

Cytokine-induced *MMP13* Expression in Human Chondrocytes Is Dependent on Activating Transcription Factor 3 (ATF3) Regulation^{*[S]}

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Chun Ming Chan¹, Christopher D. Macdonald^{1,2}, Gary J. Litherland³, David J. Wilkinson, Andrew Skelton⁴, G. Nicholas Europe-Finner⁵, and Andrew D. Rowan⁶

From the Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, Framlington Place, Newcastle-upon-Tyne NE2 4HH, United Kingdom

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Irreversible breakdown of cartilage extracellular matrix (ECM) by the collagenase matrix metalloproteinase 13 (*MMP13*) represents a key event in osteoarthritis (OA) progression. Although inflammation is most commonly associated with inflammatory joint diseases, it also occurs in OA and is thus relevant to the prevalent tissue destruction. Here, inflammation generates a cFOS AP-1 early response that indirectly affects *MMP13* gene expression. To ascertain a more direct effect on prolonged *MMP13* production we examined the potential molecular events occurring between the rapid, transient expression of cFOS and the subsequent *MMP13* induction. Importantly, we show *MMP13* mRNA expression is mirrored by nascent hnRNA transcription. Employing ChIP assays, cFOS recruitment to the *MMP13* promoter occurs at an early stage prior to gene transcription and that recruitment of transcriptional initiation markers also correlated with *MMP13* expression. Moreover, protein synthesis inhibition following early *FOS* expression resulted in a significant decrease in *MMP13* expression thus indicating a role for different regulatory factors modulating expression of the gene. Subsequent mRNA transcriptome analyses highlighted several genes induced soon after *FOS* that could contribute to *MMP13* expression. Specific small interfering RNA-mediated silencing highlighted that ATF3 was as highly selective for *MMP13* as cFOS. Moreover, ATF3 expres-

sion was AP-1(cFOS/cJUN)-dependent and expression levels were maintained after the early transient cFOS response. Furthermore, ATF3 bound the proximal *MMP13* AP-1 motif in stimulated chondrocytes at time points that no longer supported binding of *FOS*. Consequently, these findings support roles for both cFOS (indirect) and ATF3 (direct) in effecting *MMP13* transcription in human chondrocytes.

Cartilage extracellular matrix (ECM)⁷ degradation within synovial joints (especially knee, hip, hands, and spine) is a pathological process synonymous with patient disability and pain in those suffering from either osteoarthritis (OA) or rheumatoid arthritis (RA). In reality, OA and RA are not single diseases but rather pathologies with various initiating factors that often include inflammation; both diseases have the common end point of irreversible destruction of the cartilage ECM. Although more marked in RA, inflammatory mediators released by immune cells (and joint tissue cells) orchestrate the aberrant production of catabolic factors that initiate destruction in both diseases. Numerous mediators including tumor necrosis factor α (TNF α), interleukin (IL)-17, and IL-1 have been reported to constitute this inflammatory response (1, 2). Our own findings have shown that such mediators, when in combination with the IL-6 family cytokines, oncostatin M (OSM) or IL-6 itself, promote marked expression of matrix metalloproteinases (MMPs) (3–7), the enzymes known to be responsible for cartilage damage. Potent expression of MMPs is therefore very likely within most inflammatory milieux, including OA.

Of the MMPs, it is specifically the collagenases MMP1 and MMP13 that are understood to cleave the main structural component of cartilage, type II collagen, thus effecting the irreversible loss of ECM architecture and function. Current dogma indicates MMP1 (produced by synovial fibroblasts) to be key in RA (predominantly a synovium-driven pathology), whereas MMP13 (synthesized by chondrocytes) is the major effector enzyme in OA, which is thought largely to be a cartilage-mediated disease. Although this representation is somewhat over-

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[S] This article contains supplemental Tables S1–S4.

¹ Both authors contributed equally to this work.

² Present address: Arthritis Research UK, 41 Portland Place (4th floor), London, W1B 1QH, United Kingdom.

³ Present address: Institute of Biomedical and Environmental Health Research, School of Science and Sport, University of the West of Scotland, Paisley Campus, Paisley PA1 2BE, Scotland.

⁴ Present address: Bioinformatic Support Unit, Newcastle University, Newcastle-upon-Tyne, NE2 4HH, United Kingdom.

⁵ Present address: Institute of Genetic Medicine, Newcastle University, Newcastle-upon-Tyne, NE1 3BZ, United Kingdom.

⁶ To whom correspondence should be addressed. Tel.: 44-191-208-8821; Fax: 44-191-208-5455; E-mail: drew.rowan@ncl.ac.uk.

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⁷ The abbreviations used are: ECM, extracellular matrix; AP-1, activator protein-1; ATF3, activating transcription factor-3; DAPA, DNA affinity precipitation assay; IER3, immediate early responsive gene 3; IRF1, interferon regulatory factor 1; MMP13, matrix metalloproteinase 13; OA, osteoarthritis; OSM, oncostatin M; RA, rheumatoid arthritis; ANOVA, analysis of variance.

simplified, both enzymes remain key therapeutic targets for disease-modifying agents (8).

Under normal physiological conditions, chondrocytes maintain normal cartilage ECM homeostasis to enable smooth, frictionless joint articulation. In disease, a variety of stimuli such as Toll-like receptor ligands and inflammatory cytokines (3, 6, 9–11) elicit intracellular signaling cascades in chondrocytes that culminate in greatly increased *MMP13* expression. Moreover, epigenetic studies have shown promoter alterations such as methylation status (12, 13) can further contribute to the elevated *MMP13* expression, which is a hallmark of OA.

Regulation of eukaryotic gene expression represents a vital control mechanism that ensures appropriate cellular function. Transcriptional control can be supplemented at various levels, including alterations in ribosome translational efficiency and stability of mRNA transcripts and protein. Cartilage ECM proteolysis is subject to further regulation, because most MMPs are produced as inactive zymogens requiring proteolytic activation for activity (14), and a family of endogenous inhibitors (tissue inhibitors of metalloproteinases) provide additional control over the activities of these potent enzymes. Considerable research has focused on better understanding the molecular mechanisms by which transcription of MMPs is regulated, with the activator protein (AP)-1 transcription factor being considered a key factor for MMP expression (15–20). AP-1 is a protein dimer expressed ubiquitously, and is comprised of a FOS (cFOS, FOSB, FOSL1, FOS-like antigen (FRA)1 or FRA2) and JUN (cJUN, JUNB or JUND) heterodimer or a JUN homodimer. Variations in AP-1 composition dictate specificity and allow for differential gene regulation in specific tissues (21). Several studies including our own have confirmed AP-1 dependence for *MMP13* induction by diverse stimuli including phorbol ester, parathyroid hormone, TNF α , IL-1 β , and IL-17 (15–25), whereas we and others (17–19, 23, 24), have indicated cFOS-cJUN heterodimers are key regulators of *MMP13* in chondrocytes. Moreover, we have shown pro-catabolic stimuli such as IL-1 + OSM markedly enhance MMP expression compared with IL-1 alone, both *in vitro* and *in vivo* (5–7, 19), with a concomitant increase in *FOS* induction (19). However, despite a functional role of *FOS* as an immediate early gene in regulating *MMP13* expression via specific cell signaling pathways (18, 24–30), the complete mechanism underpinning transcriptional activation of this MMP remains to be defined.

In this context several studies suggest other transcriptional regulators may act, in concert with cFOS-cJUN, to effect *MMP13* transcription (23, 25, 27–29). To further understand the regulation of *MMP13* expression in the context of pathological ECM turnover, we have assessed *MMP13* transcriptional regulation following a potent pro-catabolic stimulus known to promote cartilage destruction (5, 6, 31). Here, we demonstrate that cFOS-cJUN AP-1 is not the sole AP-1 contributor to the initiation of transcription of *MMP13*. We identify, for the first time, that cFOS-cJUN also induces activating transcription factor 3 (ATF3) to modulate *MMP13* expression in chondrocytes. These data indicate a further mechanism in regulating this gene that may aid in defining more potential therapeutic targets to abrogate cartilage ECM degradation associated with OA.

Results

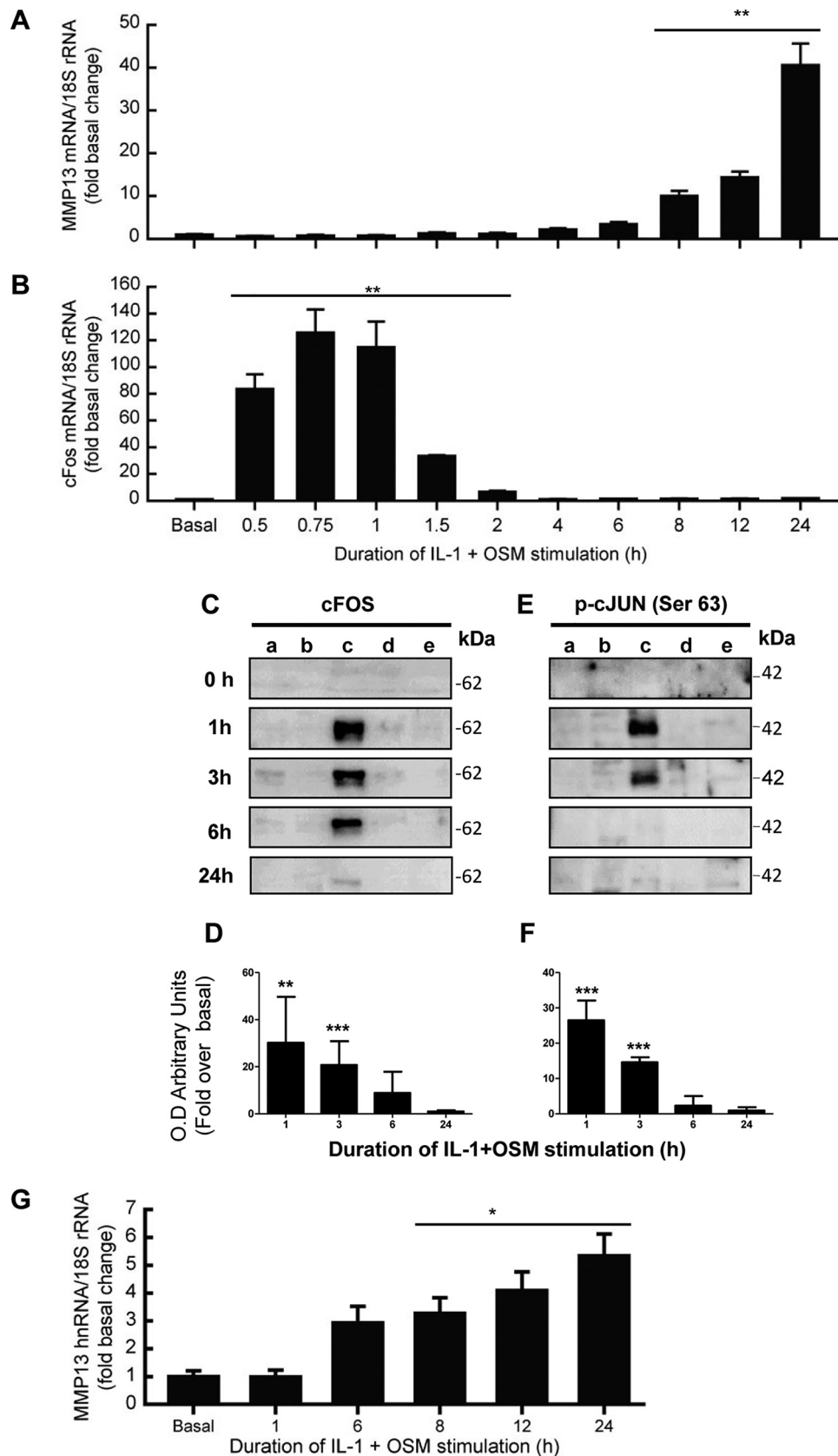
MMP13 Gene Expression Is Associated with an Early Induction of cFOS—We have previously reported that the potent cytokine combination of IL-1 + OSM induces time-dependent collagenolytic *MMP13* expression, typically reaching maximal induction at 24 h (31–33). We now confirm a detailed time course for *MMP13* induction in primary human articular chondrocytes, exhibiting detectable stimulation of mRNA expression from around 6 h post-stimulation (Fig. 1A). The expression of *MMP13* was associated with an early, rapid but transient induction of *FOS* mRNA (Fig. 1B) as we have previously reported (33). This was also reflected in detectable cFOS protein in the nuclear fraction at 1 h, which persisted up to 3–6 h (Fig. 1, C and D), and was accompanied by cJUN phosphorylation (Fig. 1, E and F). These data are consistent with the prevailing paradigm of cFOS-phospho-cJUN dependence for proinflammatory cytokine induction of *MMP13* (18, 19, 21–25, 34). Importantly, we verified that nascent hnRNA mirrored the mRNA expression indicative of transcription of the gene independent of mRNA synthesis/stability (Fig. 1G).

Enrichment of cFOS at the MMP13 Promoter Is an Early, Transient Response Event—Initially, ChIP assays revealed that cFOS enrichment was only detectable 1 h post-stimulation (Fig. 2A, inset), further suggesting an early, rapid but transient recruitment of cFOS to this promoter region. Further assays also confirmed enrichment of phospho-Ser⁵ RNA Pol II (pRNA Pol II) at the proximal *MMP13* promoter 24 h post-stimulation (Fig. 2B, inset), in line with mRNA/hnRNA expression at this time point (see Fig. 1, A and B). In this context it is worthy to note that Ser⁵ phosphorylation of RNA Pol II has been shown to be concentrated near the promoter of genes, whereas Ser² phosphorylation of RNA Pol II is observed throughout the gene (35). Consequently, no enrichment in the 3'-UTR of *MMP13* was observed in the assays presented herein (see Fig. 2B). Assays employing an anti-acetyl(Lys^{9/14})-histone H3 antibody indicated histone H3 was acetylated at the *MMP13* promoter at all time points (Fig. 2C) thus allowing appropriate regulation of transcription of the gene.

Although there was a requirement for *MMP13* induction by IL-1 + OSM via cFOS, the delay in transcription of hnRNA/mRNA combined with the rapid turnover of cFOS (see Fig. 1B–D) (36) indicated that other AP-1 regulators were synthesized following *FOS* induction, and thus contribute to transcription of the gene. A role for *de novo* protein formation was confirmed, after the early induction of cFOS, employing the protein synthesis inhibitor emetine. Here, 24 h IL-1 + OSM-stimulated cells were incubated with the inhibitor and *MMP13* mRNA expression subsequently monitored. Emetine was observed to significantly reduce *MMP13* expression, which persisted up to 6–8 h post-stimulation (see Fig. 2D). Consequently, IL-1 + OSM stimulation and induction of cFOS-p-cJUN heterodimers promote the rapid formation of transcriptional regulator(s), which further drive *MMP13* expression. To determine these novel regulators, genome-wide microarray analyses were performed on primary chondrocyte populations stimulated at specific time points with IL-1 + OSM and where *MMP13* induction occurred at 24 h. Analysis of the

data confirmed that the 24-h time point had the highest number of changes in gene expression relative to basal (Fig. 3, A and B) as previously reported for a chondrocyte cell line (33). Fur-

thermore, ingenuity pathway analysis of the 24-h dataset highlighted multiple proinflammatory signaling pathways, including IL-1 β , IL-17, and TNF α (see supplemental Table S1), as well



ATF3 Modulates MMP13 Expression

as multiple regulators and upstream regulators (supplemental Tables S2 and S3), indicating that IL-1 + OSM is indeed a potent model proinflammatory stimulus. Although heat maps (Fig. 3A) indicated relatively few changes in gene expression at the earlier time points; marked induction of several transcriptional regulators, including *FOS*, *JUN*, and *ATF3*, was observed at 1 h (Fig. 3C) and 1.25 h (Fig. 3D). Strikingly, *FOS* was one of the most highly induced genes at 1 h (Fig. 3C), which almost halved by 1.25 h (Fig. 3D) and was one of the most highly down-regulated genes at 24 h (Fig. 3E) further illustrating the transient nature of *FOS* expression. *MMP13* expression was detectable at 24 h but barely detectable at the early time points (Fig. 3, F and G), correlating with the transient expression profile of *FOS* and the more sustained expression of *ATF3* (see Figs. 3F and 4). Furthermore, *JUN* expression was elevated and sustained at both 1 and 1.25 h (Fig. 3, C and D), whereas data from the arrays also indicated increased expression from 1 to 1.25 h of *FOSL1*, *FOSB*, *JUNB*, *RELA* (p65), immediate early responsive gene 3 (*IER3*), and interferon regulatory factor 1 (*IRF1*), which were all confirmed by real-time RT-PCR in multiple chondrocyte populations (Fig. 4). Subsequent siRNA assays were employed to determine the effect of knockdown of these and cFOS/cJUN transcriptional regulators on *MMP13* expression in chondrocytes stimulated with IL-1 + OSM. Here, knockdowns of not only *FOS*/*FOSL1* but also *ATF3* resulted in substantial inhibition of *MMP13* expression ($p < 0.0001$ compared with siRNA controls (see Table 1)).

Prolonged Expression of ATF3 Modulates MMP13 Transcription—The above siRNA data thus implicated a potent role for *ATF3* in regulating *MMP13* expression. Temporal expression of *ATF3* in IL-1 + OSM-treated cells was thus determined to observe any overlap with *FOS* expression. Here, *ATF3* mRNA and nuclear protein was significantly increased at 1 h but maintained up to 24 h (see Fig. 5, A–C) in contrast to *FOS* expression (Fig. 1, B–D) where levels decreased substantially after 1 h. siRNAs knockdowns of *FOS*/*JUN* in IL-1 + OSM-stimulated cells were subsequently employed to determine any association with *ATF3* expression. Knockdown of both *FOS* and *JUN* mRNA significantly reduced *ATF3* mRNA levels (Fig. 5D). Control Western blots on nuclear extracts confirmed siRNA knockdowns of cFOS and phospho-cJUN was also mirrored at the protein level, where the latter also reflected total cJUN levels (see Fig. 5E). DNA affinity precipitation assays (DAPAs) were subsequently performed on IL-1 + OSM-stimulated cells to ensure that the temporal binding of cFOS and *ATF3* to the *MMP13* proximal promoter AP-1 element was reflective of their respective transient and sustained levels of expression. Here, specific AP-1 oligonucleotide-protein binding was initially determined in the presence of 50× excess non-biotinylated AP-1 oligonucleotide (data not shown). Further

DAPAs confirmed the ability of both of these factors to bind at 1 h; whereas only *ATF3* was still bound to this element 6 h post-stimulation (Fig. 6A). This was associated with the maintained expression of *ATF3* at 6 h in contrast to the observed decreased expression of cFOS (see Fig. 6B, Western blot input).

Discussion

Proinflammatory cytokines that are associated with cartilage destruction and arthritis have been extensively reported by several groups including our own (6, 7, 9, 37, 38). In this context, cytokine combinations such as IL-1 + OSM represent highly useful model stimuli to study complex inflammatory mechanisms in arthritis, promoting cartilage breakdown (9, 31, 38–41) that correlates with significantly enhanced collagenolytic MMP expression (4–7, 18, 19, 30–33). Indeed, ingenuity pathway analysis of the 24-h time point indicated that the stimulus of IL-1 + OSM up-regulated numerous genes, many regulators of gene expression, associated with various other proinflammatory stimuli such as IL-1, IL-17, and TNF α , indicative of IL-1 + OSM being a potent model pro-catabolic stimulus.

Such cytokine-mediated inflammation is an important contributory factor for arthritis initiation and progression. This is the case not only in RA, where anti-cytokine biologics are now a key part of therapeutic strategies (41), but is also increasingly recognized in OA (2). Pathological inflammatory milieu are unlikely to comprise only a single mediator and so induction via several cytokines may be common; this may explain, in part, how relatively low level joint inflammation can nevertheless perpetuate cartilage destruction. Thus, the cytokine-stimulated molecular pathways and regulators that drive the expression of collagenolytic MMPs are likely to be common to many proinflammatory stimuli and therefore potential therapeutic targets.

MMP13 is thought to be the key collagenase in OA (8). Despite a confirmed role for AP-1 in the transcriptional activation of many MMP genes, including *MMP13* (13, 15–19), there is a temporal discord between the expression of the rapid *FOS* response (typically 30–60 min) and the subsequent expression of collagenolytic MMPs (typically from 6 to 24 h). We have previously confirmed a role for cFOS/cJUN AP-1 regulators in *MMP13* transcription (18, 19), and here further demonstrate the rapid, early but transient induction of *FOS* in human chondrocytes. ChIP assays, performed at a time when *MMP13* mRNA was maximally detectable (24 h) confirmed no enrichment of cFOS to the proximal promoter region of the gene, although recruitment of phospho-Ser⁵ RNA Pol was observed at 24 h. This confirms there is indeed a delay between the time of *FOS* expression and when the *MMP13* gene is transcribed.

FIGURE 1. IL-1 + OSM-stimulated *MMP13* mRNA expression is preceded by nascent hnRNA formation and involves early induction of cFOS in human chondrocytes. Chondrocytes were stimulated with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for the indicated durations. Total RNA was isolated, reverse transcribed, and subjected to real-time RT-PCR for *MMP13* mRNA (A), cFOS mRNA (B), or *MMP13* nascent hnRNA (G) as described under “Experimental Procedures.” Data are expressed relative to 18S rRNA and presented as fold-increase compared with basal expression. PCR data ($n = 4$) are representative of at least three separate chondrocyte populations. Cells were subjected to subcellular fractionation and protein from the cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound (d) and cytoskeletal (e) fractions resolved using SDS-PAGE and immunoblotted with antibodies to cFOS (C) or phosphorylated cJUN (E). Combined densitometric scans of five separate blots for each protein in the soluble nuclear fraction, relative to $t = 0$ h, are shown in D and F, respectively, and were obtained from separate chondrocyte populations. All data are presented as mean (\pm S.D.), where ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ IL-1 + OSM-treated compared with control; ANOVA.

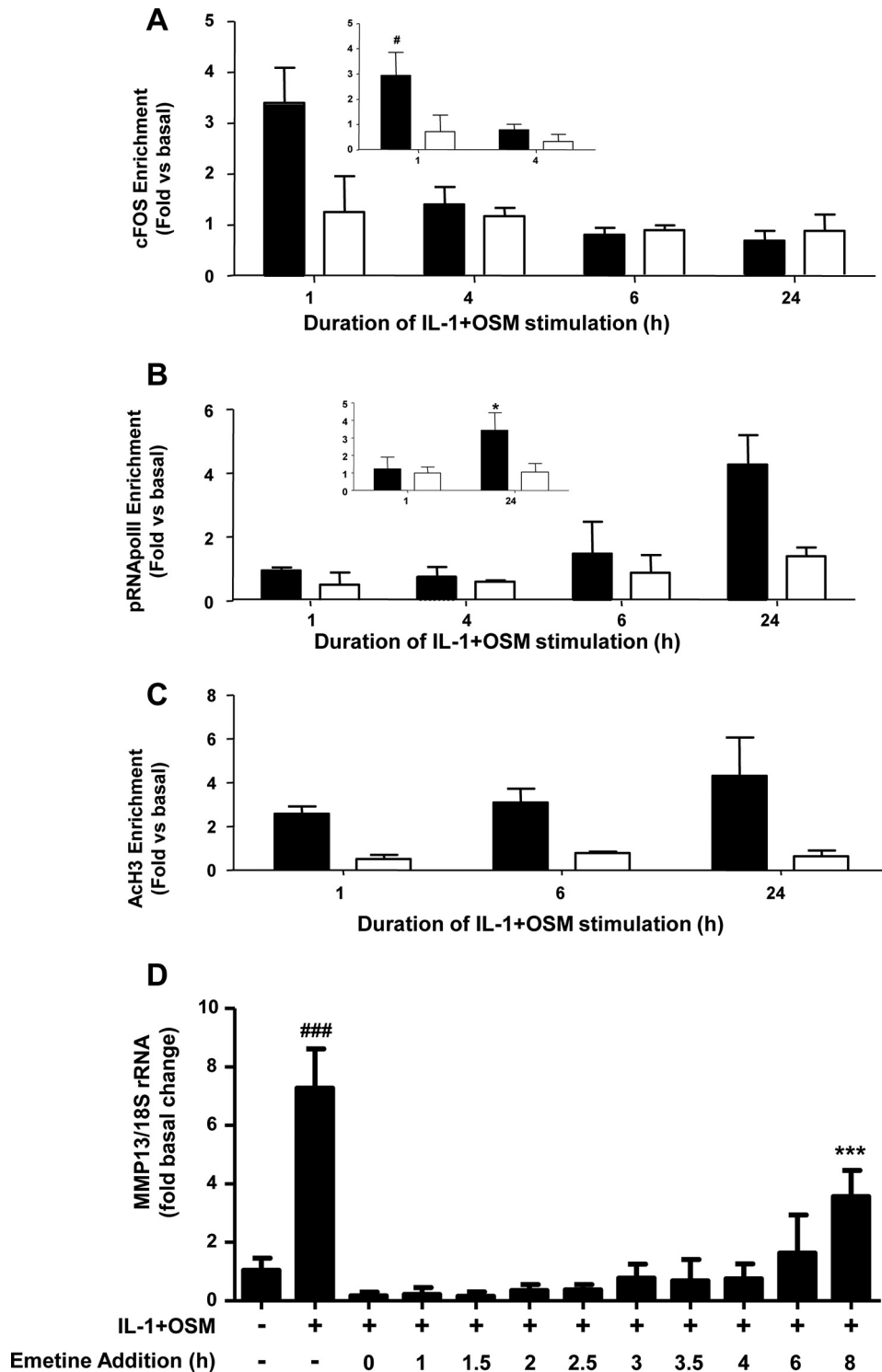


FIGURE 2. ChIP analyses of the *MMP13* proximal promoter. Human chondrocytes were treated with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for the indicated durations. Cells were then subject to DNA-protein cross-linking, lysis, and DNA shearing. Immunoprecipitation for cFOS (A), pRNA Pol II (B), or AcH3 (C) was followed by isolation of complexed genomic DNA and subsequent real-time RT-PCR for the proximal (closed bars) and 3'-UTR (used for normalization; open bars) regions of *MMP13* as indicated. Data (mean \pm S.D.) are pooled from three (five for inset data) separate chondrocyte populations. Statistical comparisons are: *, $p < 0.05$ (24 h IL-1 + OSM stimulation versus basal); #, $p < 0.05$ (1 h IL-1 + OSM stimulation versus basal). D, chondrocytes were treated with IL-1 (0.05 ng/ml) and OSM (10 ng/ml) for 24 h. Emetine (10 μ M final concentration) was added at the indicated times after IL-1 + OSM stimulation and real-time RT-PCR performed on extracted RNA relative expression levels of *MMP13* mRNA were normalized to 18S rRNA, where ***, $p < 0.001$ (IL-1 + OSM + emetine versus basal); ###, $p < 0.001$ (IL-1 + OSM versus basal). Data (mean \pm S.D., $n = 6$) are representative of three separate experiments each using chondrocyte cultures from different donors.

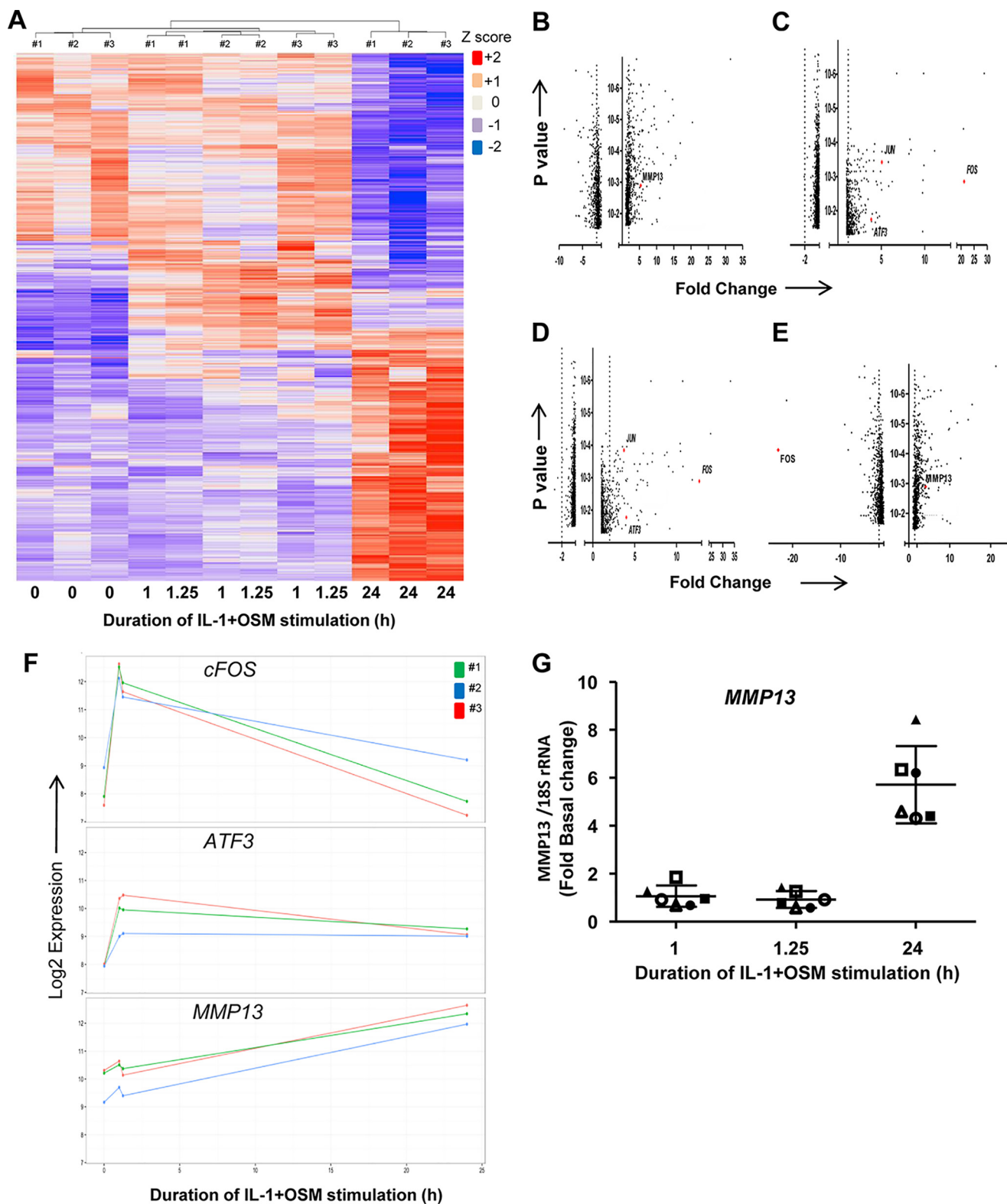


FIGURE 3. IL-1 + OSM stimulation induces expression of several transcriptional regulators prior to MMP13 expression. Human chondrocytes were treated with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for the indicated time points and total RNA isolated. A–F, RNA from three separate populations (numbers 1, 2, and 3) was profiled using the Human HT-12v4 Expression Beadchip. A heat map of all significantly regulated genes is presented. It should be noted that the modest differences in gene expression between 1 and 1.25 h are reflected in these time points being grouped by chondrocyte population (A). Volcano plots are presented depicting genes significantly regulated > ±2-fold, as indicated by the vertical dotted lines, at 24 (B), 1 (C), and 1.25 h (D), as well as a plot of change in fold-expression from 24 versus 1 h (E); genes of note are highlighted in red. F, expression profiles for *MMP13*, *FOS*, and *ATF3* from the microarray dataset for each chondrocyte population are shown. G, RNA from six separate chondrocyte populations was subjected to real-time RT-PCR for *MMP13*, and relative expression levels (normalized to 18S rRNA) at the indicated times determined. The plots show mean ± S.D. (n = 4). Data from the populations used in the microarray analyses are highlighted by the solid symbols (#1, ▲; #2, ■; #3, ●).

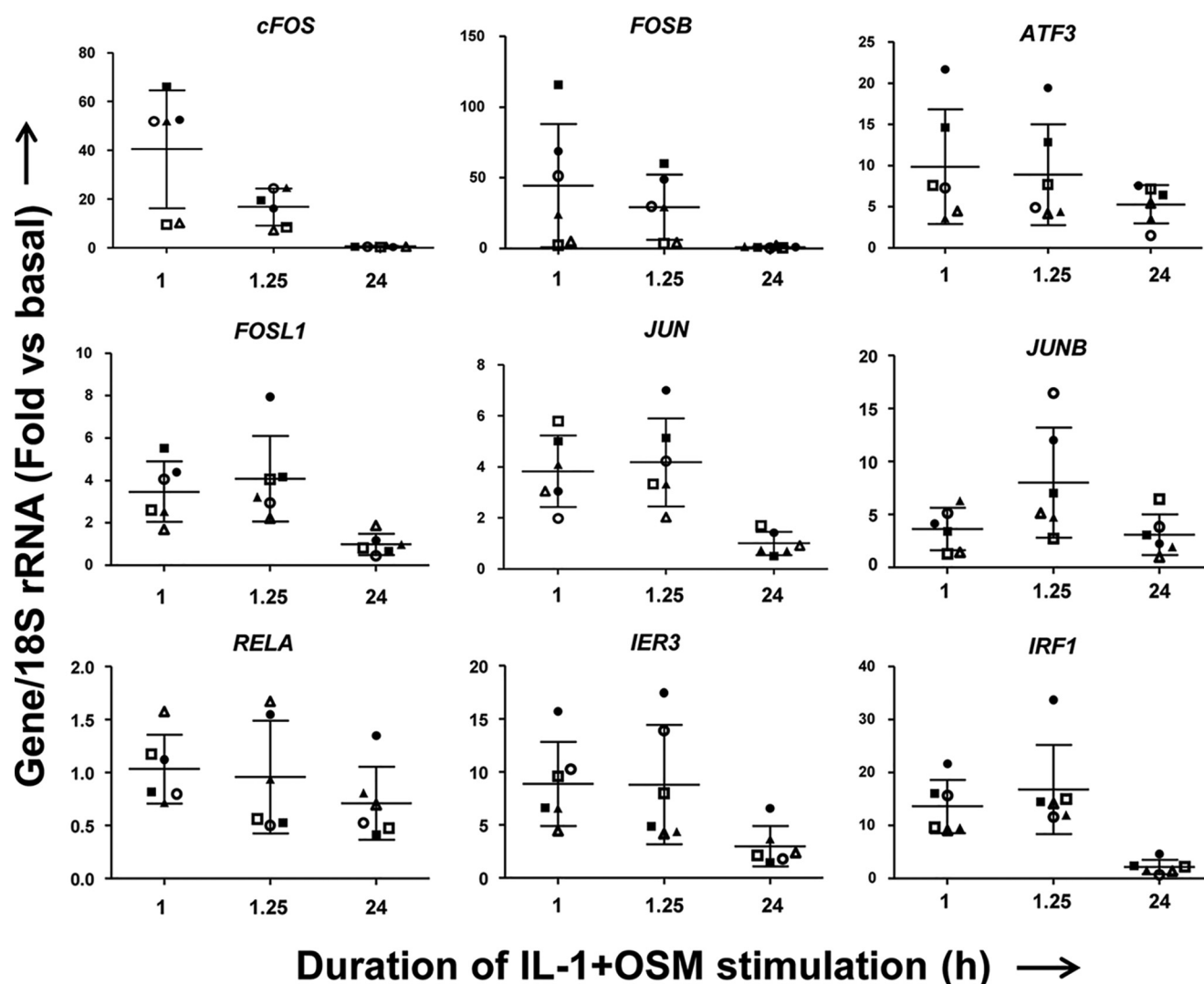


FIGURE 4. **Expression of potential transcriptional *MMP13* regulators following IL-1 + OSM stimulation.** Six separate human chondrocyte populations were treated with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for the indicated durations. Total RNA was isolated, reverse transcribed, and subjected to real-time RT-PCR for the indicated genes as described under "Experimental Procedures." Data are expressed relative to 18S rRNA and presented as fold-increase compared with basal expression (mean \pm S.D., $n = 4$). Note: the same symbols have been used as in Fig. 3E to denote individual populations.

TABLE 1

***MMP13* induction is primarily dependent on FOS family members and ATF3 following IL-1 + OSM stimulation of chondrocytes**

Prior to stimulation with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml), human chondrocytes were transfected with siRNA specific for the indicated genes, or a non-targeting control (siCon; all 100 nM), and mRNA expression levels (mean \pm S.D., $n = 6$) of *MMP13* were measured at 24 h post-stimulation, relative to siCon-treated cells, normalized to 18S rRNA. The percentage inhibition of *MMP13* expression relative to IL-1 + OSM + siCon was then calculated. Statistical comparisons are *versus* IL-1 + OSM + siCon (Student's two-tailed unpaired *t* test), where ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. The data are pooled from three separate experiments, each using chondrocyte cultures from different donors.

Gene (siRNA)	MMP13	
	% Inhibition	<i>p</i> value
	\pm S.E.	
<i>ATF3</i>	42.4 (15.2)	****
<i>cFOS</i>	42.2 (9.5)	****
<i>FOSB</i>	29.8 (19.9)	**
<i>FOSL1</i>	39.1 (8.9)	****
<i>IER3</i>	18.4 (11.7)	*
<i>IRF1</i>	19.5 (14.8)	*
<i>JUN</i>	23.9 (13.3)	**
<i>RELA</i>	17.7 (10.9)	*

Although cFOS was recruited transiently to the promoter at early time points, no pRNA Pol II was detected thus suggesting the gene remained transcriptionally silent but potentially poised as acetylation of H3-histones was observed at all time points studied. This indicates that at early time points, recruitment of a cFOS-cJUN AP-1 complex may not be sufficient to effect *MMP13* gene transcription. Moreover, this suggests that further transcriptional regulators are required for the marked *MMP13* expression observed following IL-1 + OSM stimulation. Inhibition of protein synthesis after IL-1 + OSM stimulation and, importantly, following expression of cFOS protein (as well as cJUN phosphorylation) confirmed a role for the subsequent expression of different regulatory proteins in the transcriptional activation of *MMP13*. To determine these effectors, genome-wide microarray analyses of stimulated human chondrocytes was employed: this confirmed that *FOS* was one of the most highly induced genes following IL-1 + OSM stimulation at 1 h, and that this expression was very transient. Although heat maps indicated relatively few changes in gene expression

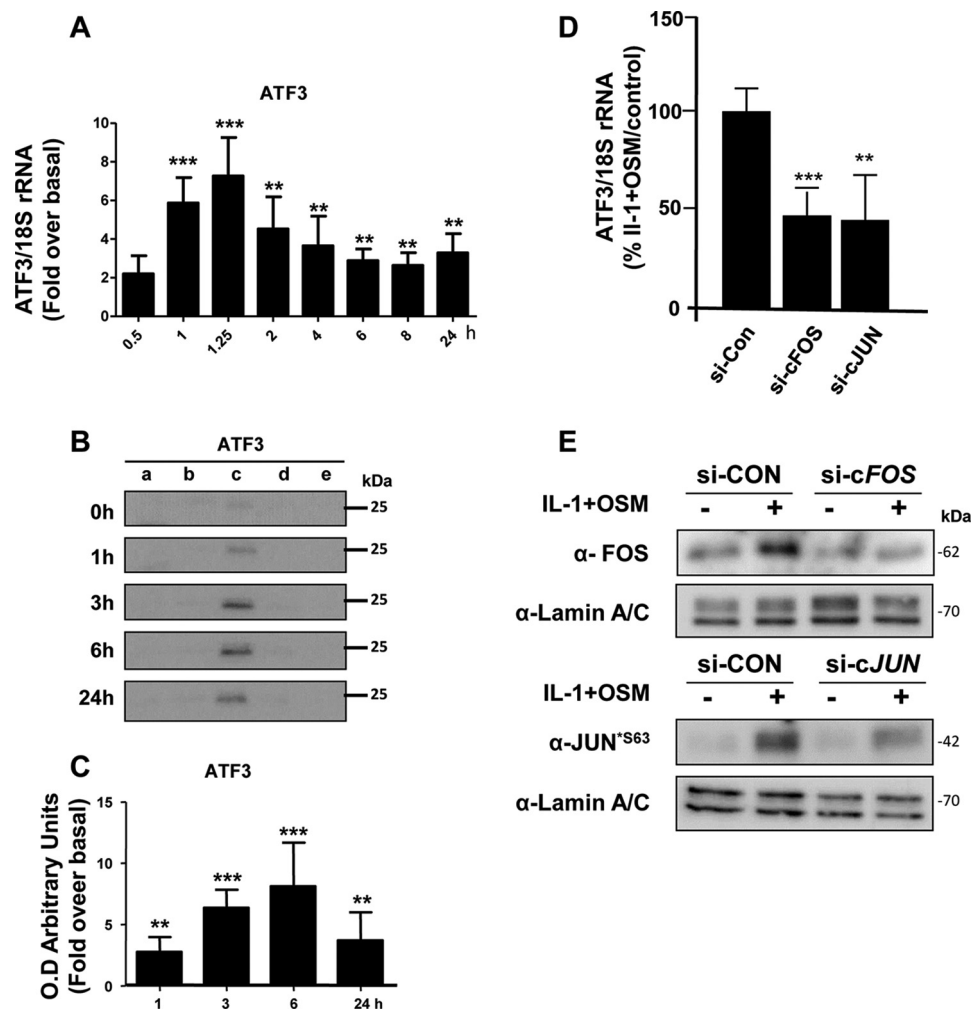


FIGURE 5. IL-1 + OSM induces sustained expression of ATF3, which is cFOS/cJUN-dependent. *A*, human chondrocytes were treated with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for the indicated durations. Total RNA was isolated, reverse transcribed, and subjected to real-time RT-PCR for *ATF3* as described under "Experimental Procedures." Data are expressed relative to 18S rRNA and presented as fold-increase compared with basal expression (mean \pm S.D., $n = 4$). All data are representative of three separate experiments each using chondrocyte cultures from different donors. *B*, cells were subjected to subcellular fractionation and proteins from the cytosolic (*a*), membrane-bound (*b*), soluble nuclear (*c*), chromatin-bound (*d*), and cytoskeletal (*e*) fractions resolved using SDS-PAGE and immunoblotted with an antibody to ATF3. *C*, relative density (compared with $t = 0$ h) data of combined densitometric scans for the soluble nuclear fractions of three separate blots (mean \pm S.D.) are presented. All statistical comparisons are IL-1 + OSM-treated compared with basal (ANOVA), where $***, p < 0.001$; $** , p < 0.01$. *D*, human chondrocytes were treated with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for 1.25 h. Prior to stimulation, chondrocytes were transfected with siRNA specific for *FOS*, *JUN*, or a non-targeting control (*siCon*; all 100 nM). Following stimulation, total RNA was isolated, reverse transcribed, and subjected to real-time RT-PCR for *ATF3*. Data are expressed relative to 18S rRNA and plotted relative to the IL-1 + OSM + *siCon* stimulation (set to 100%; mean \pm S.D., $n = 8$). Statistical comparisons are specific siRNA versus *siCon* (ANOVA), where $***, p < 0.001$; $** , p < 0.01$. *E*, nuclear fractions were also isolated following stimulation and immunoblotted for cFOS and p-cJUN^{S63} or the soluble nuclear marker protein lamin A/C to assess protein expression following siRNA knockdown.

between 1 and 1.25 h, probably reflecting in part the heterogeneity of primary chondrocyte populations, several genes (encoding known modulators of gene transcription) were nevertheless expressed at 1 h and appeared to have a more sustained gene expression profile at 1.25 h. These included *ATF3*, *FOSL1*, *FOSB*, *JUNB*, *RELA*, *IER3*, and *IRF1*. However, siRNA knockdowns of these factors indicated that only FOS family members and ATF3 had the most potent inhibitory effect on *MMP13* expression. It is most probable that both FOSB and FOSL1 could also contribute to AP-1-dependent *ATF3* expression, but herein we have focused on cFOS as this is considered a prime AP-1 effector protein for *MMP* expression (15–25) and silencing of this AP-1 member had the most marked effect on *MMP13* induction. Our data thus implicate an important role for ATF3 in affecting *MMP13* expression subsequent to the

transcription of *FOS*. Expression of ATF3 at both the mRNA and protein levels indicated increased expression at the early 1-h time point as for cFOS but in contrast this was sustained over 24 h where maximum *MMP13* levels were observed. Importantly, siRNA knockdowns of both *FOS* and *JUN* significantly reduced *ATF3* expression, further confirming a transcriptional relationship between these factors and the continued expression of *ATF3* in regulating *MMP13* transcription. ATF3, like other bZIP transcriptional regulators, can form both homo- or heterodimers to either activate or repress gene expression, and in many instances is dependent on cell type and the promoter sequence of the target gene (42, 43). Indeed, the proximal AP-1 motifs for *MMP1* and *MMP13* are different, and whereas silencing ATF3 reduced *MMP13* expression suggesting a transcriptional activator role, *MMP1* expression

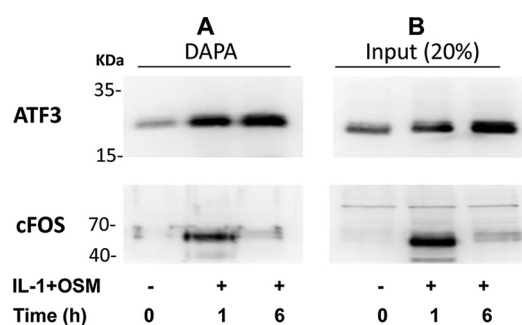


FIGURE 6. **ATF3, but not cFOS, binds the proximal *MMP13* AP-1 motif at a transcriptionally active time point in IL-1 + OSM-stimulated chondrocytes.** Human chondrocytes were treated with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for the durations indicated. Nuclear lysates were prepared and subjected to either DAPA with the ATF3 or cFOS antibodies (A) or Western blotting alone (input) (B), as described under "Experimental Procedures." All data are representative of three separate experiments each using chondrocyte cultures from different donors.

was unaffected.⁸ Furthermore, binding to the proximal AP-1 promoter element of *MMP13* was observed for both cFOS and ATF3 at the early 1-h time point with only ATF3 bound at the much longer time point of 6 h. Consequently, these studies provide strong evidence that ATF3 is transcriptionally dependent upon the initial inflammation-induced expression of cFOS·cJUN so as to mediate prolonged *MMP13* expression. In this context, ATF3 is a well established stress-inducible gene (44), considered a hub for cellular adaptive responses (44, 45), which has previously been shown to be involved in chondrocyte differentiation (46). Moreover, very recent studies have shown that ATF3 regulates AP-1-dependent gene expression in murine osteoclast precursors (47), and that increased expression of ATF3 in mouse and human chondrocytes is a prime mediator of OA development (48). Importantly, deletion of *ATF3* was observed to abrogate the onset of OA (48). In conclusion, data presented herein indicate that cytokine-induced expression of *MMP13* is dependent on the initial transient formation of cFOS·cJUN dimers, which subsequently promote sustained formation of ATF3 to modulate expression of the gene.

Experimental Procedures

Materials—All chemicals were obtained from Sigma (Poole, UK) unless otherwise stated and of the highest purity available. All cytokines were recombinant human. IL-1 α was a generous gift from Dr. Keith Ray (GlaxoSmithKline, Stevenage, UK). OSM was prepared in-house as described (49). siRNA reagents were screened for toxicity using the Toxilight assay of adenylate kinase release (Lonza, Wokingham, UK).

Chondrocytes—Human chondrocytes were isolated by enzymatic digestion of intact articular cartilage from OA patients undergoing joint replacement surgery as described (50). All subjects gave informed consent and the study was approved by the Newcastle and North Tyneside Joint Ethics Committee. Chondrocytes were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum,

100 IU of penicillin, 100 μ g/ml of streptomycin, 40 units/ml of nystatin.

Cell Fractionation and Immunoblotting—Chondrocyte lysates were prepared as described previously (30, 50). In some experiments chondrocytes were subjected to subcellular fractionation using the NE-PER Nuclear and Cytoplasmic Protein Extraction Kit or Subcellular Protein Fractionation Kit (both from ThermoFisher Scientific, Loughborough, UK). Lysates or fractions were resolved by SDS-PAGE, transferred to PVDF membranes, and subsequently probed with the following antibodies: lamin A/C (number 4777) was purchased from Cell Signaling Technology (Danvers, MA); cFos (sc7202), cJun^{S63} (sc7980), and ATF3 (sc188) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Transfection and RNA Interference—Primary human chondrocytes were prepared and cultured as above, and transfected as described previously (30). As an initial screen for modulation of *MMP13* expression, Qiagen FlexiPlate small interfering RNA (siRNA) reagents were used. Dharmacon ON-TARGETplusTM SMARTpools[®] (ThermoFisher Scientific) of 4 specific siRNA duplexes (total of 100 nM siRNA; see supplemental Table S1) were used for follow-up studies where appropriate; silencing efficacy was $\geq 90\%$ as assessed by Western blotting (data not shown). After transfection, cells were stimulated for 1.25 h to measure expression of transcriptional regulators, or 24 h for MMP genes. Changes in mRNA expression were calculated by comparison with cells transfected with 100 nM siCONTROL (siCon: non-targeting siRNA #2, catalog number 001210-02; Dharmacon).

Chromatin Immunoprecipitation (ChIP)—ChIP experiments were performed according to the standard protocol detailed in the EZ-ChIP kit (Merck-Millipore). Briefly, human chondrocytes were cultured until 70–80% confluent, at which point they were treated with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for various durations. Cellular components were then cross-linked using formaldehyde for 5 min with agitation, and the reaction quenched with 0.125 M glycine for 5 min (with agitation). Cells were then washed twice and scraped into PBS supplemented with protease inhibitors. Cells were pelleted, resuspended in lysis buffer, and lysates were sonicated in a Bioruptor Plus (Diagenode) sonicating water bath (15 cycles of 30 s on, 30 s off; full power) to shear chromatin. Samples were then pre-cleared with the appropriate agarose beads for 1 h and subjected to overnight immunoprecipitation with relevant antibodies (10 μ g) or corresponding isotype controls: anti-phospho-RNA polymerase II CTD repeats YSPTSPS (Ser⁵ pRNA Pol II; ab5131; Abcam, Cambridge, UK), anti-acetyl-(Lys^{9/14})-histone H3 (AcH3, number 06-599; Millipore, Dundee, UK), and anti-cFos (Santa Cruz). Antibody·protein·DNA complexes were extracted with agarose beads and washed sequentially with kit-supplied buffers. DNA was then eluted and cross-links reversed (4 h incubation at 65 °C with 5 M NaCl, and then a 1-h incubation at 37 °C with RNase and proteinase K to degrade RNA and protein, respectively). DNA was purified by spin column and then used as input for SYBR Green real-time PCR, using the following *MMP13* primers: proximal promoter/transcription start site: For, 5'-GAAAAAGTCGC-CACGTAAGC-3' and Rev, 5'-CGACAATGAGTCCAG-

⁸ C. D. Macdonald, G. J. Litherland, G. N. Europe-Finner, and A. D. Rowan, unpublished data.

CTCAA-3'; 3'-untranslated region (3'-UTR): For, 5'-TCGG-CACAAAATACAGGTCA-3' and Rev, 5'-GCCTCCCCTT-TTTAGACCAC-3'. Data were normalized to background using isotype-control antibodies and variations in amount of DNA were normalized using the percent input method and shown as fold-change over basal in unstimulated cells at $t = 0$ h.

DAPA—Chondrocyte nuclear lysates were generated as described above (ThermoFisher Scientific). Nuclear protein (80 μ g) was incubated with double-stranded biotinylated oligonucleotide (35 pmol) containing the proximal *MMP13* AP-1 binding site (5'-[biotin]-TAAGTGATGACTCACCATTGC-3'; 5'-GCAATGGTGAGTCATCACTTA-3') in 500 μ l of binding buffer (12 mM HEPES, pH 7.9, 4 mM Tris-HCl, 60 mM KCl, 5% (w/v) glycerol, 0.5 mM EDTA, 1 mM DTT, and 1 mini-protease inhibitor mixture tablet (Roche)/10 ml of buffer) for 45 min at 4 °C. Streptavidin-coated agarose beads, pre-cleared with BSA, were then added and incubated for 2 h with rotation at 4 °C. Beads were pelleted by brief centrifugation, supernatants were discarded, and pellets washed three times with 1 ml of binding buffer for 5 min at 4 °C with rotation. Beads were finally resuspended in 50 μ l of sample buffer, incubated at 100 °C for 5 min, pelleted, and supernatants removed for SDS-PAGE (as above).

Gene Expression Analyses—RNA was typically stabilized in cell lysates in a 96-well format and cDNA synthesized using the Cells-to-Signal™ kit (Life Technologies) as directed. Real-time PCR assays were conducted using primers (30) and conditions described previously (18, 34). TaqMan assays used Universal Probe Library probes (Roche Applied Sciences) as directed (see supplemental Table S4). Nascent hnRNA transcript expression of *MMP13* was determined via reverse transcription and TaqMan (37) employing 5'-AGCACCCTTCTCATGACCTC-3' (For) and 5'-TCCCCTGGTCTTGTGTGAG-3' (Rev) primers spanning the Exon7/Intron7 boundary with probe number 82.

For genome-wide analyses, RNA was isolated from chondrocytes using RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. RNA from three individual patient samples was stimulated with IL-1 + OSM for three durations. Changes in whole genome expression were then profiled using the Human HT-12v4 Expression Beadchip (Illumina, Saffron Walden, UK). Amplification, labeling, hybridization, and detection were all performed by Cambridge Genomic Services (Cambridge University, UK). Raw expression data (GEO accession GSE86578) were analyzed using Agilent GeneSpring GX11 (Agilent Technologies, Santa Clara, CA). Raw data were normalized with a quantile algorithm and the baseline transformed to the median of all samples. Relative gene expression of the probe set was determined by normalizing raw expression values for each probe set to the unstimulated control from each chondrocyte population. The selection of genes for further investigation was based on detailed functional analysis of statistically significant gene expression performed with Ingenuity Pathways Analysis (Ingenuity Systems). Analysis of RNA expression data in the context of known biological response and regulatory networks allowed for the identification of genes with relevant biological function.

Statistical Analyses—Statistical differences between sample groups were assessed using one-way analysis of variance (ANOVA) with a post hoc multiple comparison test or Student's two-tailed unpaired t test, where ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. For clarity, only selected comparisons are presented in some figures.

Author Contributions—A. D. R., G. J. L., and G. N. E.-F. designed the research; C. M. C., C. D. M., and D. J. W. performed the research; C. M. C., C. D. M., G. J. L., A. S., G. N. E.-F., and A. D. R. analyzed the data; G. N. E.-F. and A. D. R. wrote the paper.

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