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RESEARCH ARTICLE OPEN ACCESS

A Network-Based Approach to Understanding Key Signaling Pathways in Intervertebral Disc Biology

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ABSTRACT

Background: Intervertebral disc degeneration (IDD) reflects a poorly characterized shift from anabolic matrix renewal to catabolic breakdown. Fragmentary experimental and modeling efforts leave many signaling routes unresolved; a consolidated map is required to guide hypothesis-driven studies.

Methods: A literature-curated regulatory network model (RNM) for human nucleus pulposus (NP) cells was assembled from PubMed and enriched with interactions from STRING, KEGG and R&D Systems. The static graph was converted to a semi-quantitative dynamical system. Simulations examined responses to interleukin-1 β (IL-1 β) and the Toll-like receptor (TLR) and were benchmarked against time-resolved proteomics from 2D NP monolayers and 3D alginate cultures representing healthy and degenerated discs. Single-node perturbations assessed the impact of targeted inhibition on catabolic output.

Results: The final network contains 82 proteins and 193 directed edges; 41.4% of supporting evidence derives from NP-specific studies, the highest for any cell type. Baseline activity depicts a non-degenerate disc, with high expression of anabolic mediators and low expression of catabolic enzymes. Both IL-1 β and TLR elicited strong c-Jun and p65 NF- κ B activation in silico and in vitro. The model simulated I κ B α depletion, whereas its increase in the experimental study shows the biological agreement among in silico and in vitro simulations.

Conclusions: This RNM unifies scattered IDD data into a validated dynamic framework that mirrors NP signaling. The publicly available model provides a foundation for multiscale studies linking molecular events to disc mechanics and for prioritizing therapeutic interventions.

1 | Introduction

Intervertebral disc degeneration (IDD) is a key pathological process implicated in low back pain (LBP), one of the leading

causes of disability worldwide [1]. Although LBP has a complex etiology and pathology, mounting evidence indicates that IDD has a central role in the development and progression of LBP. The development of IDD is based on cellular and biochemical

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changes in the microenvironment of IVD, which leads to progressive impairment of the function and structure of the IVD [2, 3], the pathogenesis of which has not been fully elucidated. Yet, progressive destruction of the extracellular matrix (ECM), changes in the phenotype of IVD cells, loss of active IVD cells, increased cell senescence and death, and excessive inflammatory responses have been proposed as key factors that aggravate the disruption of normal disc function [4, 5]. Current clinical treatments for IDD primarily address pain management and symptom relief [6]. In the case of severe chronic pain, surgical treatments of degenerated discs, such as discectomy, spinal fusion, lumbar disc herniation decompression surgery, and nucleotomy, are generally performed [7–9]. However, these methods alter the flexibility and/or mechanical stability of the spine, and their success remains very limited in the long term. Some studies suggest that inducing ECM remodeling might mitigate mild to moderate disc degeneration, but this requires good control of the biological processes to halt and restore, and both the efficacy and robustness of such interventions have yet to be confirmed in the clinic [10–15]. A deeper understanding of IDD pathogenesis is, therefore, essential to identify effective therapeutic targets to cope with the degradation and the functional alterations of the IVD structure.

The IVD comprises a central nucleus pulposus (NP), a surrounding annulus fibrosus (AF), and cartilaginous endplates (CEP) [16]. The NP contains type II collagen and abundant proteoglycans, particularly aggrecans (ACAN), enabling the tissue to retain water and keep functional intradiscal pressure levels. The AF, composed of type I collagen fibers in the outer AF and gradually turning into a higher proportion of type II collagen in the inner AF, confines the NP and contributes to the maintenance of the intradiscal pressure levels, and it provides both tensile and shear strength to the disc [17, 18]. As per the CEP, they act as a cranial and caudal filter, mainly between the NP and the adjacent vertebrae, contributing to the dynamic control of the intradiscal fluid balance, and to the transport of solutes necessary for the metabolism of the IVD cells [19, 20]. IDD does not only alter disc tissues, but also the functional interactions among these tissues, involving hereditary, age-related, metabolic, and biomechanical risk factors. Accordingly, the biological degenerative mechanisms of the IVD are complex and heterogeneous and defining IDD endotypes stands for a real challenge.

IDD progression is characterized by a spectrum of molecular, cellular, and biomechanical alterations, including disrupted ECM homeostasis [21, 22], emergence of immunomodulatory microenvironments [23], altered biomechanics and mechanotransduction [24], cell senescence [25, 26], and cell death [27, 28]. In latter stages, pathological neo-vascularization and neo-innervation occur [29]. Already in early to mild degeneration, because the IVD is an avascular organ, the accumulation of degradation products that are difficult to metabolize occurs, and a closed acidic environment may gradually develop. This homeostatic imbalance propagates inflammatory signals and causes a release of inflammatory mediators (TNF, IL-1 α , IL-1 β , IL-6, IL-8, IL-17) and matrix-degrading enzymes (MMPs, ADAMTS) [21, 30–34].

There is a great need for understanding these complex interactions among signaling pathways, and cascades of molecular

events that allow cells to sense and respond to internal and external cues, before advanced degeneration steps. Essential to grasp the mechanisms of disc homeostasis and of the disruption thereof, extensive experimental models have been developed to explore and manipulate specific molecular targets. Such research revealed numerous pathways that regulate normal IVD cell survival, matrix synthesis, and overall tissue homeostasis, but that often become dysregulated in IDD [35–38]. As per the promotion of anabolic activity in the IVD, IGF signaling was reported to boost proteoglycan and collagen expression while inhibiting ECM degradation [39]. Furthermore, TGF- β and BMP pathways are crucial for both embryonic disc development and adult disc maintenance, enhancing collagen and proteoglycan synthesis; however, excessive or dysregulated TGF- β /BMP signaling can paradoxically lead to ectopic ossification in aging animal discs [40–42]. Although individual miRNAs can target multiple genes across diverse pathways, a subset of miRNAs has been shown to converge specifically on the TGF- β signaling cascade. Recent studies indicate that modulating these TGF- β targeting miRNAs may be a novel target for IVD degeneration [43, 44]. During the progression of IDD, pro-inflammatory cytokine IL-1 β activates MAPK/ERK and NF- κ B signaling, which shifts the balance toward ECM degradation rather than synthesis, drives further release of IL-1 β and TNF- α , promotes disc cell apoptosis [45, 46] while restricting NP cell proliferation, and enhances autophagy [47]. MAPK/ERK inhibition was proposed, therefore, as a promising avenue for therapeutic intervention [48]. It should be noted that during IDD, MAPK/P38 and MAPK/JNK, which directly activate AP-1, are more dominant than MAPK/ERK [49]. The NF- κ B pathway is also understood as a key regulator of IDD, driving the expression of catabolic enzymes (e.g., MMPs and ADAMTSs) in NP cells, in response to pro-inflammatory stimuli, a process that can be mitigated by inhibitors such as BAY11-7082, siRNA-mediated p65 knock-down, or IKK-targeting peptides [50]. Another key pathway with context-dependent behavior is Wnt / β -catenin signaling. At physiological levels it supports NP-cell survival and stimulates ACAN and COL2A synthesis, contributing to normal disc development and maintenance [51]. On the other hand, aberrant Wnt activity can accelerate the degenerative cascade by promoting catabolic mediators [36, 52]. Although each pathway has its own unique molecular cascade, significant cross-talks exist, reflecting the complex regulatory network that maintains, or disrupts, disc integrity. While apprehending such cross-talks looks necessary, it also appears overwhelmingly complex, largely out of reach of experimental models and measurements alone.

Accordingly, several cell signaling-based computational models have been proposed so far. The first mathematical model of NF- κ B dynamics was developed by Carlotti et al. [53], and many teams have improved it since then by incorporating reactions that govern I κ B dynamics [54–56] and introducing the p100 protein, which acts as an inhibitor of NF- κ B activation [57]. Pogson et al. took the modeling of the pathway's stochastic behavior one step further by developing an agent-based model of the NF- κ B signalling [58]. Regarding the ERK signaling pathway, over 30 computational models exist to investigate various aspects of the biological behavior of this cascade, such as bistable feedback loops [59], oscillations [60], feedback inhibition [60], autocrine loops [61, 62], Ras activation [63], receptor regeneration [64], and temporal dynamics [65]. Niarakis

et al. developed a comprehensive dynamic, logical model of the main signaling pathways involved in mast cell activation that integrates expert feedback to elucidate the pathway's responses to various stimuli systematically [66]. More recently, models have explored the YAP/TAZ signaling pathway for cellular mechanotransduction in response to matrix stiffness [67]. Others have focused on TNFR1-mediated signaling pathways for NF- κ B activation, apoptosis, and necroptosis in human cancer therapy [68]. Several groups have also modeled the Wnt and VEGF pathways involved in the pancreatic ductal adenocarcinoma cancer [69], and the cross talk between SARS-CoV-2 infection and the renin-angiotensin-aldosterone [70]. Finally, the effects of varying mechanical forces and inflammation on knee articular chondrocytes have been examined [71].

Despite these advancements for heterogeneous sets of diseases, cells, and biological systems, IDD research still lacks a comprehensive model that integrates all the crucial signaling pathways involved in its regulation. Thus, this study aims to develop a literature-based regulatory network model that captures the most important signaling pathways implicated in IDD. To achieve this, ligand-mediator interactions identified from existing cell-network model [72] will be integrated to map how specific molecules activate or inhibit downstream soluble factors, thereby providing a holistic framework for modeling IVD signaling dynamics. Furthermore, experimental validations will be carried out to confirm the model's predictive capabilities in recapitulating key aspects of IDD pathology.

2 | Materials and Methods

2.1 | Corpus and Static Signaling Pathway Representation

To build a comprehensive map of the signaling pathways regulating the IVD, PubMed was queried systematically with a series of targeted searches of keywords to capture studies focused on the molecular cascades initiated by soluble protein ligands (Table 1). Queries marked with “nucleus pulposus” were restricted to that compartment to reduce an otherwise excessive number of results. Based on a previously established model that identified key soluble proteins [72], these proteins served as ligands for tracing corresponding downstream signaling events. For each ligand, the activated pathway was delineated in three steps (Figure 1): from receptor binding (1. Reception) through intracellular signaling events (2. Transduction) until it either activates or inhibits another soluble protein (3. Response).

This systematic approach yielded 1238 articles, from which 45 peer-reviewed studies, taht is corpus, that provided detailed insights into the molecular cascades in NP cells were identified. The aim was to construct a directed protein-protein interaction model that maps both the identified activations and inhibitions, that is links, among proteins, that is nodes, within the pathways extracted from the corpus. By examining the sequence from ligand binding to receptor engagement, adaptor protein recruitment, and the subsequent

TABLE 1 | PubMed systematic search for signaling pathways in IVD research.

Boolean relationships	# of results
{TGF-beta} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	87
{GDF5} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	6
{IGF-1} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	22
{CCN2} $\vee$ {CTGF} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	18
{PROGRANULIN} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	1
{IL-1beta} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc} $\wedge$ {nucleus pulposus}	208
{IL-6} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	95
{IL-17} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	26
{IL-18} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	6
{IL-12} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	1
{IFN-gamma} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	2
{TNF} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc} $\wedge$ {nucleus pulposus}	192
{IL-10} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	8
{IL-4} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	2
{IL-1Ra} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	3
{IL-8} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	26
{NF-kB} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc} $\wedge$ {nucleus pulposus}	225
{MAPK} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc} $\wedge$ {nucleus pulposus}	118
{JAK-STAT} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	8
{PI3K/AKT} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc} $\wedge$ {nucleus pulposus}	99
{WNT} $\wedge$ {signaling pathway} $\wedge$ {intervertebral disc}	85

Note: Each row shows the Boolean query used to identify studies addressing a particular signaling molecule or pathway in the context of the intervertebral disc. The symbols $\wedge$ and $\vee$ denote logical AND and OR, respectively. Where indicated, the term “nucleus pulposus” was added to focus on that disc compartment. The “# of results” column lists the total number of PubMed hits retrieved as of the date of querying. Searches were conducted between 2 and 20 of March 2024.

kinase-driven signaling events, we sought to delineate the precise regulatory mechanisms that drive NP cell responses central to IDD: oxidative-stress handling, pro-inflammatory cytokine production, anabolic extracellular-matrix synthesis, and programmed cell death.

The topology of the NF- κ B pathway in the model established IkBa as an inhibitor of Nf-kB. Biologically, IkB α binds directly

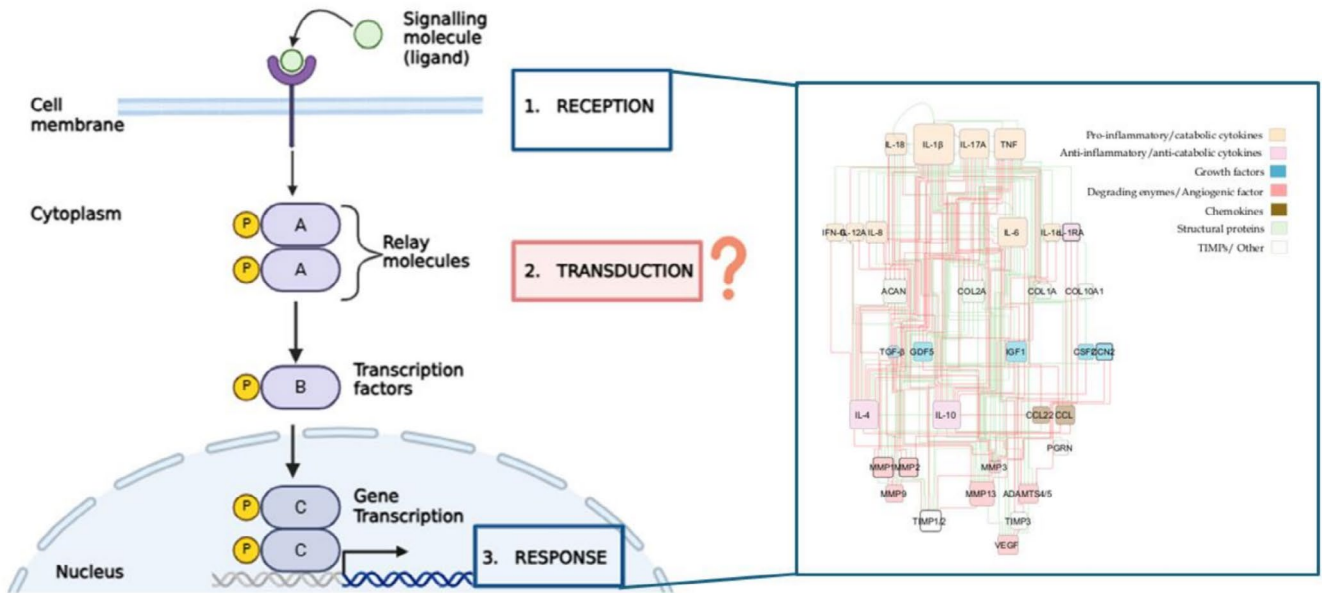


FIGURE 1 | Static representation of a signaling pathway network in IVD regulation. On the left, the diagram highlights three general stages of a signaling pathway: reception (ligand binding), transduction (intracellular relay), and response (transcriptional changes). On the right, the PPI network displays soluble proteins implicated in IVD regulation, showing how ligands and their downstream targets are interconnected. Although this network captures ligand-response relationships, the detailed intracellular signaling steps connecting them are not depicted in the p-p model.

to the p65-p50 NF- κ B dimer and sequesters the latter in the cytoplasm, preventing DNA binding and transcriptional activity in resting cells (Figure 2A). Phosphorylation of I κ B α by the IKK complex marks I κ B α for ubiquitination and proteasomal degradation. Once I κ B α is degraded, the “brake” on p65 is removed and the liberated NF- κ B complex translocates into the nucleus (Figure 2A). In the model, I κ B α was represented as an inhibitory node upstream to p65, to capture its role in preventing NF- κ B translocation (Figure 2B). Yet, this representation must be correctly interpreted in contrast to phosphorylation data: a phosphorylation event on I κ B α in biology corresponds to the degradation of the molecule, and it was translated into an inhibition of the I κ B α node in the network, which relieved the inhibition of the node p65. Thus, measured increases in I κ B α phosphorylation in experiments should correspond to a decreased activity of the I κ B α node in the model.

2.2 | Mathematical Approach

After gathering the corpus information that defined the nodes and the oriented links, we transformed the static, knowledge-based RN into a dynamic model that captures the evolution of protein activity, on an artificial timeline. This transformation was achieved using a method originally proposed by Mendoza et al. which translates static Boolean solutions into a continuous system of ordinary differential equations (ODEs) [73]. Briefly, the time derivative of each node's activation depends on the set of activating and inhibiting regulatory links, as well as on the current activation states of the corresponding upstream nodes, following a sigmoidal integration of the total regulatory input (Equation 3). This framework enables a semi-quantitative representation of the steady states (SS) imposed by the knowledge-based network topology.

Consider a network of proteins such as X , Y , Z , and so forth. Each protein's expression level (or concentration) is expressed as a function of time:

$$X(t), Y(t), Z(t) \quad (1)$$

The overall state of the system at time t is given by the vector:

$$x(t) = [X(t), Y(t), Z(t), \dots] \quad (2)$$

For each protein X , its temporal behavior is modeled by an ODE of the form:

$$\frac{dX}{dt} = (\text{production terms}) - (\text{decay terms})$$

where the production terms capture the activation due to incoming signals, and the decay terms reflect the natural degradation of the protein.

In our dynamic framework, each node x_n , representing a soluble protein, is governed by an ODE that integrates activation by other proteins and intrinsic decay, and is expressed as follows:

$$\frac{dx_n}{dt} = \frac{-e^{0.5h_n} + e^{-h_n(\omega_n-0.5)}}{(1 - e^{0.5h_n}) + (1 + e^{-h_n(\omega_n-0.5)})} - \gamma_n x_n \quad (3)$$

Here the term on the left, $\frac{dx_n}{dt}$, represents the rate of change of the protein's expression level. h_n represents the gain, that is how fast the total node activation will change, as the aggregated input, ω_n , changes. High gain values make the SS tend to Boolean solutions. The fraction on the right is an activation function, formulated as a sigmoid, which is modulated by the aggregated input ω_n . In contrast to Boolean activation rules,

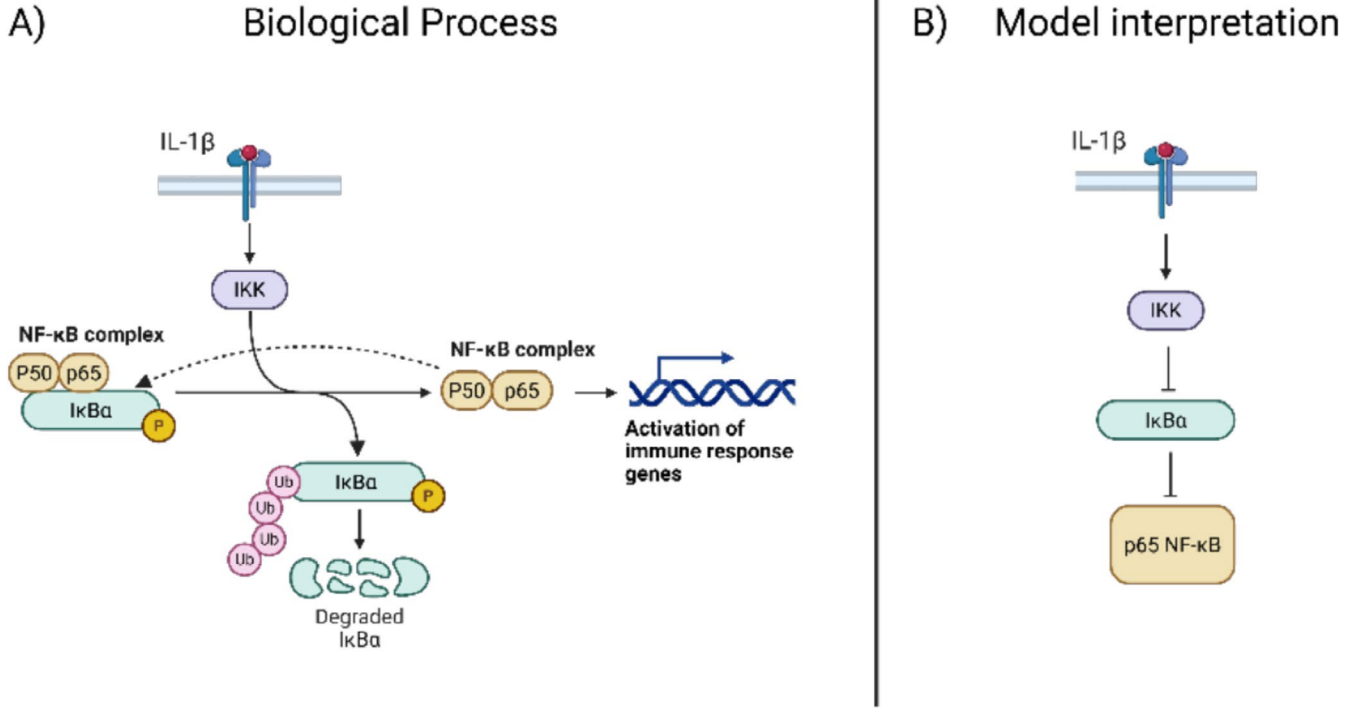


FIGURE 2 | Biological versus model depiction of the IκBα–NF-κB switch. (A) IL-1β activates IKK, which phosphorylates and ubiquitinates IκBα, leading to its degradation and release of the p50–p65 NF-κB dimer for gene activation; resynthesised IκBα later restores inhibition (dashed arrow). (B) The in silico model abstracts this as IKK inhibiting IκBα, which in turn inhibits p65 NF-κB, a double-negative path that reproduces net NF-κB activation after IL-1β stimulation.

this sigmoid function allows capturing a continuous spectrum of intermediate node activation, between 0 and 1, for given concentrations of activators and inhibitors. The final term, $-\gamma_n x_n$, accounts for the linear decay of the protein, with $\gamma_n > 0$ being the decay constant.

The variable ω_n encapsulates the net regulatory input for node x_n by integrating the contributions of both activators and inhibitors. Since the regulatory influences can vary in number and type, ω_n is computed using distinct formulations depending on whether a node is influenced solely by activators, solely by inhibitors, or by a combination of both. The equation is as follows:

$$\omega_n = \begin{cases} \left(\frac{1 + \sum \alpha_{nk}}{\sum \alpha_{nk}} \right) \left(\frac{\sum \alpha_{nk} x_{nk}^a}{1 + \sum \alpha_{nk} x_{nk}^a} \right) & \bar{\sigma} \\ \left(1 - \left(\frac{1 + \sum \beta_{nl}}{\sum \beta_{nl}} \right) \left(\frac{\sum \beta_{nl} x_{nl}^i}{1 + \sum \beta_{nl} x_{nl}^i} \right) \right) & \bar{\sigma}\bar{\sigma} \\ \left(\frac{1 + \sum \alpha_{nk}}{\sum \alpha_{nk}} \right) \left(\frac{\sum \alpha_{nk} x_{nk}^a}{1 + \sum \alpha_{nk} x_{nk}^a} \right) \left(1 - \left(\frac{1 + \sum \beta_{nl}}{\sum \beta_{nl}} \right) \left(\frac{\sum \beta_{nl} x_{nl}^i}{1 + \sum \beta_{nl} x_{nl}^i} \right) \right) & \bar{\sigma}\bar{\sigma}\bar{\sigma} \end{cases} \quad (4)$$

$$0 \leq x_n \leq 1.$$

$$0 \leq \omega_n \leq 1.$$

$$h_n, \alpha_{nk}, \beta_{nl}, \gamma_n > 0.$$

$\{x_{nk}^a\}$ is the set of activators of x_n .

$\{x_{nl}^i\}$ is the set of inhibitors of x_n .

$\bar{\sigma}$ is used if x_n has only activators.

$\bar{\sigma}\bar{\sigma}$ is used if x_n has only inhibitors.

$\bar{\sigma}\bar{\sigma}\bar{\sigma}$ is used if x_n has both activators and inhibitors.

The weights α and β quantify the influence of each regulatory input. In the absence of any information regarding protein sensitivity to its mapped activators and inhibitors, we adopted $\alpha = 1$ and $\beta = 1$, which translated into sigmoidal node activation with average steepness at inflexion [73]. The design of ω_n ensures that its values remain within the interval [0, 1], preserv-

ing the Boolean asymptotic behavior of the activation function, in case of high gains. For all nodes, an intermediate gain value was selected, that is $h = 10$, to preserve average sigmoidal total node activations, for given α , β , activator, and inhibitor values. According to the set of model parameter values, a decay constant $\gamma = 1$, maintained a consistent rate of protein degradation, for the interpretation of the semi-qualitative SS of the modeled network [73].

The network dynamics and SS behavior were computed using a continuous-time regulatory network model formulated as a system of ODEs and solved in MATLAB using the variable-step Runge–Kutta method (ode45). To characterize baseline and stimulus-induced responses, a paired simulation design was employed. For each of $n=200$ independent runs, node activation levels were initialized with random values uniformly sampled from $[0, 1]$ and simulated under basal (unclamped) conditions over a fixed time interval ($t=0-100$). The final state of each basal simulation was assumed to represent the SS and was subsequently used as the matched initial condition for two perturbation simulations, in which either IL-1 β or TLR was clamped to an activity level of 1 throughout the integration.

Final SS activation values were collected for all nodes and conditions, and summary statistics (mean, median, and standard deviation) were computed across paired runs. To further characterize SS distribution shapes, we quantified per-node skewness and assessed unimodality by estimating the number of modes using kernel density estimation (KDE)-based peak counting with a minimum peak-prominence threshold. Histograms of final SS activation levels, normalized as probability density functions and overlaid with KDE curves for basal, IL-1 β , and TLR conditions, are provided in the (Figure S2).

Condition-dependent effects were evaluated per node using paired t -tests and Wilcoxon signed-rank tests on the final SS values, preserving within-run pairing. Three comparisons were assessed independently (Basal vs. IL-1 β , Basal vs. TLR, and IL-1 β vs. TLR), and multiple testing across nodes was controlled using the Benjamini–Hochberg false discovery rate (FDR) procedure. All simulations, statistical analyses, and visualizations were implemented in MATLAB. The regulatory network model and associated code are publicly available at Zenodo (DOI: [10.5281/zenodo.18785769](https://doi.org/10.5281/zenodo.18785769)), with source code maintained at GitHub [74].

2.3 | Enrichment

The original network baseline failed to capture the homeostatic IVD state, marked by high levels of structural proteins and growth factors alongside low, but non-zero, expression of inflammatory mediators and degradative enzymes. Equilibrium requires both anabolic and catabolic components, with catabolic factors present at modest levels; their complete absence in the initial model made the enrichment step indispensable. This was achieved initially through the inclusion of 11 additional nodes (Act1, BMP, BMPR, ROS, FOXO, SIRT1, SIRT2, SOCS-3, STAT1, and STAT6). These nodes were added based on their critical roles in signaling pathways relevant to IVD biology, identified through focused literature searches in PubMed. Each candidate was identified through targeted PubMed queries (keywords listed in Table 2), with “Act1 and CGPR” queried using only “signaling pathway” due to a lack of IVD-specific results. Table 2 summarizes search terms and hit counts for all added regulators.

The next step of the enrichment has been done through databases by looking for links among nodes presented in Tables 1 and 2. The STRING database was first used, which integrates extensive evidence from protein–protein interaction (PPI) literature,

TABLE 2 | PubMed systematic search for additional nodes related to signaling pathways in IVD research.

Boolean relationships	# of results
{ACT1} $\wedge$ {signaling pathway}	196
[[BMP] $\wedge$ {signaling pathway}]] $\wedge$ {intervertebral disc}	27
[[ROS] $\wedge$ {signaling pathway}]] $\wedge$ {intervertebral disc}	118
[[{FOXO} $\vee$ {CTGF}]] $\wedge$ {signaling pathway}]] $\wedge$ {intervertebral disc}	8
[[SIRTUINS] $\wedge$ {signaling pathway}]] $\wedge$ {intervertebral disc}	44
[[SOCS3] $\wedge$ {signaling pathway}]] $\wedge$ {intervertebral disc}	5
[[STAT1] $\wedge$ {signaling pathway}]] $\wedge$ {intervertebral disc}	7
[[CGPR] $\wedge$ {signaling pathway}]]	4
[[RUNX2] $\wedge$ {signaling pathway}]] $\wedge$ {intervertebral disc}	9

Note: Each row shows the Boolean query used to identify studies addressing a particular signaling molecule or pathway in the context of the intervertebral disc except for the proteins Act1 and CGRP. The symbol $\wedge$ denotes logical AND. The “# of results” column lists the total number of PubMed hits retrieved as of the date of querying. Searches were conducted between 10 September 2024 and 20 December 2025.

computational predictions, and experimental results. The process aimed to expand the links among nodes by targeting general PPIs, and that their inclusion could represent a normal signaling pathway of the IVD. A stringent approach was applied, selecting interactions supported by high-confidence scores (≥ 0.900), ensuring strong and reliable biological relevance. To mention, 82 proteins were utilized, encompassing structural ECM proteins, growth factors, cytokines, receptors, proteases, protease inhibitors, signaling intermediates, transcription factors, and neuropeptides. For each potential new link, we reviewed the relevant sources (literature or databases) provided by STRING to determine the directionality of the interactions, identifying which proteins acted as activators or inhibitors of others. If clear directionality was not established for a link, we chose not to add that information to the model, ensuring that only well-supported directed interactions were represented.

Further validation and cross-verification were conducted using signaling pathway information from KEGG [75] and the R&D Systems [76] database, specifically reviewing the following pathways:

- TGF- β signaling pathway
- MAPK signaling pathway
- ERK signaling pathway
- Toll-like receptor signaling pathway
- PI3k-Akt signaling pathway
- JAK–STAT signaling pathway

- Nf-Kb signaling pathway
- TNF signaling pathway
- FOXO signaling pathway

Each identified link was critically evaluated to ensure consistency with established biological mechanisms. Importantly, links derived solely from experiments involving non-physiological conditions, such as artificial overexpression studies or cancer cells, were avoided to maintain biological realism. Similarly, the addition of proteins and links respected the contextual relevance specific to IVD physiology. Therefore, this enrichment approach provided a robust and biologically meaningful expansion of the regulatory network, accurately capturing signaling dynamics relevant to IDD and homeostasis.

2.4 | Assessment Against Experimental Studies

To evaluate the performance of the cell signaling network model, two independent experimental datasets derived from human IVD NP cells were utilized [3, 77]. These datasets were intentionally selected to represent complementary experimental contexts rather than parallel or directly comparable culture systems, allowing validation of the network under distinct but biologically relevant conditions.

In the first experimental dataset, human NP cells obtained from surgical samples ($n = 7$ donors) were expanded in monolayer culture until passage 3. Although monolayer culture may promote partial dedifferentiation relative to native NP tissue, it was used here to expand cells for subsequent stimulation experiments [78]. Cells were then stimulated for 7 days with selected pro-inflammatory factors including Pam2CSK4 (100 ng/mL) and IL-1 β (100 pg/mL and 1 ng/mL), with media replenished on Days 1, 3, and 6. Cells cultured in complete medium without stimulation served as controls. After the stimulation period, cell lysates were collected and analyzed using a Luminex-based multiplex phosphoproteomic assay targeting 21 phosphorylated proteins. Data were reported semi-quantitatively as median fluorescence intensities (MFI), normalized to unstimulated controls from the same donor. Statistical significance was determined using non-parametric Kruskal-Wallis tests followed by Dunn's post hoc analysis with Bonferroni correction. A threshold of ± 1.5 fold-change and a p value ≤ 0.05 was considered biologically significant [77].

In the second experimental dataset, NP cells from traumatic human IVD tissues (mean age: 44.42 ± 10.41 years, $n = 7$) were expanded to passage 3 in monolayer, encapsulated at a density of 4×10^6 cells/ml in alginate beads, and cultured for 2 weeks under hypoxic conditions (5% O₂, 5% CO₂, 37°C) to restore native NP cell phenotype³. This 3D hypoxic alginate culture system was selected to better preserve NP-specific characteristics and to validate network predictions under conditions that more closely resemble the in vivo NP microenvironment. These re-differentiated cells were then stimulated for 2 days with IL-1 β (1 ng/mL). Cell lysates were subsequently analyzed using the Luminex multiplex phosphoproteomic assay targeting the same 21 phosphorylated proteins as in the first

experiment. Normalization was performed against unstimulated control samples, and statistical analysis employed the non-parametric Mann-Whitney rank-sum test with significance established at $p \leq 0.05$. Together, these two experimental datasets provided rigorous validation for assessing the predictive accuracy and biological relevance of the computational signaling network model.

3 | Results

3.1 | IVD NP Cell Signaling Regulatory Network

A consolidated schematic of the pathways is presented in Figure 3, summarizing findings from 45 peer-reviewed studies that provided detailed descriptions of the signaling cascades active in NP cells (Table S1). With a total of 89 links and 66 nodes, the model topology represented how extracellular ligands (TGF- β , GDF5, IGF1, IL-1 β , IL-6, IL-17, IL-18, IL-12, IFN- γ , TNF, IL-10, IL-4, CSF2, IL-8, and CGRP) engage with their respective receptors to initiate key intracellular cascades. The schematic further highlights critical knowledge gaps with bold red lines and nodes, which denote dead ends and missing links in our understanding. For instance, the JNK and ERK nodes remain unresolved, leaving unclear how signals propagate downstream. Moreover, the pathway leading to β -catenin activation is not fully defined, and the mechanism that activates SIRT1 is also ambiguous. In addition, the diagram shows that c-Jun directly activates IL-1 β and MMP3, while several soluble proteins (such as GDF5, IL-18, IL-12, IFN- γ , IL-4, CSF2, and IL-8) are located at the cell membrane with signaling pathways that are yet to be elucidated.

Using the computational framework described in the Materials and Methods, we first computed the baseline SS activation profile of the initially curated, non-degenerate NP cell regulatory network under basal (unclamped) conditions. As shown in Figure 4, the resulting SS landscape was dominated by a limited subset of constitutively active signaling and transcriptional regulators, while the majority of ECM-related genes, inflammatory mediators, and stress-responsive pathways remained weakly activated or inactive. Boxplots across the 200 simulation runs for each molecular component are provided in Figure S1.

In particular, most canonical NP anabolic markers, including ACAN, COL2A, TGF- β , IGF1, and Sox9, exhibited low-to-moderate activation levels, and inflammatory cytokines (e.g., IL-1 β , IL-6) were largely suppressed, consistent with a non-inflammatory basal state. However, this baseline configuration did not capture distinctive features of the NP cellular environment, such as coordinated anabolic activity, balanced matrix turnover, or physiologically relevant regulatory coupling between signaling hubs and downstream effector genes. The baseline SS profile exhibited limited separation of functionally distinct signaling and regulatory processes. This indicated that the initially curated topology lacked sufficient regulatory depth and contextual specificity to reproduce meaningful NP-specific signaling states, thereby motivating a subsequent topology enrichment step to improve biological expressiveness and predictive capacity.

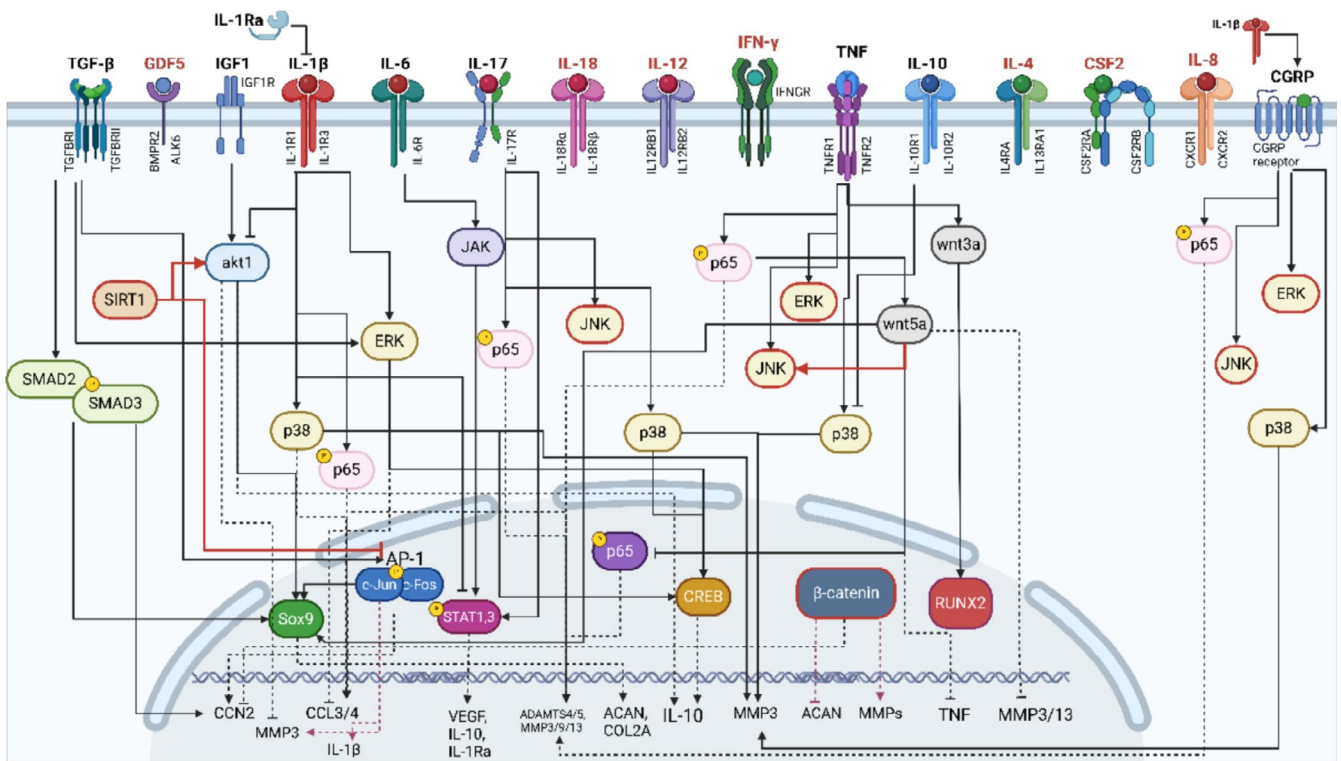


FIGURE 3 | Initial Topology of the Cell Signaling Pathway. Signaling pathways of the soluble proteins regulating the intervertebral disc in NP cells. Dashed lines indicate transcription steps, and bold red nodes or red lines mark missing links needing further clarification.

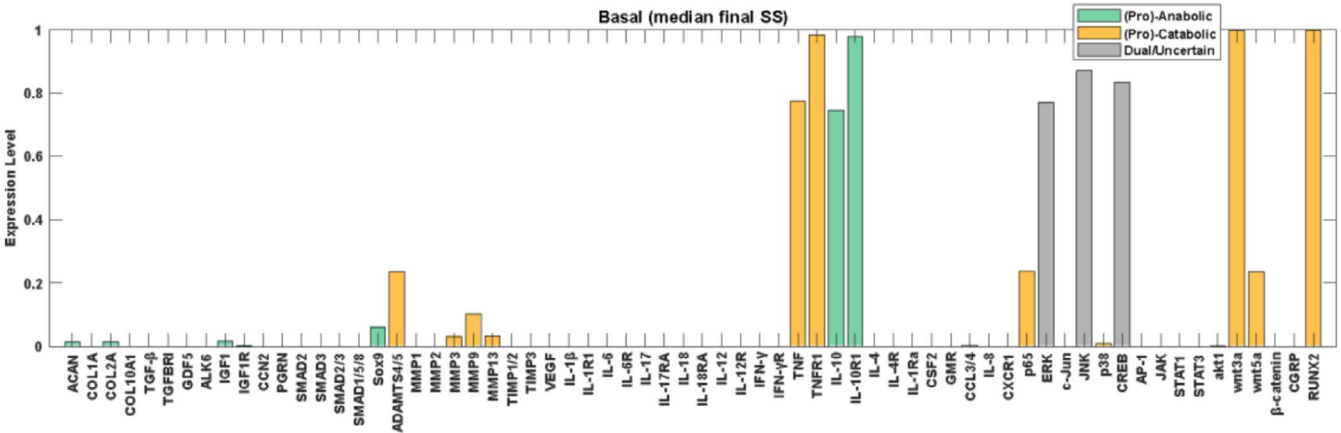


FIGURE 4 | Baseline SS activation profile of the initially curated NP regulatory network. The bar chart shows the median SS activation values (vertical axis) for each network node (horizontal axis) under basal conditions. Activation levels range from 0 (inactive) to 1 (fully active).

3.2 | Enriched IVD NP Cell Signaling Regulatory Network

The enriched signaling regulatory network comprised 82 molecular components interconnected by 193 directed regulatory interactions, capturing key processes relevant to NP cell biology. Detailed information on the activators and inhibitors of each component is provided in Table S2, whereas a schematic representation of the enriched network topology is shown in Figure 5. Simulation of the enriched network under basal conditions resulted in convergence toward a stable attractor characterized by the selective activation of anabolic

and homeostatic signaling pathways (Figure 6). In this basal state, strong activation was observed for major growth factor pathways, including TGF- β , GDF5, and IGF1, along with their downstream intracellular mediators such as SMAD-family and akt1. Consistent with an NP-like anabolic phenotype, the transcription factor Sox9 exhibited high activation, accompanied by robust expression of key extracellular matrix components, ACAN and COL2A.

In contrast, several pathways associated with fibrotic differentiation and degeneration remained effectively suppressed. Notably, COL1A1 and COL10A1 displayed near-zero activation.

Basal (median final SS)

Expression Level

Legend: (Pro)-Anabolic (Green), (Pro)-Catabolic (Yellow), Dual/Uncertain (Grey)

Gene	Expression Level (approx.)	Category
ACAN	1.0	(Pro)-Anabolic
COL1A	1.0	(Pro)-Anabolic
COL2A	0.98	(Pro)-Anabolic
COL10A1	1.0	(Pro)-Anabolic
TSP-1	1.0	(Pro)-Anabolic
TGF- β	0.92	(Pro)-Anabolic
TGFBR1	1.0	(Pro)-Anabolic
BMP	1.0	(Pro)-Anabolic
BMPR	1.0	(Pro)-Anabolic
GDF5	1.0	(Pro)-Anabolic
ALX3	1.0	(Pro)-Anabolic
IGF1R	1.0	(Pro)-Anabolic
CNN2	0.98	(Pro)-Anabolic
PCRN	1.0	(Pro)-Anabolic
SMAD2	1.0	(Pro)-Anabolic
SMAD3	1.0	(Pro)-Anabolic
SMAD2/3	1.0	(Pro)-Anabolic
SMAD1/5/8	0.92	(Pro)-Anabolic
Sox9	1.0	(Pro)-Anabolic
ADAMTS4/5	1.0	(Pro)-Anabolic
MMP1	0.0	(Pro)-Anabolic
MMP2	0.0	(Pro)-Anabolic
MMP3	0.0	(Pro)-Anabolic
MMP9	0.0	(Pro)-Anabolic
MMP13	0.0	(Pro)-Anabolic
TIMP1/2	1.0	(Pro)-Anabolic
VEGF	0.95	(Pro)-Anabolic
IL-1 β	0.0	(Pro)-Anabolic
IL-1 α	0.0	(Pro)-Anabolic
IL-6	0.0	(Pro)-Anabolic
IL-8R	0.0	(Pro)-Anabolic
IL-17	0.0	(Pro)-Anabolic
IL-17RA	0.0	(Pro)-Anabolic
IL-18	0.0	(Pro)-Anabolic
IL-18RA	0.0	(Pro)-Anabolic
IL-12	0.0	(Pro)-Anabolic
IL-12R	0.0	(Pro)-Anabolic
IFN- γ	0.0	(Pro)-Anabolic
IFN- γ R	0.0	(Pro)-Anabolic
TNF	0.0	(Pro)-Anabolic
TNFR1	0.98	(Pro)-Anabolic
IL-10	1.0	(Pro)-Anabolic
IL-10R1	1.0	(Pro)-Anabolic
IL-4	0.05	(Pro)-Anabolic
IL-4R	0.0	(Pro)-Anabolic
IL-1Ra	0.22	(Pro)-Anabolic
CSF2	0.0	(Pro)-Anabolic
CSF2R	0.0	(Pro)-Anabolic
CCL3/4	0.0	(Pro)-Anabolic
IL-8	0.0	(Pro)-Anabolic
CXCR1	0.0	(Pro)-Anabolic
TLR	0.0	(Pro)-Anabolic
INOS	0.02	(Pro)-Catabolic
p65	0.0	(Pro)-Catabolic
Ikk β	1.0	Dual/Uncertain
Ikk	1.0	Dual/Uncertain
TRAF6	0.0	Dual/Uncertain
Act1	0.0	Dual/Uncertain
ERK	0.92	Dual/Uncertain
c-Jun	0.0	Dual/Uncertain
JNK	0.0	Dual/Uncertain
p38	0.0	Dual/Uncertain
CREB	1.0	Dual/Uncertain
AP-1	0.0	Dual/Uncertain
JAK	0.25	Dual/Uncertain
STAT1	0.1	Dual/Uncertain
STAT3	0.25	Dual/Uncertain
STAT6	0.25	Dual/Uncertain
SOCS-3	0.22	Dual/Uncertain
akt1	0.92	(Pro)-Anabolic
SIRT1	0.0	(Pro)-Anabolic
SIRT2	0.0	(Pro)-Anabolic
ROS	0.0	(Pro)-Anabolic
FOXO	0.0	(Pro)-Anabolic
wnt3a	0.0	(Pro)-Anabolic
wnt5a	0.0	(Pro)-Anabolic
β -catenin	0.0	(Pro)-Anabolic
CGRP	0.0	(Pro)-Anabolic
GATA3	0.12	Dual/Uncertain
RUNX2	0.0	(Pro)-Anabolic

Likewise, catabolic, osteogenic, and stress-associated transcriptional programs, including Wnt/ β -catenin signaling, FOXO activity, and RUNX2 were minimally active. The majority of matrix-degrading enzymes (MMPs and ADAMTS) and pro-inflammatory cytokines (IL-1 β , IL-6, IL-17, IL-18, IL-12, IFN- γ , TNF) and chemokines (CCL3/4) also remained close to zero activation. Boxplots and detailed SS distributions across the 200 simulation runs for each molecular component are provided in Figures S2–S5.

unique cell type represented. Yet, more than half of the included studies targeted other cell types, with a mix of phenotypes (Figure 7A). Immune cells accounted for the largest proportion of studies after NP cells, representing 25.3%. Other distinct cell types included chondrocytes (12.6%), fibroblasts (8.6%), and osteoblasts (1.7%). Finally, a further 10.3% was classified as “Others,” covering a broad range of cell lines and primary cells. Within the NP/NPC group itself, 54% of the studies involved human samples, whereas 46% are based on animal models.

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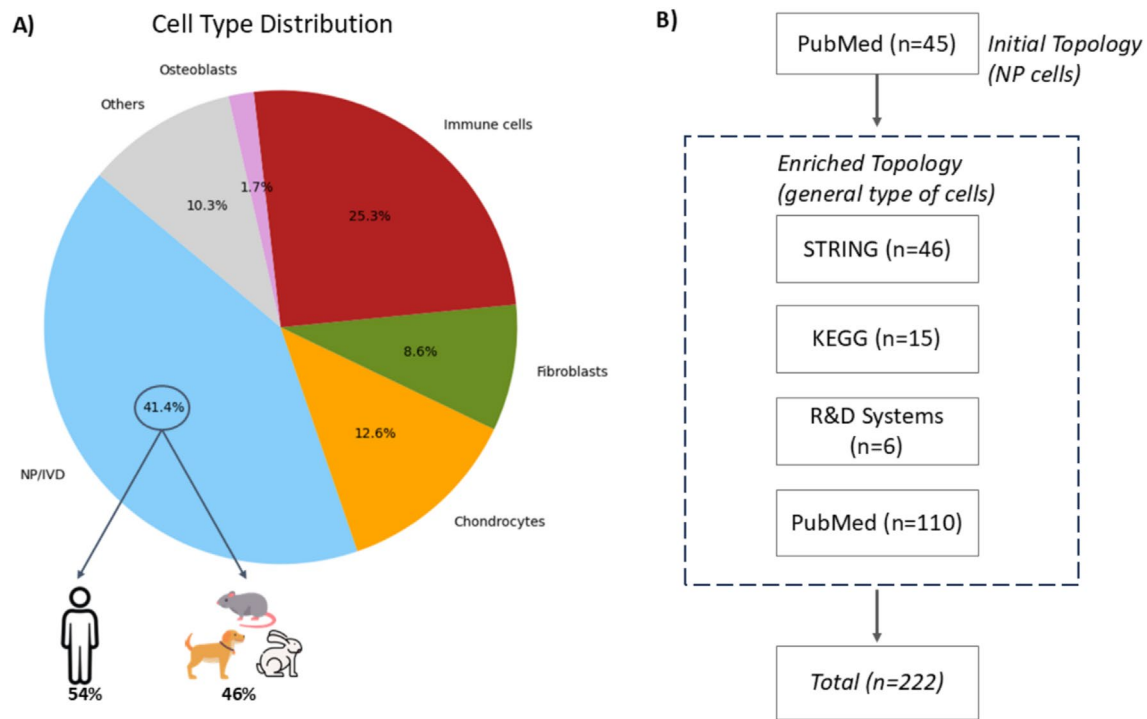


FIGURE 7 | Corpus results. (A) Distribution of cells depending on their type and origin, (B) Data sources and number of links among nodes before and after the enrichment process.

many new connections unexplored in the original NP-only dataset based on the initial 45 PubMed articles (Figure 7B).

3.4 | Assessment Against Experimental Studies

After finalizing the network and establishing a baseline that reflects normal IVD conditions, we assessed its predictive performance using two independent experimental datasets [3, 77]. In the first 2D study, NP cells were stimulated with either IL-1 β or Pam2CSK4, the latter being a ligand that specifically activates TLR [77]. We compared computational predictions with the in vitro findings, treating Pam2CSK4 as representative of TLR activation. Both IL-1 β and Pam2CSK4 stimulation resulted in a significant increase in c-Jun and p65 (NF- κ B) levels in the experiments (Figure 8B) as well as in the model ($p < 0.05$) (Figure 8A). As per I κ B α , the experimental data indicated a significant increase in I κ B α phosphorylation following IL-1 β and Pam2CSK4 stimulation (Figure 8B), and the computational model coherently predicted a significant decrease ($p < 0.05$) of I κ B α node activation (Figure 8A). The qualitative comparisons of computational simulations and experimental proteomic measurements under IL-1 β and TLR stimulation are provided in Figure 8C. For such comparisons, any node the predicted level of which changed by more than 10%, upon stimulation (orange and green bars, Figure 8A) compared to the unstimulated baseline (blue bars, Figure 8A), was designated as increasing or decreasing, in the in silico simulations. Boxplots and detailed SS of the 200 runs for each protein under IL-1 β and TLR stimulation can be found in Figure S2–S5.

In the second 3D-cultured experimental study involving trauma-induced NP cells, the observed changes in c-Jun, NF- κ B, and I κ B α trended upward ($p = 0.07$) (Figure 9B), though these were

not statistically significant [3]. Nevertheless, the trend of c-Jun and NF- κ B increasing upon IL-1 β comes in agreement with the computational simulations (Figure 9A,C). I κ B α increased phosphorylation, in the experiments (Figure 9B) and decreased node activation in the simulations (Figure 9A), again meeting the expectations, in terms of biological coherence (Figure 9C).

4 | Discussion

This study aimed to investigate the key signaling pathways involved in IVD regulation, building on a previously developed RNM for soluble proteins [79]. Mapping these pathways is crucial not only to understand which routes lead to anabolic or catabolic outcomes but also to guide experimental design. By identifying specific gaps and missing links, researchers can tailor their studies to address unresolved questions, thereby optimizing experimental approaches and improving the reliability of the results. Moreover, delineating the key regulatory nodes that maintain tissue homeostasis lays the groundwork for the eventual identification of potential therapeutic targets.

RNMs have emerged as indispensable tools in regenerative medicine because of their ability to integrate vast amounts of biological data, thereby offering unparalleled scalability [80, 81]. This capacity has led to the development of various computational frameworks that simulate cell signaling processes [71, 82–85]. Despite these advancements, no current model comprehensively captures the signaling pathways associated with the key soluble mediators involved in IVD regulation.

The NP-specific network for cell signaling was derived from a comprehensive PubMed search and ultimately comprised 66

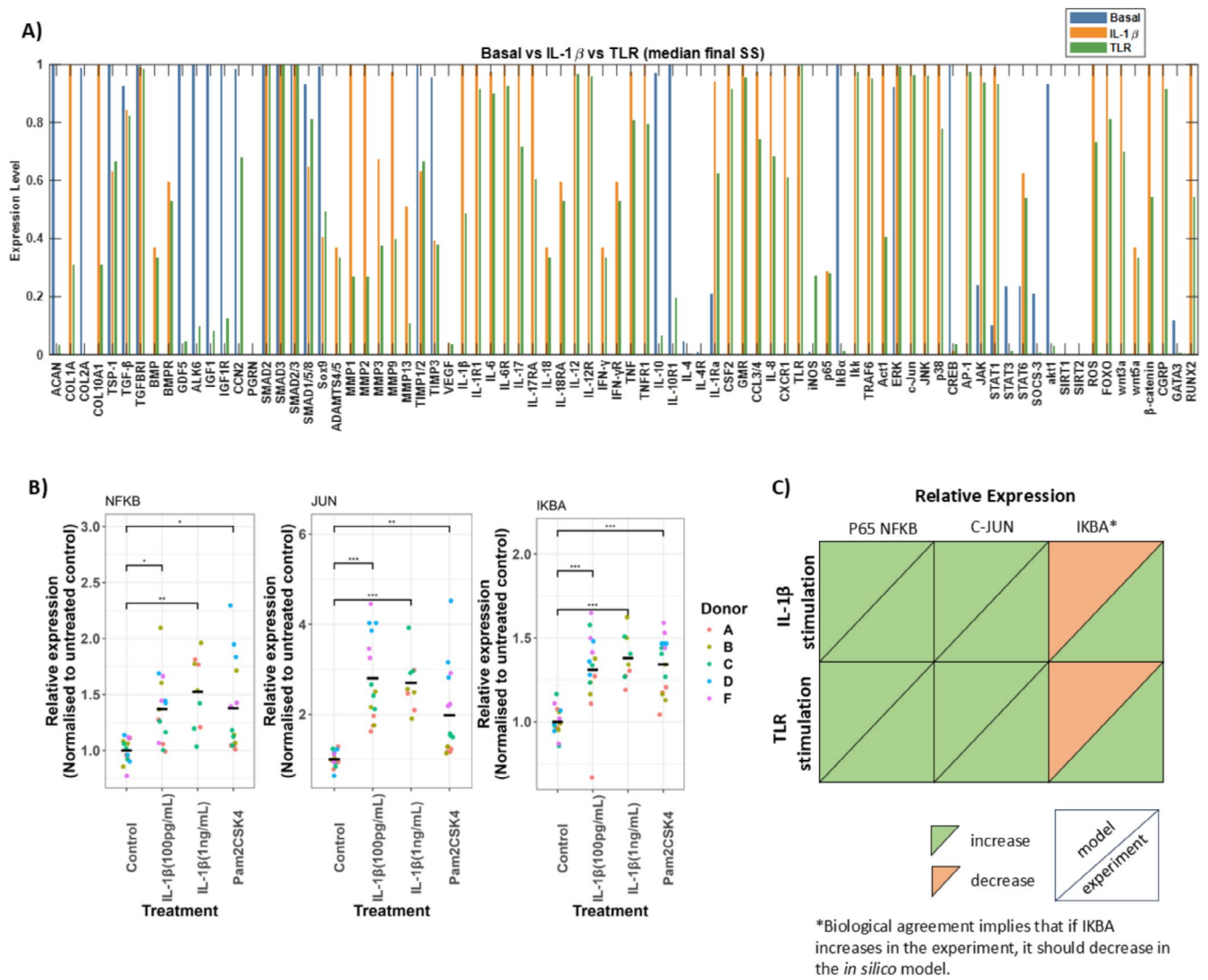


FIGURE 8 | Computational assessment against 2D-cultured experimental study. Network simulations after (A) IL-1 β and TLR stimulation are validated against experimental data, (B) obtained from five human NP cell donors treated with IL-1 β (100pg/mL and 1 ng/mL) and Pam2CSK4 (TLR). The qualitative comparison of IL-1 β and TLR-induced responses between computational simulations and proteomic experiments are shown in (C).

nodes and 89 regulatory links. Baseline network behavior was summarized using the median SS activation level of each protein, while the complete set of 200 SS values and the relative frequency of each attractor are reported in the (Figures S6 and S7). Analysis of the baseline SS profile showed that the initial network captured activity in a limited subset of signaling and transcriptional regulators associated with growth factor, inflammatory, and pain-related pathways. However, canonical pro-anabolic markers, including ACAN, COL2A, IGF1, Sox9, and akt1, exhibited only low-to-moderate activation levels, and coordinated anabolic regulation across extracellular matrix-related components was not observed. This imbalance suggested that the baseline network, although biologically consistent, was incomplete with respect to pathways known to sustain NP homeostasis. The initial baseline therefore appeared to be enriched for a small number of signaling pathways, suggesting that other critical regulatory elements were either underrepresented or absent.

Several omitted cascades have well-documented roles in disc biology: GDF-5 maintains the structure and function of the IVD [86]; TGF- β /SMAD2-3 is the canonical pro-anabolic axis that boosts ACAN and COL2A while restraining inflammation [87], yet chronic over-activation promotes fibrosis and ectopic ossification [88]; IL-10 counterbalances NF- κ B driven catabolism, dampening IL-1 β /TNF- α signaling and rescuing matrix synthesis in degenerate NP cells; IL-18, IL-12 and IFN- γ drive catabolic gene expression and NP-cell apoptosis [33, 89]; IL-4 suppresses IL-1 β -induced matrix degradation but can switch to pro-inflammatory behavior in degenerated cells [90]; GM-CSF (CSF2) and IL-8 exacerbate discogenic pain by activating spinal glia and promoting neuroinflammation [91, 92]; TNF- α /JNK-ERK and IL-17/ERK signaling sharply up-regulate MMP-2, MMP-13 and COX-2 [93, 94]; CGRP/ERK triggers NP apoptosis and inflammation [95]; and missing regulators for SIRT1, β -catenin and downstream targets of RUNX2 critically modulate senescence [96], Wnt activity [97] and hypertrophic

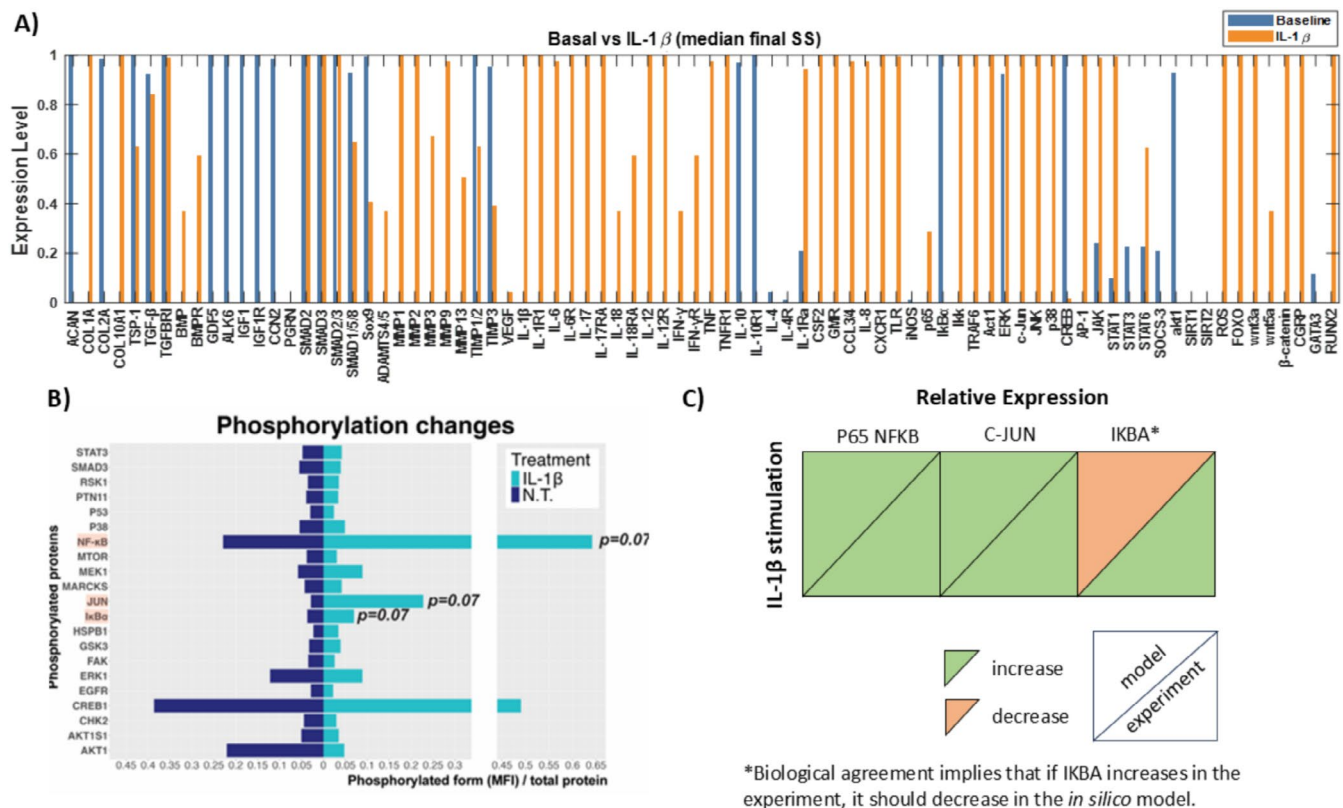


FIGURE 9 | Computational assessment against 3D-cultured experimental study. Network simulations after (A) IL-1 β stimulation are validated against experimental data, (B) obtained from three human NP cell donors treated with IL-1 β (1 ng/mL). A qualitative comparison of IL-1 β -induced responses between computational simulations and proteomic experiments are shown in (C).

matrix remodeling [98], and enhance FOXO's nuclear translocation therein facilitating transcription of antioxidant and autophagy genes [99]. Incorporating these pathways would restore the anabolic-catabolic balance, capture neuro-immune drivers of pain, and reveal additional therapeutic nodes in the NP signaling network. To address these limitations, the initial network was enriched by integrating additional data from general cell studies, drawing from databases such as STRING, KEGG, and R&D Systems, as well as supplementary evidence retrieved from PubMed.

After such enrichment, pro-anabolic components, including ACAN, COL2A, TGF- β /SMAD signaling, GDF5, IGF1/akt signaling, TIMPs, IL-10 signaling, IL-1Ra, and SOX9, exhibited high expression levels. In contrast, pro-catabolic elements such as COL1A, COL10A, ADAMTS4/5, MMPs, VEGF, inflammatory cytokines (IL-1 β , IL-6, IL-17, IL-18, IL-12, IFN- γ , TNF, IL-8), TLR, NF- κ B/p65, p38 MAPK, Wnt/ β -catenin, and CGRP signaling were very close to zero. This profile suggests that while NP cells retain the capacity for remodeling and inflammatory responses, they predominantly favor anabolic signaling under these baseline conditions, reflecting a more physiologically stable and matrix-protective state. Altogether, these results reflected well established findings: TGF- β , via the SMAD pathway and SOX9, is essential for the growth and maintenance of IVD tissue and exerts anti-inflammatory effects by inhibiting I κ B α phosphorylation and subsequent NF- κ B activation in NP cells [37, 41]. In the context of IGF signaling, decreased IGF and IGF1R mRNA levels have been reported in highly degenerated

human NP tissues [100], whereas research by Pratsinis and Kletsas demonstrated that IGF1 enhances IVD cell proliferation through the ERK and Akt pathways [12], although caution is warranted, as abnormal IGF activation may accelerate degeneration [101].

The very low expression of (pro)-inflammatory cytokines is also expected, given the role of these proteins in activating catabolic mediators via p38 MAPK and NF- κ B/p65 pathways [38, 102] in IVD cells. Moreover, TNF is known to activate the Wnt3a/ β -catenin pathway, which increases during degeneration [103] and drives the expression of MMPs, COL1A, and COL10A in osteoblasts [104–106]. The inclusion of TLR reflects its elevated expression in degenerative disc cells and its involvement in stimulating catabolic pathways through p38 and ERK signaling [107, 108]. Regarding ROS inclusion, in IVD homeostasis, ROS levels are kept low to prevent the disruption of cellular balance [109], but when ROS increases, it triggers FOXO to move into the nucleus and activate antioxidant defenses to mitigate oxidative damage [110]. Lastly, low levels of CGRP, a pain-associated factor expressed in IDD cells [111] that promotes inflammation and catabolic phenotypes via p65 and p38 signaling [95], potentially inducing chondrocyte senescence and apoptosis [112], are also in line with the need to represent a non-degenerate disc.

For model validation, two experimental studies in human NP cells were conducted, one using 2D cultures derived from degenerated IVDs [77] and another using 3D cultures from

non-degenerated IVDs [3]. Monolayer cultures of degenerated NP cells expanded to P3 may not fully reflect the native phenotype, potentially amplifying catabolic responses. This design enabled standardization of cell numbers and donor comparability, with results normalized to donor-matched controls [113]. Consequently, the 2D data represent relative, directionally consistent responses rather than absolute *in vivo* magnitudes. In both experiments, while IL-1 β and TLR stimulation produced results that aligned with the activation of c-Jun and p65, we observed a disagreement with I κ B α . In our computational simulation, I κ B α levels decreased following IL-1 β stimulation, whereas the experimental data showed an increase. This “disagreement”, as introduced in the Methods section, can be explained by the dynamic nature of I κ B α regulation: upon IL-1 β stimulation, IKK phosphorylates I κ B α , leading to its degradation and the subsequent release of p65. However, activated p65 then induces the transcription and re-synthesis of I κ B α as part of a negative feedback mechanism [114, 115]. The experimental results capture this later phase of I κ B α resynthesis, whereas our simulation, which does not incorporate a time-dependent feedback loop, only reflects the initial degradation phase.

Unlike earlier computational frameworks for the IVD and cartilage [71, 79], the model presented here is the first to our knowledge that embeds the intracellular signaling circuitry that governs IVD homeostasis. Several existing IVD models emphasize tissue mechanics and transport, for example, poroelastic finite-element simulations of human lumbar discs that track load redistribution and nutrient diffusion but omit intracellular signaling [116–120]. More recent disc-specific systems-biology efforts, such as the NP RNM we previously developed, begin to map soluble mediators, yet did not include the downstream cascades that actually connect cytokine stimuli to matrix turnover and pain signaling [79]. An agent-based model built with the Parallel Network (PN) methodology can forecast NP-cell behavior in pro-inflammatory and mechanosensitive settings [121, 122], but its protein palette is limited. Interestingly, the exploratory research allowed by the presented pathway model could nicely support the development of data-driven high level molecules such as the PN methodology. Most recently, Bermudez-Lekerika et al. assembled static, knowledge-based PPI maps for NP cells under IL-4, IL-10 and IL-1 β stimulation; these networks excel at prioritizing candidate proteins from secretome mass-spectrometry data but lack directionality, dynamics and links to downstream functional read-outs [123]. In articular-cartilage modeling, a mechano-inflammatory chondrocyte network has been developed with over 100 nodes capturing integrin/osmotic pathways [71], and a coupled chemo-mechano-biological FE scheme links joint loading to spatiotemporal enzyme activity in early osteoarthritis [124], capabilities that have yet to be translated to the disc.

The enriched NP network expands the biochemical map, but several limits remain. Remarkably, the validated enriched model incorporated 41.4% originated from studies on NP cells, and within this subset, 54% were derived from human research while 46% were derived from rabbits, mice and canine. These non-NP data points were indispensable for plugging obvious gaps, effectively complementing, but also highlighting, the scarcity of dedicated NP information, and they remind us how urgently the field needs more human IVD signaling studies.

Importantly, the enriched network integrates evidence derived from multiple species, including human, mouse, rabbit, and canine models. Although species-specific differences in cytokine sensitivity, matrix composition, and signaling dynamics are well documented, the incorporated interactions represent conserved signaling mechanisms that have been repeatedly reported across musculoskeletal tissues. Wherever possible, human NP data were prioritized, and non-human evidence was used only when human-specific information was unavailable, reflecting a pragmatic but biologically grounded modeling strategy. This cross-species integration is common in systems biology models of cartilage and connective tissues and is justified here by the high conservation of core pathways such as TGF- β /SMAD, NF- κ B, MAPK, and Wnt signaling [125]. Consequently, model predictions should be interpreted as reflecting conserved NP signaling logic rather than species-specific quantitative responses, particularly when extrapolating toward human pathophysiology. Mechanotransduction is missing because integrin, TRPV-channel, and PIEZO pathways are not yet modeled, and key metabolic regulators such as glucose, lactate, pH, and oxygen (with their AMPK, HIF-1 α , and sirtuin loops) are still absent. All reactions are encoded in qualitative logic without time constants. Hence, transient behavior and oscillations are calculated on a virtual time, just to reach the steady state. The latter is the result of the network, interpretable as a stable phenotype, but biological transient effects or dose dependencies are not captured *per se*, and the current network topology has been developed, only focusing on expected NP cell phenotypes. Thus, annulus fibrosus, end-plate, immune, or neural actors as well as spatial gradients across the disc would require novel topological and solver model developments.

In conclusion, this study represents the first comprehensive model that captures the most important signaling pathways involved in IVD regulation, integrating both anabolic and catabolic processes in NP cells. The presented enriched network, validated through 2D and 3D experimental studies, accurately reflected key mediators such as c-Jun, p65, and I κ B α upon IL-1 β and TLR stimulation. This work lays a robust foundation for understanding tissue homeostasis in IVDs and offers a valuable framework for identifying potential therapeutic targets. Future directions include refining the model to incorporate time-dependent dynamics and extending it to include mechanotransduction pathways and metabolites, which will further enhance our understanding of IVD pathophysiology and regenerative strategies.

Author Contributions

S.T., J.N., J.P., and C.L.L.M. performed the conceptualization. S.T., Z.G.W., M.S.-Q., F.K.C. and J.N. developed methods. S.T. wrote the main manuscript, run the *in silico* simulation, prepared the figures and tables, and contributed to running experiments. Z.G.W. developed the MATLAB computational pipeline used for the *in silico* simulations, performed literature review and data curation, conducted the *in silico* initial simulations, reviewed and edited the manuscript. P.B.-L. and E.K. ran the main experiments and performed their data analysis. E.K., S.T. and K.B.C. executed Luminex measurements. J.N., J.P., C.L.L.M. reviewed and edited the manuscript and sourced funding. All authors approved the final version of the manuscript.

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Conflicts of Interest

Exarchos Kanelis and Leonidas G. Alexopoulos were employed by Protavio Ltd. Christine Lyn Le Maitre and Benjamin Gantenbein are members of the editorial board of JOR Spine. All other authors declare no conflicts of interest. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The regulatory network model and associated code are publicly available at Zenodo (DOI: [10.5281/zenodo.18785769](https://doi.org/10.5281/zenodo.18785769)), with source code maintained at GitHub.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Boxplots of the initial RNM network. The baseline of the initial RNM for every protein is represented by black boxplots and indicates its median value. **Figure S2:** Histograms and kernel density estimates of final steady-state activation levels across the enriched regulatory network model. Each panel displays the distribution of final steady-state activation values obtained across simulations for a given network component under three conditions: basal (blue), IL-1 β stimulation (orange), and TLR stimulation (green). Shaded bars represent normalized histograms, while solid curves correspond to kernel density estimates (KDEs), providing smooth approximations of the underlying distributions. Annotated skewness and modality metrics summarize distribution asymmetry and the number of dominant attractor states, respectively. **Figure S3:** Boxplots of the enriched RNM network. The baseline of the enriched RNM for every protein is represented by black boxplots and indicates its median value. **Figure S4:** Boxplots of the enriched RNM network after IL-1 β stimulation. The baseline of the enriched RNM after IL-1 β stimulation for every protein is represented by black boxplots and indicates its median value. **Figure S5:** Boxplots of the enriched RNM network after TLR stimulation. The baseline of the enriched regulatory network model (RNM) after TLR stimulation for every protein is represented by black boxplots and indicates its median value. **Figure S6:** Median activation levels and cluster composition of final steady states under IL-1 β stimulation. Horizontal bars show the median final activation level of each node across simulations under basal conditions (blue) and IL-1 β clamp (orange). Black dots indicate the centers of distinct clusters (attractor groups) in the distribution of final states obtained across runs under IL-1 β stimulation, while

adjacent percentage labels denote the fraction of simulations converging to each cluster. This representation highlights nodes exhibiting robust activation/repression (single 100% cluster) as well as nodes displaying multistability or heterogeneous responses to IL-1 β perturbation, reflecting alternative regulatory outcomes within the network. **Figure S7:** Median activation levels and cluster composition of final steady states under TLR stimulation. Horizontal bars represent the median steady-state activation of each node across simulations in the basal condition (blue) and following TLR clamp (orange). Black dots mark the cluster centers of final activation levels across runs under TLR stimulation, with percentage labels indicating the proportion of simulations associated with each cluster. Compared with basal behavior, several nodes exhibit multi-cluster distributions, revealing increased response heterogeneity and attractor diversification induced by TLR signaling, while other nodes remain highly robust with convergence to a single dominant state. **Table S1:** Initial topology. Summary of interactions in the IVD knowledge-based regulatory network model derived from the literature via PubMed. **Table S2:** Enriched topology. Summary of interactions in the IVD knowledge-based regulatory network model derived from the literature via PubMed enriched by using the STRING, KEGG and R&D database and manual enrichment via PubMed.