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Transcriptional response of human articular chondrocytes treated with fibronectin fragments: an *in vitro* model of the osteoarthritis phenotype

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Abstract

Objective: Fibronectin is a matrix protein that is fragmented during cartilage degradation in osteoarthritis (OA). Treatment of chondrocytes with fibronectin fragments (FN-f) has been used to model OA *in vitro*, but the system has not been fully characterized. This study sought to define the transcriptional response of chondrocytes to FN-f, and directly compare it to responses traditionally observed in OA.

Design: Normal human femoral chondrocytes isolated from tissue donors were treated with either FN-f or PBS (control) for 3, 6, or 18 hours. RNA-seq libraries were compared between

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AUTHOR CONTRIBUTIONS

K.S.M.R. and C.K. carried out the analysis and interpretation of the data.
K.S.M.R. and V.U. collected and assembled the data.
K.S.M.R., D.H.P., and R.F.L. drafted the manuscript.
C.K. and V.U. revised the article for important intellectual content.
R.F.L. and D.H.P. obtained funding for, conceived, and designed the experiments.
All authors provided final approval of the article prior to submission.

CONFLICT OF INTEREST

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time-matched FN-f and control samples in order to identify changes in gene expression over time. Differentially expressed genes were compared to a published OA gene set and used for pathway, transcription factor motif, and kinome analysis.

Results: FN-f treatment resulted in 3,914 differentially expressed genes over the time course. Genes that are up- or downregulated in OA were significantly up- ($p < 0.00001$) or downregulated ($p < 0.0004$) in response to FN-f. Early response genes were involved in proinflammatory pathways, whereas many late response genes were involved in ferroptosis. The promoters of upregulated genes were enriched for NF- κ B, AP-1, and IRF motifs. Highly upregulated kinases included *CAMK1G*, *IRAK2*, and the uncharacterized kinase *DYRK3*, while growth factor receptors *TGFBR2* and *FGFR2* were downregulated.

Conclusions: FN-f treatment of normal human articular chondrocytes recapitulated many key aspects of the OA chondrocyte phenotype. This *in vitro* model is promising for future OA studies, especially considering its compatibility with genomics and genome-editing techniques.

Keywords

osteoarthritis; chondrocytes; cartilage; fibronectin; RNA-seq

INTRODUCTION

Osteoarthritis (OA) is the most common form of joint disease and affects over 250 million people worldwide, including over 10% of those older than 60 years¹. There is no known cure, and treatments are currently limited to symptom management. One major reason for the lack of treatments is an incomplete understanding of the mechanisms that promote OA and its progression. While mouse models and human samples have provided valuable insights into OA biology, new human disease models amenable to manipulation and high-throughput screening would improve our ability to understand and potentially better treat this painful and disabling disease.

OA involves many, if not all, of the tissues that comprise articular joints, with degradation and loss of articular cartilage noted as a central feature². Studies of potential OA pathways often compare chondrocytes isolated from normal cartilage obtained from various animal species, including humans, to chondrocytes obtained from OA tissue. A limitation, particularly with human tissue, is that the OA chondrocytes are most often isolated from cartilage obtained at the time of joint replacement, resulting in comparisons being made to cells at an advanced stage of disease. Animal models, including mice, have been critical for mechanistic studies, but major differences in genomes, body structures, and OA prevalence limit the relevance to human biology³.

A commonly used option for modeling the chondrocyte OA phenotype has been to stimulate primary cells or cell lines *ex vivo* with cytokines such as IL-1 or TNF α ⁴. A major limitation of these studies is that the cells are treated with levels of cytokines in the ng/ml range to obtain a desired response, while (at least in the synovial fluid) IL-1 and TNF α are only present in pg/ml amounts⁵. In addition, recent studies, including failed clinical trials of IL-1

and TNF α inhibition in OA, suggest that multiple pro-inflammatory mediators contribute to OA development, and IL-1 or TNF α may not be the driving factors^{6–8}.

An alternative *in vitro* model for simulating a chondrocyte OA phenotype utilizes fragments of fibronectin. Fibronectin is an extracellular matrix protein present in cartilage that is upregulated in OA tissue and subsequently degraded by several proteases^{9,10}. Fibronectin fragments (FN-f) of various sizes and at levels in the μ M range have been detected in OA cartilage and synovial fluid as well as in cartilage from patients with rheumatoid arthritis^{11–13}. Injection of FN-f into rabbit joints was found to induce cartilage proteoglycan loss, which is a feature of early OA¹⁴. Treatment of isolated human chondrocytes or cartilage explants with FN-f has been shown to recapitulate many known features of OA, including production of multiple matrix-degrading enzymes and proinflammatory cytokines found in OA joints^{9,15,16}. While these results demonstrate the value of FN-f treatment for studying OA, the global similarity between FN-f-treated chondrocytes and OA chondrocytes has not been fully explored.

The purpose of this study was to characterize the transcriptional response to acute FN-f stimulation of *ex vivo* human chondrocytes and to compare this response to those previously observed in OA. The goal was to have a model system where small molecule inhibitors or methods to alter expression of specific genes could be tested in short-term studies. Acute rather than chronic stimulation was also used because primary chondrocytes do not maintain their phenotype in long-term culture. We found that FN-f triggers a robust transcriptional response in primary chondrocytes, which correlates with changes observed during OA. Analysis of gene ontology terms, signaling pathways, and transcription factor motifs revealed that known regulators of OA progression also play a role in the FN-f response, as do a host of genes and pathways that had not previously been implicated in OA. These results support FN-f treatment as a viable model for studying transcriptional control of OA progression and provide a valuable resource for future studies.

METHODS

Sample collection and treatment

Primary articular chondrocytes were isolated by enzymatic digestion from normal human femoral cartilage obtained from three tissue donors, aged 50–61 years and without a history of arthritis, as previously described¹⁷. Cells were cultured to confluency in DMEM/F12 media with 10% fetal bovine serum, and then made serum-free for 2 hours prior to treatment with either purified 42 kDa endotoxin-free recombinant FN-f (1 μ M in PBS), prepared as previously described, or PBS as a control¹⁸. The FN-f used here consists of domains 7–10 in native fibronectin, which contains the RGD cell-binding domain recognized by the α 5 β 1 integrin. After 3, 6, or 18 hours of treatment with FN-f or PBS, media was removed, cultures were quickly rinsed with cold PBS, and RNA was immediately isolated using the RNeasy kit from Qiagen.

RNA-seq library preparation

Prior to library preparation, all RNA samples were analyzed using a TapeStation RNA HS tape to confirm RNA integrity numbers (RIN) within 8.5–10, indicating high-quality, intact RNA. Ribosomal RNA was removed using the New England Biolabs NEBNext rRNA Depletion Kit (Human/Mouse/Rat), and libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit and NEBNext Multiplex Oligos for Illumina. Final libraries were then quantified using a Qubit 4 Fluorometer and run on a TapeStation D1000 HS tape, to confirm average fragment sizes were within 260–320 base pairs and calculate molarity for pooling.

Processing of RNA-Seq libraries

RNA-seq libraries were sequenced to an average depth of approximately 58 million reads per sample (50 bp, paired-end reads) on an Illumina HiSeq 4000 (High Output). Low-quality reads and adapters were trimmed using Trim Galore! (v. 0.4.3), and trimmed reads were then mapped to the hg19 transcriptome and quantified using Salmon (v. 0.8.2)^{19,20}. Both programs were run with default settings.

Identifying differential genes

Gene-level quantifications were summarized from each sample using tximport (v. 1.2.0)²¹. Differential analysis was conducted in R with DESeq2 (v. 1.22.2) using a design adjusting for donor variability when calculating differences between treatment groups (~ donor + treatment)²². The log₂ fold change (L2FC) values were shrunken using the “apeglm” method²³. Differential genes were defined as genes with an FDR-adjusted p-value below 0.01 (likelihood ratio test; LRT) and an absolute L2FC above 1 when comparing FN-f treated samples to their time-matched controls.

Temporal clustering of genes

To assign temporal response classes for the 3,914 differential genes, first a z-score was calculated from the variance-stabilized counts, centering the counts in each sample relative to the average counts among all samples for each gene. Then, for each donor and time point, the untreated control score was subtracted from the FN-f treated score for every gene. The difference in z-score was then averaged over the three donors, ultimately providing three values for each gene representing the normalized expression relative to the control at each time point (3, 6, and 18 hours). This matrix was then clustered using k-means clustering with a *k* of 4. These clusters were labeled “Up Early”, “Up Late”, “Down Early”, and “Down Late” based on their expression relative to untreated controls at each time point.

Comparing with genes differentially expressed in OA cartilage

The previously published RAAK study identified genes that were up- or downregulated in OA-affected cartilage compared to preserved cartilage in the same joint²⁴. To focus on only the genes that exhibited the strongest changes in OA, the genes from the RAAK study were filtered for those with a p-value less than 0.01 and an absolute L2FC of greater than 0.585 (equivalent to 1.5 up- or down-fold). A Mann-Whitney U test was used to determine if the

FN-f-induced L2FC of each set of OA-responsive genes were significantly higher or lower, respectively, than the FN-f-induced L2FC of genes outside of each gene set.

GO, KEGG, and transcription factor motif enrichment analysis

The “findMotifs.pl” tool in the HOMER software suite (v. 4.10.4) was used on each cluster of genes in order to identify significantly enriched Gene Ontology (GO) terms (p-value < 0.01), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (p-value < 0.01), and transcription factor motifs (p-value < 1×10^{-12} , per software recommendations)^{25,26}. *De novo* motifs were compared to known motifs from the HOCOMOCO dataset (v. 11, p-value < 0.001). Motifs with best match scores below 0.9 were classified as “unannotated”, in that they did not appear to have a conclusive known motif match.

Kinome visualization

To identify and visualize protein kinases present in each cluster, cluster assignments were plotted using the human kinome visualization tool, Coral²⁷. Flat text files were created listing the ENSEMBL identifier, k-means cluster, and maximum absolute L2FC among all three time points for each differential gene. These lists were then used to plot both categorical (cluster, encoded in branch/node color) and qualitative data (maximum absolute L2FC, encoded in node size) on the protein kinase tree, originally published by Manning et al²⁸.

Data availability

Data is made publicly available at GEO accession GSE150411, including; raw sequencing data; transcript-level quantification output from Salmon; and a table containing gene-level summaries of read counts in each sample, cluster assignments and FDR-adjusted p-values (LRT) for each gene, as well as L2FC and the difference between FN-f treated and control normalized count z-score at each time point.

RESULTS

FN-f induces global changes in chondrocyte gene expression

To determine the extent to which FN-f treatment alters transcription in human chondrocytes, we performed a three-point RNA-seq time course of 3, 6, and 18 hours (Fig S1). We used principal component analysis (PCA) of the 18 samples to determine the extent to which each attribute (donor, time in culture, and time treated with FN-f) contributed to transcriptional state. Untreated samples clustered largely by donor rather than by time in culture, suggesting that time in culture had relatively minor impacts on gene expression (Fig 1A). Conversely, samples treated with FN-f clustered by treatment time, suggesting that FN-f treatment alters transcriptional state consistently, independent of the donor of origin. Statistical analysis of differential expression patterns confirmed these results. Untreated samples exhibited 748 differentially expressed genes between time points (FDR < 0.01, absolute L2FC > 1; Fig 1B, Table S1). In contrast, comparison of FN-f-treated samples to their time-matched controls revealed 3,914 genes that changed significantly in response to FN-f treatment in at least one of the three time points (Table S2). Increased treatment time correlated with increased numbers of differentially expressed genes, with 1,005, 2,561, and 2,636 genes affected at 3,

6, and 18 hours, respectively. Together, these results demonstrate that FN-f treatment has a profound effect on transcription that is distinct from the effects of *ex vivo* culturing, and this effect is robust when accounting for variation in response among biological replicates.

Investigation of specific differential genes revealed expected changes for many known regulators of OA. Genes upregulated in response to FN-f included cytokines and chemokines such as *CXCL2* (178-fold), *LIF* (147-fold), and *IL6* (292-fold). Interleukin-1 β (*IL1B*), a proinflammatory cytokine with elevated expression in OA chondrocytes^{29,30}, was upregulated at all time points, peaking at 110-fold. Upregulated genes also included proteases such as *MMP13* and *MMP10* (both 11-fold). Matrix metalloproteinase 13 (*MMP13*) is an enzyme that degrades type II collagen and is thought to play a critical role in cartilage degradation in OA^{12,15,31–34}. Interestingly, among the downregulated genes were the collagen-binding integrins *ITGA10* and *ITGA11*, which decreased by 3.6- and 3.4-fold, respectively. Additionally, the BMP and WNT antagonists *GREM1* and *DKK1*, which are downregulated in osteoarthritic cartilage, decreased by 40-fold and 5.8-fold, respectively³⁵.

FN-f treatment induces transcriptional changes similar to OA

Changes in gene expression in response to FN-f were compared to the differential expression reported in chondrocytes isolated from OA and preserved tissue in the RAAK study²⁴ using a subset of differential genes with the strongest effects (p-value < 0.01, absolute L2FC > 0.585) (Table S3; Fig 2). Genes upregulated in the OA tissue were also upregulated in response to FN-f treatment, and these effects grew more pronounced with longer exposure to FN-f (Fig 2; Mann-Whitney U test, Bonferroni-adjusted p-value < 0.01). Genes that were downregulated in OA tissue were similarly downregulated in response to FN-f, though only exhibiting statistical significance after 18 hours of treatment. These results suggest that FN-f induces similar transcriptional changes to those found in OA chondrocytes.

To further evaluate FN-f treatment as a model of the OA chondrocyte phenotype, we intersected our differentially expressed genes with 64 OA-associated variants that have been recently identified through Genome Wide Association Studies (GWAS)³⁶. These single-nucleotide polymorphisms (SNPs) represent loci with genotypes statistically associated with the OA phenotype, but most occur in non-coding regions of the genome. This suggests that the SNPs directly impact regulatory regions, and the gene(s) that they affect—which in turn promote the OA phenotype—could be up to hundreds of thousands of base pairs away, mediated through short- or long-range regulatory interactions^{37–42}. In order to identify genes that could be affected by these variants, we overlapped the SNP coordinates with our differentially expressed genes and found that 175 of the differential genes identified in this study were within 400 Kb of a GWAS SNP. These genes are summarized in Figure 3 and include many genes with previously reported connections to OA, such as *NFKB1*⁴³, *SOX7*⁴⁴, *IL11*⁴⁵, *GDF5*^{46,47}, *FGF18*⁴⁸, *TNFSF15*, and *NKX3-2*⁴⁹. While differential genes were not enriched near OA GWAS loci (permutation test p-value = 0.61), the genes within this range represent a preliminary identification of target genes potentially affected by OA-associated genetic variants.

FN-f triggers both early- and late-response genes

To investigate the temporal patterns of transcriptional changes in response to FN-f, we performed k-means clustering of differentially expressed genes (Fig 4). This revealed four distinct temporal patterns. Early-response genes (both up- and downregulated) exhibited changes in expression as early as 3 hours but generally peaked at 6 hours of FN-f treatment. This response class includes many upregulated genes that have been previously implicated in OA, including: AP-1 components *FOS* and *JUN*^{30,50}; interleukins such as *IL1B*, *IL11*, *IL6* and *IL8*^{29,32,51–55}; interleukin-receptor-associated kinases *IRAK2* and *IRAK3*; *NOD2* and *RIPK2*⁵⁶; aggrecanases *ADAMTS1* and *ADAMTS4*^{30,51,57,58}; metalloproteinase *MMP1*^{50,51}; chemokines *CXCL1*, *CXCL2*, and *CXCL3*¹⁶; *NGF*²⁴; *LIF* and *TNF*^{53,59,60}; *TNFAIP6* and *TNFRSF11B*²⁴; and *PTGES*²⁴. Among genes that decreased early were: transcription factor *SP7*, known to be downregulated by TNF⁶¹; WNT antagonist *DKK1*³⁵; and regulators of differentiation such as *DLX5* and *SOX12*^{62,63}. Late-response genes showed maximum absolute L2FC after 18 hours of FN-f treatment. Many of these genes have also been implicated in OA and/or matrix remodeling, including: *MMP10* and *MMP13*^{15,51,56,64}; *CD55* and *PAPPA*²⁴; interleukins such as *IL17C*^{51,52,55}; chemokine receptor *CXCR4*⁶⁵; signaling protein WNT5A^{66–68}; bone morphogenetic protein BMP6^{57,69,70}; IL1 receptor antagonist *IL1RN*; and collagens *COL13A1* and *COL7A1*^{57,71}. Among the genes downregulated at later time points were: cartilage-specific integrin $\alpha 10\beta 1$ (*ITGA10*), as well as integrin $\alpha 11\beta 1$ (*ITGA11*); BMP antagonist *GREM1*^{35,72}; WNT receptor *FZD8*⁷³; differentiation factor *GDF10*⁷⁷; and oxidative defense gene *GPX3*⁷⁴. These results highlight the value of looking at FN-f response across a time course as it both reveals transient events that are not observed at every time point, and provides insight into the temporal order and possibly even causal relationships between regulatory events.

We investigated whether the length of genes—and the corresponding time it would take to transcribe them—could account for the difference in response time. While the mean gene length for the late-response genes was significantly longer than that of early-response genes (Mann-Whitney U test, p-value = 1.3×10^{-11} ; Fig S2), the two distributions exhibited substantial overlap. Therefore, gene length is not likely to be the primary determinant of early versus late response.

FN-f induces transcription of proinflammatory genes and pathways

To understand the likely phenotypic impact of the changes induced by FN-f, we performed Gene Ontology (GO) enrichment analysis for the genes in each of the four clusters (Fig 5A, Table S4). Both early- and late-response genes that were upregulated in response to FN-f were strongly enriched for proinflammatory biological processes including “response to cytokine” and “immune system process”. Genes in these categories include NF- κ B subunits, chemokine receptors, interleukins, MAP kinases, TNF ligands, and other proinflammatory cytokines. This is consistent with previous studies that have demonstrated that FN-f treatment stimulates a proinflammatory response via MAP kinases and NF- κ B signaling^{15,16,31,53,75}, as well as the established role of inflammation in the progression of OA^{30,43,51,52,55,59,60,76,77}. Genes downregulated in response to FN-f were more weakly enriched for GO terms for development, transcriptional regulation, and cell adhesion, and included HOX genes, *TGFBR2*, and *COL8A2*, among others.

To determine pathways that were affected by FN-f treatment, we identified Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were enriched in each of the four gene sets (Fig 5B, Table S5). Early-response upregulated genes were enriched for TNF and NF- κ B pathways, both of which have been implicated in OA and even targeted for therapeutic OA treatments^{43,60,78}. Intriguingly, late-response upregulated genes were strongly enriched for the ferroptosis pathway, a form of programmed cell death dependent on iron and accumulation of lipid peroxides induced by reactive oxygen species, which have been implicated in OA⁷⁹. The late-response upregulated genes involved in ferroptosis included *ACSL1*, *ACSL4*, *ACSL5*, *GCLM*, *SLC39A8*, *SLC7A11*, *TFRC*, *HMOX1*, and *FTH1*. Downregulated genes were also weakly enriched for Rap1 and Hippo signaling pathways, as well as ECM-receptor and carcinogenesis genes.

To visualize how specific kinases were regulated in response to FN-f, we generated kinome tree maps using the human kinase visualization tool Coral (Fig 6). Kinase branches and nodes were colored to indicate time course clusters. This analysis identified many kinases with suspected roles in OA progression, including the p38 pathway member *MAP2K3*, which was upregulated 9.3-fold in response to FN-f^{53,80}. In contrast, we found that *TGFBR2* was downregulated 3.4-fold in response to FN-f, which is consistent with recent findings that decreased *TGFBR2* was correlated with increased OA severity in mice⁸¹. These studies also uncovered a number of kinases that have not been previously implicated in OA or chondrocyte dysfunction. For example, *DYRK3*, which was upregulated 3.6-fold in response to FN-f, is relatively poorly annotated and has thus been characterized as part of the “dark kinome”. *DYRK3* and other understudied kinases identified in this study provide novel targets for further study with regard to their involvement in OA.

Transcriptional drivers of FN-f response include NF- κ B and AP-1

To determine the transcription factors responsible for the global transcriptional changes induced by FN-f treatment, we used the HOMER software suite to identify *de novo* transcription factor motifs enriched in the promoters of up- or downregulated genes (Fig 7). Upregulated genes exhibited a strong enrichment for NF- κ B, Activator Protein 1 (AP-1), and interferon regulatory factor (IRF) binding motifs. In addition to NF- κ B, AP-1 and IRF-8 have both been shown to contribute to OA and cartilage matrix degradation^{30,82}. These results are also consistent with our findings that many AP-1 and NF- κ B subunit genes are upregulated early in response to FN-f.

This analysis also revealed the enrichment of several unannotated motifs in the promoters of up- and downregulated genes (Fig S3). The presence of these *de novo* motifs may suggest that transcription factors with currently uncharacterized motifs also play a role in FN-f response. Additional investigation of the proteomic and phosphoproteomic landscape of chondrocytes responding to FN-f treatment may help to further characterize this model system and identify other key regulators in this response, which could also play a role in OA progression.

DISCUSSION

Developing cell culture systems that model key aspects of diseases can be incredibly valuable for deciphering mechanisms and testing therapeutic interventions, particularly when high-throughput screens are necessary. Using a fragment of the matrix protein fibronectin, we investigated a human cell-culture model of the acute chondrocyte response to cartilage matrix breakdown, a key trigger of OA. Our transcriptome-wide analysis confirmed a similarity between this system and changes observed in OA tissue, suggesting that this is a powerful system with which to study the OA chondrocyte phenotype. We classified over a thousand FN-f-responsive genes by their direction and timing of regulation and confirmed that many genes and pathways upregulated in response to FN-f have previously been characterized as a part of the OA phenotype. This includes inflammatory cytokines and chemokines such as *IL1B* and CXC ligands, matrix-degrading proteinases such as *MMP13*, and members of the NF- κ B signaling pathway.

The presence of early- and late-response gene clusters in this system is reminiscent of primary and secondary responses observed in other systems, such as the inflammatory response in immune cells^{83–88} and the growth factor response promoting differentiation and proliferation⁸⁹. Variation in response times can stem from differences in genomic and regulatory features, including the degree to which products are regulated at transcriptional, post-transcriptional and translational levels, the baseline differences in RNA polymerase II occupancy at transcription start sites, and the dependency of target gene regulation on epigenetic modifications⁸⁹. Further exploration of these and other regulatory mechanisms in this system may provide a deeper understanding of how chondrocytes are phenotypically altered in OA.

This study also revealed many genes and transcription factors that have not been previously associated with OA. One example is the finding of over-representation of the ferroptosis pathway in the upregulated late-response genes. Ferroptosis is a relatively recently described mechanism of cell death that involves iron and excessive levels of lipid peroxides generated by oxidation of lipids⁷⁹. Ferroptosis can result from disturbances in the glutathione-dependent antioxidant system, release of excessive reactive oxygen species (ROS) from the mitochondria, and oxidation of lipids by lipoxygenases and cyclooxygenases⁹⁰. Previous studies have demonstrated that FN-f treatment of chondrocytes generates ROS that regulate signaling involved in MMP expression¹⁸. Although ferroptosis per se has not been described in OA cartilage, studies have demonstrated lipid peroxidation⁹¹, glutathione oxidation⁹², mitochondrial dysfunction⁹³, and increased activity of lipoxygenases and cyclooxygenases⁹⁴, indicating ferroptosis could contribute to chondrocyte death in OA cartilage. This finding may also be relevant to osteoarthritis associated with hemochromatosis where excessive iron is present⁹⁵.

The intersection between FN-f-responsive genes and OA GWAS loci provides a subset of genes that could be affected by OA-associated genomic variants, offering potential targets for follow-up studies. Identifying eQTLs and mapping the three-dimensional chromatin architecture in chondrocytes would allow us to more accurately and specifically identify target genes of OA risk variants. In addition to the NF- κ B family, which has been considered

as an OA target for quite some time, *FGF18* was a FN-f-responsive gene (up early) present in the GWAS dataset. Unlike NF- κ B, *FGF18*, which is an anabolic growth factor, is in clinical trials for knee OA as an intra-articular agent that may promote cartilage growth⁹⁶. Additional growth factors present in both datasets were the BMP family members *GDF5* (down early) and *BMP5* (up early) as well as the BMP signaling protein *SMAD6* (down early). Consistent with the FN-f-induced chondrocyte phenotype, allelic variation in the *GDF5* gene has been associated with reduced expression⁹⁷. *BMP5* is a regulator of bone and cartilage formation during development, but its role in OA is not clear⁶⁹. *SMAD6* is an inhibitor of *SMAD1/5*, and its overexpression in mice was associated with a reduction in osteophyte formation, suggesting that decreased *SMAD6* expression could be detrimental in OA^{98,99}.

While FN-f treatment of *ex vivo* chondrocytes represents a powerful tool to understand some of the events that promote OA, it does not recapitulate all aspects of OA, nor does it serve to replace animal models nor analysis of human tissue. Osteoarthritis is a complex disease involving multiple tissues and arises due to both genetic and environmental factors. A more complete mechanistic understanding of OA therefore requires orthogonal approaches with offsetting advantages and limitations. This *ex vivo* FN-f treatment model does, however, fill a valuable gap and provide a flexible and manipulatable system with which to understand the behavior of chondrocytes in both healthy and disease conditions. By combining this system with recent advances in genomics and genome editing (including the ability to edit primary human chondrocytes¹⁰⁰), this FN-f model offers incredible promise for study of OA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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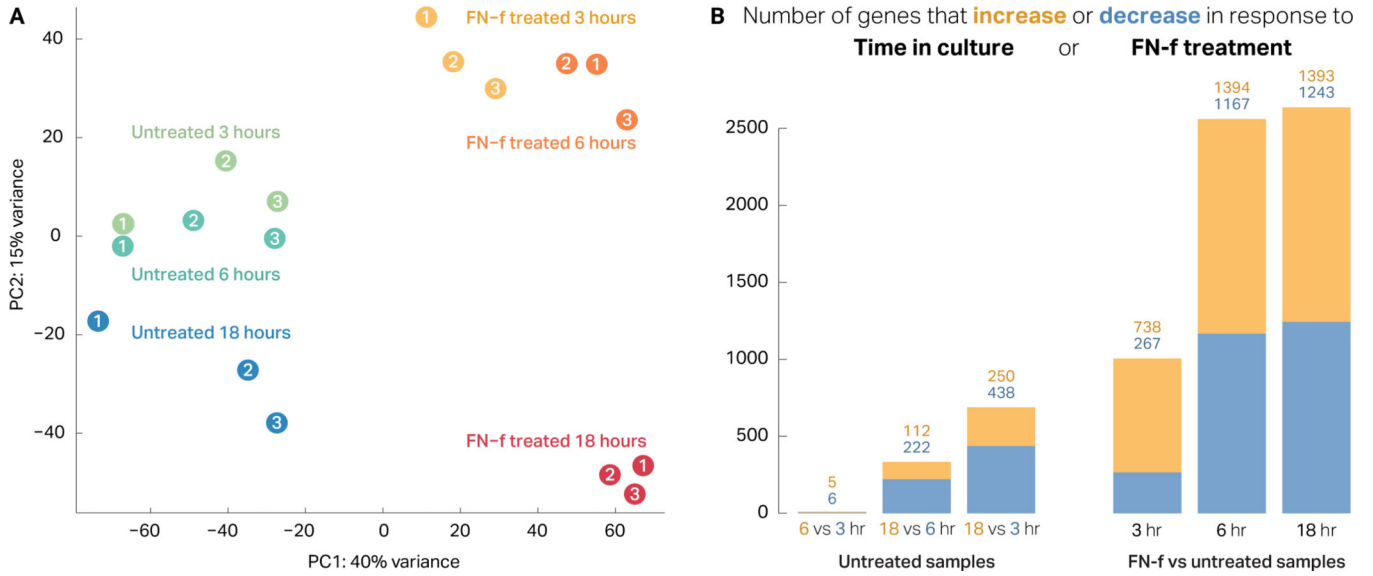


Figure 1. FN-f treatment induces a robust transcriptional response in human chondrocytes. (A) Principal component analysis (PCA) of each sample (colors indicate condition, numbers indicate donor) reveals a distinct separation between FN-f-treated and untreated samples along the first principle component (explaining 40% variance). Additionally, FN-f-treated samples cluster by length of treatment rather than by donor, whereas untreated samples cluster more based on donor (particularly along PC1). (B) Bar plots depicting the number of genes that exhibit significant differences in expression due to time in culture (left) or FN-f treatment (right; FDR > 0.01, absolute L2FC > 1). Yellow and blue bars represent the number of genes that increase or decrease in each comparison. Above each bar, the exact number of up- and downregulated genes are labelled in yellow and blue, respectively. (Left) A bar plot depicting the number of genes that change significantly between untreated control samples reveals that time in culture has only a minimal impact on gene expression. (Right) A bar plot depicting the number of genes that change significantly in FN-f-treated samples compared to time-matched controls demonstrates that FN-f induces a robust transcriptional response in chondrocytes.

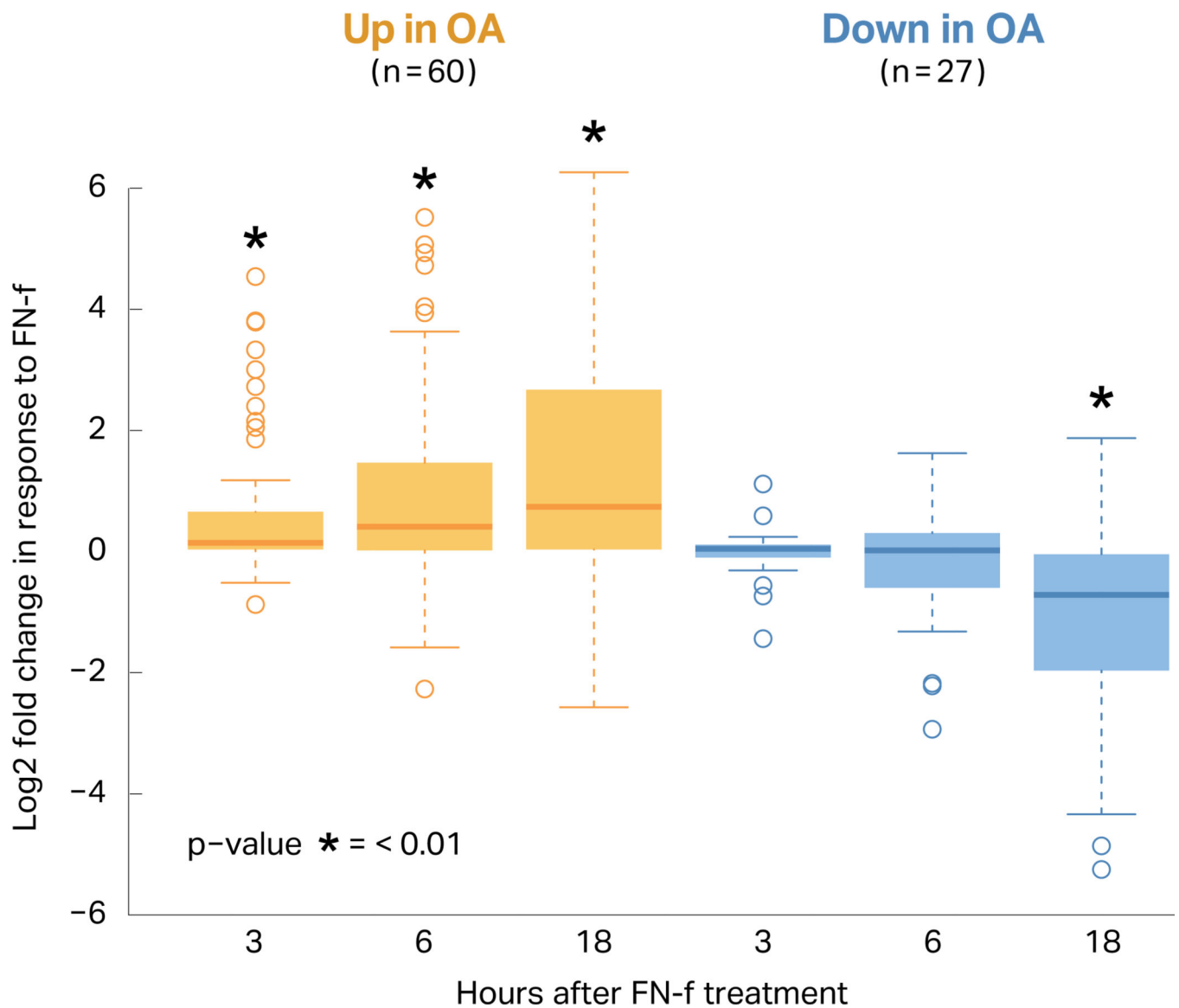


Figure 2. FN-f treatment induces changes similar to osteoarthritis.

The previously published RAAK study compared osteoarthritis cartilage to preserved cartilage in the same joint, and of the differential genes reported, 60 were upregulated and 27 were downregulated with a high degree of change and significance (FDR p-value < 0.01, absolute L2FC > 0.585). The boxplot shows the log2 fold change between FN-f treated and control samples at each time point for both the up- (yellow) and downregulated (blue) genes. These genes were ranked by fold change and compared to a ranked list of fold changes for genes outside of the OA set (* = Mann-Whitney p-values < 0.01).

Up Early		Up Late	Down Early		Down Late	
ALAS1	NIP7	AHCYL2	AC020922.1	NPRL2	ABHD14A	KIF12
ALCAM	PINX1	AQP9	ACBD4	PDE4A	ABHD14B	LBH
ARF6	PML	BTN2A1	AKTIP	PHF7	AC007461.1	LGALS2
ARHGAP27	PSMB8	BTN3A1	AP000769.1	PLCD3	ADAP2	LOXL1
BCL6	PSMB9	BTN3A2	AP003068.23	POC1A	ATP5S	NAT8L
BMP5	RELA	BTN3A3	B3GNT8	PPM1M	BANK1	NEIL1
BTN2A2	RHOG	FILIP1	BSN	PPP1R3B	COL11A1	NIPSNAP3B
CCDC85B	RP11-368I7.4	GMPPB	C22orf29	RGL1	COL21A1	OR7A17
CDC42EP2	RP1L1	GPR56	C3orf18	RPP25	CPA4	PCBP4
DAXX	RTP4	HIC1	CCDC106	RUNX2	CUTA	QTRT1
DCP1A	SLC15A4	LPP	CDC42EP1	SEPT5	CYP2A6	RTN4RL1
FAM110C	SLC30A10	NACC1	CLDN23	SERTAD4	DYNC111	SCAMP5
FGF18	SOCS2	NQO1	CLEC18A	SMAD6	EIF3L	SGK223
FOSL1	SOX7	ORM1	CLEC18C	SUV420H2	ERG	SLC44A2
FRMD8	SOX9	OVCA2	CYP1A1	TBX4	FGFR3	SNTB2
GNL3	SPATA2L	PAPPA	DCAKD	USP17L2	FLNC	STAB1
ICAM1	STRA6	PDXP	FAM212A	WWP2	GCOM1	
ICAM4	SVIL	POLA2	FAM53A	ZMYND10	HIST1H1A	
ICAM5	TAP1	RAC2	FAM86B1	ZNF524	HIST1H2AB	
IER2	TAPBP	RILPL2	GDF5		HIST1H2BB	
IL11	TGFA	SLC39A7	H1F0		HIST1H2BE	
ISLR2	TLR4	SLC39A8	HIST1H1D		HIST1H2BI	
JUNB	TNFSF15	SMCO2	HOOK2		HIST1H3A	
LDLR	TSR1	TAP2	ICAM3		HIST1H3B	
MICALL1	UBE2H	TBX2	LRIG3		HIST1H3E	
MTMR9	UBE2N	TENM3	MNT		HIST1H3G	
NFKB1	UBE2S	TKT	NAT14		HSD17B8	
NFKBIE	VPS37B	TSPAN33	NAT6		ISLR	

Figure 3. FN-f responsive genes near osteoarthritis GWAS loci.

This table lists all differential genes that change in response to FN-f-treatment and that have a transcriptional start site within 400 Kb of a GWAS SNP as identified in Tachmazidou et al, 2019³⁶. Each gene is listed under the appropriate FN-f response class (as determined by k-means clustering of all differential genes).

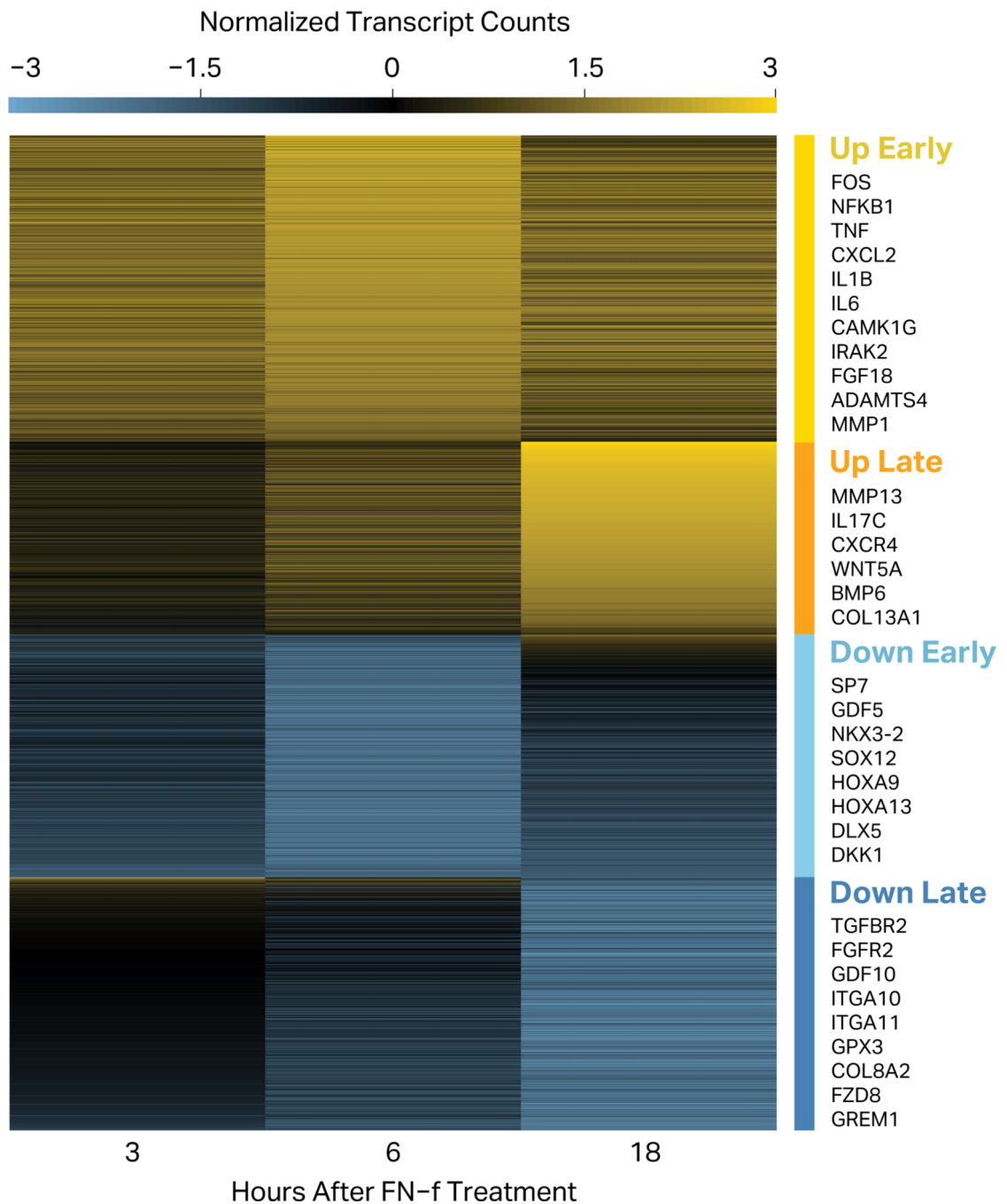


Figure 4. FN-f treatment regulates both early- and late-response genes.

The genes that changed significantly in response to fibronectin fragment treatment were clustered according to their difference in z-score normalized counts between FN-f-treated and untreated samples at each time point. This separated the differential genes into four classes: “Up Early” (yellow; n=1,205), “Up Late” (orange; n=759), “Down Early” (light blue; n=956), and “Down Late” (dark blue; n=994). Selected genes are highlighted in each cluster.

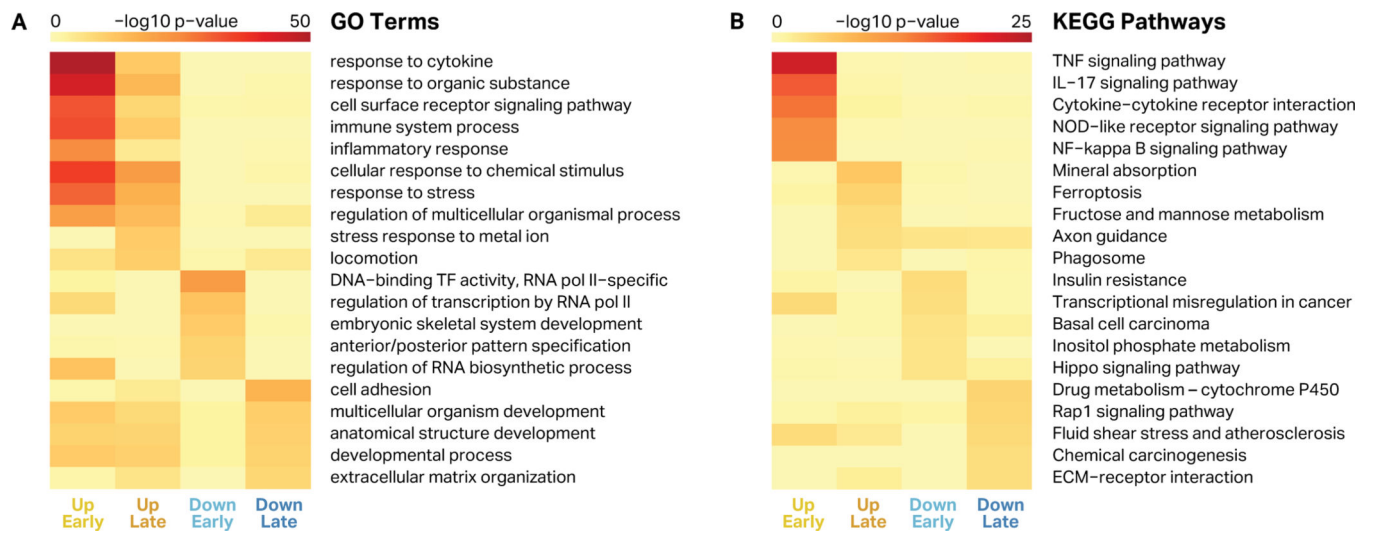


Figure 5. Gene ontology terms and KEGG pathways enriched in response to FN-f. For every cluster of differential genes, five representative GO terms (A) and KEGG pathways (B) are shown, based on enrichment p-value and redundancy with other terms. Heatmap color represents the -log₁₀ of the enrichment p-value for each term or pathway for genes in each cluster (up early, up late, down early, and down late).

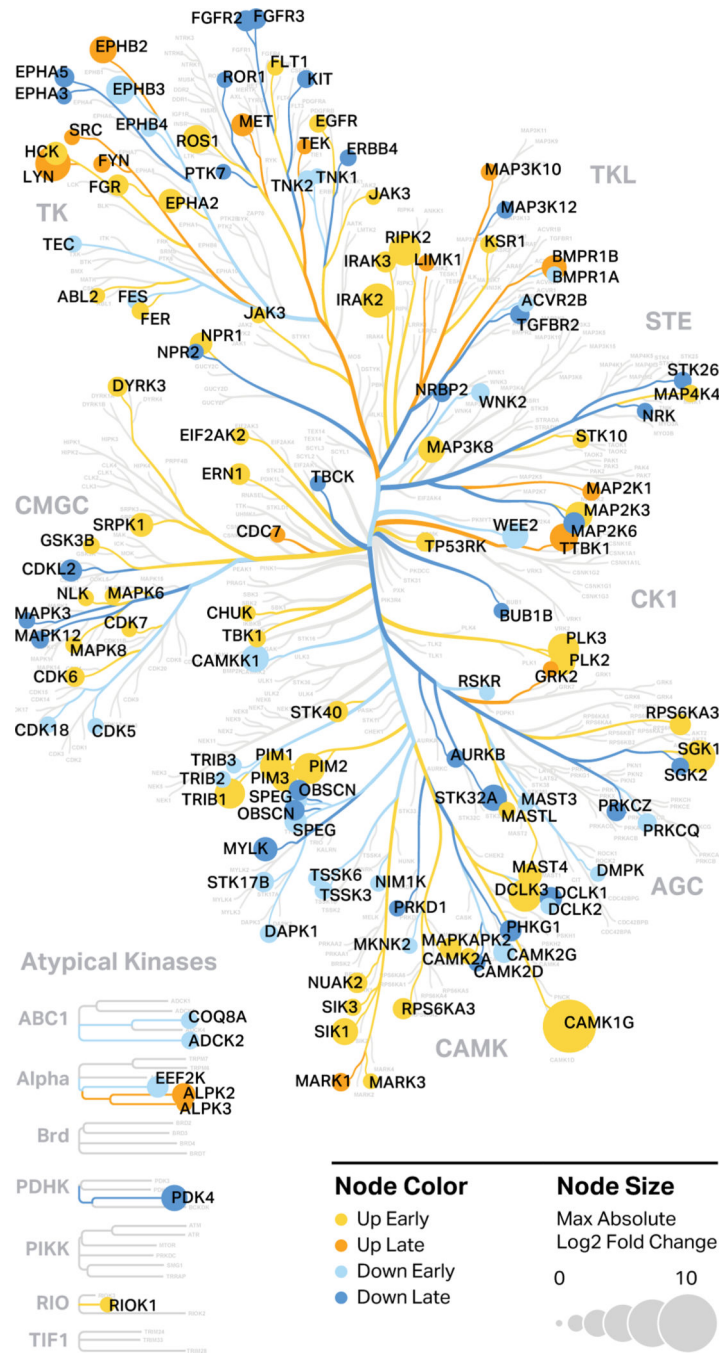


Figure 6. Protein kinases transcriptionally regulated by FN-f. Protein kinases that are differentially expressed in response to FN-f treatment were highlighted in a human kinome map generated by Coral. Color represents the temporal response class of the genes as determined by k-means clustering, while node size represents the maximum absolute L2FC between time-matched FN-f-treated and control samples among the three time points.

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Motif 1 (Up)P-value: 8.4×10^{-24}

Occ: 4.6% vs. 1.2%

NF- κ B

Match Score: 0.931

**Motif 2 (Up)**P-value: 1.1×10^{-18}

Occ: 19.3% vs. 12.0%

AP-1

Match Score: 0.925

**Motif 3 (Up)**P-value: 2.7×10^{-17}

Occ: 3.9% vs. 1.1%

IRF

Match Score: 0.923



Figure 7. Transcription factor motifs enriched in promoters of genes upregulated by FN-f. Transcription factor motifs were identified *de novo* from the promoters of genes either upregulated or downregulated by FN-f, as determined by their k-means clustering assignment. Each *de novo* motif (top) lists the p-value of enrichment, and the percent of promoters occupied by the motif both within the gene set and in the background. The bottom motifs are the best match from the HOCOMOCO database, and are reported along with the match score as determined by HOMER. Only factors with enrichment p-values below 1×10^{-12} and a match score of 0.90 or higher to a known motif are reported here. The best matches for the top three motifs among upregulated genes are NF- κ B (TF65_HUMAN.H11MO.0.A), Activator Protein 1 (AP-1; FOSB_HUMAN.H11MO.0.A), and interferon regulatory factor (IRF; IRF9_HUMAN.H11MO.0.C).