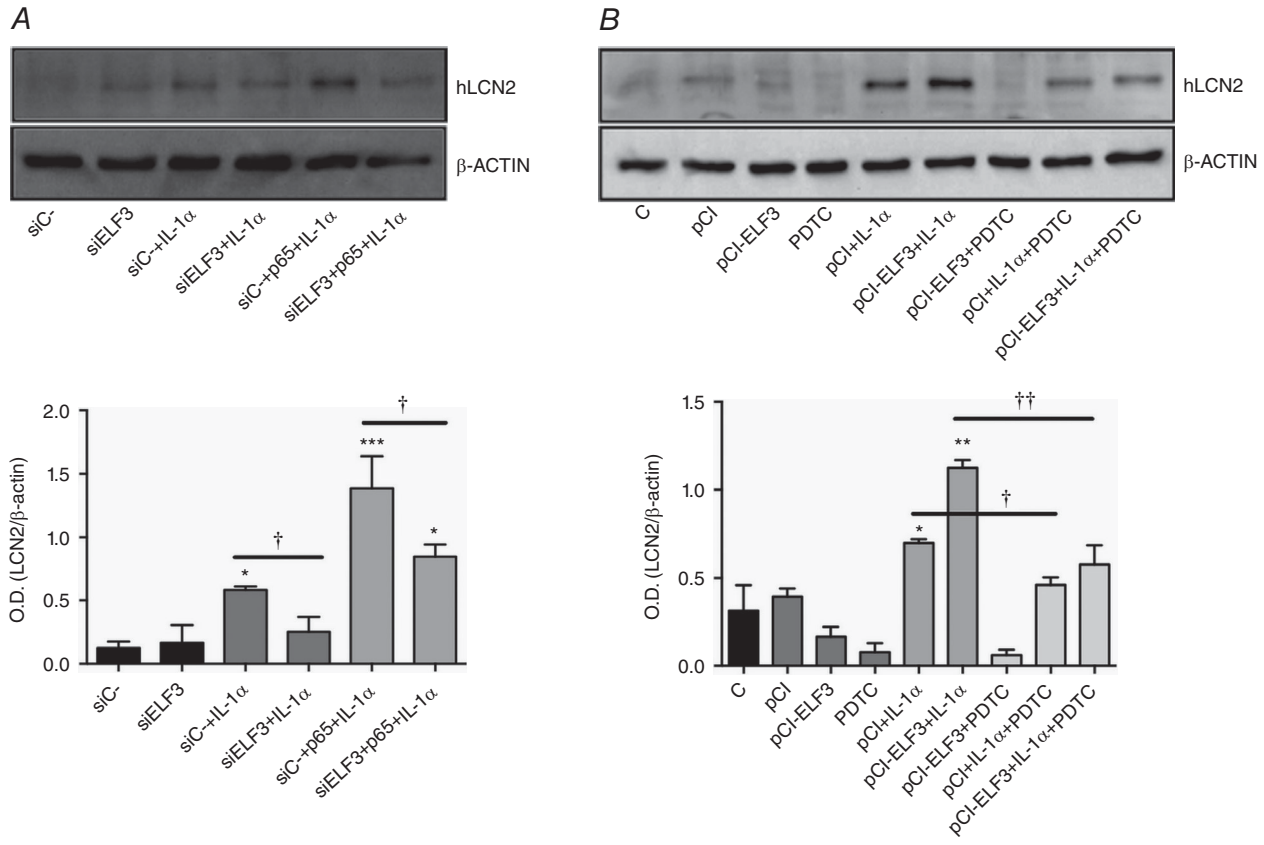

Figure 6. LCN2 expression in T/C-28a2 transfected human chondrocytes
A and *B*, top, LCN2 protein in T/C-28a2 cells after transient transfection with the pCI-ELF3 (100 ng) expression vector or the empty vector (pCI), ELF3 gene knockdown with siELF3 or non-targeting siRNA (siC–) (20 nM), and IL-1α treatment (1 ng ml^{-1}) for 24 h. Inhibitor PDTC (30 μM) was added 1 h before cytokine stimulation. Bottom, data showing densitometric analysis of all performed Western blots.

mediators, called collectively adipokines, which are involved in the regulation of multiple physiological and pathological processes, particularly impacting on immune and inflammatory responses. Accumulating evidence has highlighted the influence of adipokines on inflammation, and most of these peptides are characterized as having predominantly pro-inflammatory actions (Gómez *et al.* 2011; Scotece *et al.* 2011; Conde *et al.* 2013). Adipokines include a wide range of disparate molecules that are mostly, but not exclusively, produced by white adipose tissue. Actually, other joint tissues including cartilage, synovial tissues and bone not only synthesize adipokines, but are also targets of adipokine actions (Gómez *et al.* 2011; Scotece *et al.* 2011; Conde *et al.* 2013).

Recently, one of these adipose tissue-derived factors, LCN2, has emerged as a potential new factor involved in the regulation of cartilage homeostasis (Conde *et al.* 2011). Our group demonstrated that mouse and human chondrocytes express LCN2, particularly when pro-inflammatory conditions are at play. Namely, IL-1 and LPS, as well other pro-inflammatory adipokines such as leptin, are able to significantly increase LCN2 gene expression in human chondrocytes (Conde *et al.* 2011). The overexpression of this adipokine decreases cell proliferation and increases the expression of hypertrophy markers in chondrocytes (Owen *et al.* 2008). Note, we showed that LCN2 expression increases gradually in murine ATDC5 chondrogenic cells with formation of cartilage nodules and enhancement of mineralization, suggesting that LCN2 is a marker of late-phase chondrogenic differentiation and hypertrophy potentially involved in endochondral ossification (Conde *et al.* 2011). Moreover, transgenic mice overexpressing LCN2 exhibit alterations in the growth plate, characterized by chondrocytes with more immature phenotype and a low proliferation rate (Costa *et al.* 2013). These data, together with the fact that LCN2 is able to stabilize the activity of MMP9 and to increase proteoglycan loss in cartilage, suggest a potential role for this adipokine in the degeneration of articular cartilage occurring during OA.

Although the regulation of LCN2 has been described in other cells types, such as pancreatic islet β-cells and kidney fibroblasts (Glaros *et al.* 2012; Chang *et al.* 2013), the cellular mechanism(s) and the signalling pathways involved in the IL-1-driven induction of this adipokine in chondrocytes are practically unknown. For this reason, we wished to describe, in detail, how LCN2 is induced by IL-1α in chondrocytes, focusing our attention on the role played by the transcription factors ELF3 and NFκB.

The participation of ELF3 in the transcriptional regulation of different genes involved in inflammation and in cartilage catabolism is a recent advance. In fact, ELF3 expression is enhanced in OA cartilage and its protein levels co-localize with MMP13 expression in the same tissue. So, in agreement with other published articles (Grall *et al.* 2005; Wu *et al.* 2008; Otero *et al.* 2012), we found that ELF3 regulates the expression of different pro-inflammatory and catabolic factors such as TNF-α, IL-6, COX2 and MMP13 in chondrocytes, which further highlights the role of this transcription factor in mediating inflammatory responses. Thus, in the present study we sought to analyse whether the ETS transcription factor ELF3 participates in the induction of LCN2 in human chondrocytes.

Recent studies have shown that different transcription factors such as STAT1, STAT3, NFκB and C/EBPδ are required for LCN2 induction in adipocytes, hepatocytes and kidney fibroblasts. More precisely, binding of these transcription factors to the LCN2 promoter was required for its transcriptional activation after stimulation with different pro-inflammatory agents (Glaros *et al.* 2012; Chang *et al.* 2013; Zhao *et al.* 2014; Xu *et al.* 2015). However, the participation of ELF3 in this process has not been investigated previously in chondrocytes or other cell types. Thus, we examined the involvement of ELF3 in the cytokine-induced LCN2 gene expression in human chondrocytes. Promoter analyses revealed the presence of conserved ETS factor binding sites in the LCN2 promoter, and co-transfection experiments clearly showed how ELF3-overexpressing chondrocytes have higher levels of LCN2 mRNA and protein in response to IL-1α. In addition, both *in vitro* knockdown and *in vivo* knockout

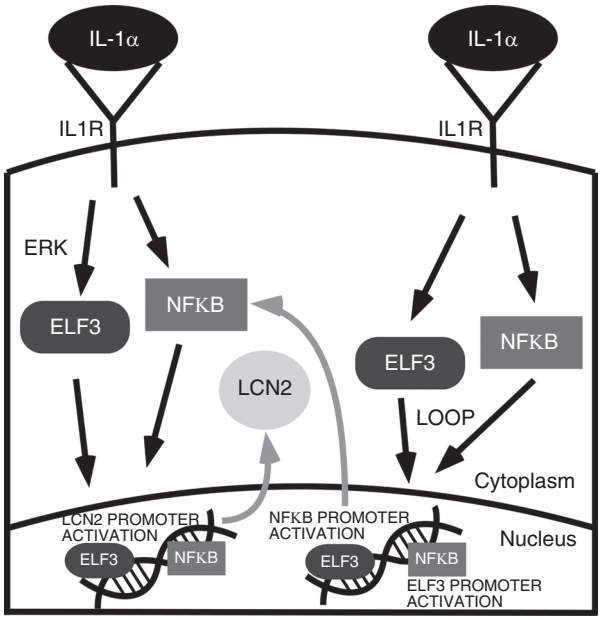

Figure 7. Schematic representation of the most relevant findings of the study
IL-1α is able to activate ELF3 and NFκB transcription factors, which in turn lead the induction of LCN2. At the same time, the activation of both transcription factors by IL-1α results in a positive feedback loop between them, leading to the induction of LCN2 expression.

of ELF3 resulted in significantly reduced LCN2 levels upon IL-1α challenge, and ELF3 overexpression transactivated the LCN2 promoter. These results demonstrate that ELF3 is a relevant transcription factor able to regulate the IL-1α-induced transcription of LCN2 in chondrocytes. Importantly, the ELF3-driven LCN2 promoter transactivation was regulated by the activity of ERK1/2. The ETS transcription factors are MAPK effectors (Sharrocks, 2001; Tootle & Rebay, 2005), and we recently showed that ERK signalling enhances the ELF3-driven transactivation of the MMP13 promoter in chondrocytes and its binding to the endogenous promoter sequence (Otero *et al.* 2012). In the present study, we observed the participation of ERK signalling in both ELF3-induced activation of LCN2 promoter, as well as in the up-regulation of LCN2 protein mediated by IL-1α or by the combination of this pro-inflammatory cytokine with ELF3 overexpression. The regulation of the activity of ELF3 by different kinases, including p21-activated kinase-1 and ERK, was studied previously (Manavathi *et al.* 2007; Otero *et al.* 2012). In addition, potential phosphorylation sites for ERK have been identified in the central region of ELF3 (Oettgen *et al.* 1997). Our observation that pharmacological inhibition of this kinase decreased the ELF3-driven LCN2 promoter transactivation and protein levels provides new insight on the contribution of ERK to the regulation of ELF3 activity. However, whether this kinase modulates the activity of ELF3 via direct phosphorylation of the ELF3 protein or by affecting the activities of potential ELF3 interacting partners remains unknown and merits further investigation.

In line with other published studies in different cell types (Borkham-Kamphorst *et al.* 2011; Chang *et al.* 2013; Zhao *et al.* 2014), we also show here the participation of NFκB in the induction of LCN2 in human chondrocytes. Noteworthy, we demonstrated that ELF3 and NFκB collaborate to transactivate the LCN2 promoter, which is consistent with the ability of ETS transcription factors to act as co-factors with other transcriptional regulators. For instance, ELF3 acts in conjunction with AP-1 in the transcriptional regulation of MMP13 in chondrocytes (Otero *et al.* 2012).

We demonstrated that ELF3 and NFκB, not only cooperate in the activation of LCN2 transcription, but also possibly in post-transcriptional regulation (Fig. 7). As far as we are aware, this is a new mechanistic link in the pathophysiology of chondrocytes. Similar results were reported using prostate cancer cells, where ELF3 and NFκB constituted a positive feedback loop that resulted in enhanced nuclear translocation of different subunits of NFκB and binding to different target genes such as COX2 and IL-6 (Longoni *et al.* 2013).

In conclusion, our paper reveals new signalling pathways in the regulation of LCN2 gene expression in human chondrocytes, demonstrating for the first time the participation of the ETS transcription factor ELF3 in this process. Moreover, we also describe a novel mechanism involving the cooperation between ELF3 and NFκB in the control of LCN2 expression in chondrocytes. Thus, in addition to uncovering a novel mechanistic link between ELF3 and NFκB in LCN2 regulation in cartilage, the present study supports the critical role of LCN2 in inflammatory conditions, particularly in those related with obesity co-morbidities such as osteoarthritis. Broadening our knowledge about novel mechanisms elicited by adipokines such as LCN2 will provide new avenues for progressing with therapeutic approaches to control endogenous molecules to prevent inflammatory responses.

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Additional information

Competing interests

None.

Author contributions

All experiments were carried out in the Santiago University Clinical Hospital, NEIRID Lab. J.C. and M.O. participated in acquisition and interpretation of the data and drafting the manuscript. M.S., R.G., V.A., V.L., J.P. and F.L. participated in acquisition of data. M.G. participated in drafting the manuscript and revising it critically. O.G. participated in conception and design of the study, analysis and interpretation of data, drafting and critical revision of the manuscript, and scientific supervision of experiments. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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