

TEAD4 integrates IFN- γ and TGF- β signaling to drive pathogenic synovial fibroblast programs in difficult-to-treat rheumatoid arthritis

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Abstract

Difficult-to-treat rheumatoid arthritis (D2T RA) remains a major unmet clinical challenge, with synovial fibroblasts increasingly recognized as key drivers of persistent inflammation and treatment resistance. Recent single cell and spatial transcriptomic studies have identified pathogenic fibroblast subsets, including sublining DKK3⁺, THY1^{high}, ITGA5⁺, and COMP^{high} populations, associated with multidrug-resistant disease states. However, transcriptional regulators integrating inflammatory and profibrotic signaling in these fibroblast states remain poorly defined. In addition, inference-based approaches using transcriptomic data are limited in accurately capturing transcription factor activity. Here, we applied nuclear proteomics using data-independent acquisition mass spectrometry (DIA-MS) to directly identify transcription factors induced in cytokine-stimulated rheumatoid arthritis synovial fibroblasts. We identified TEAD4 as a transcription factor selectively upregulated by IFN- γ signaling. Transcriptomic and functional analyses demonstrated that TEAD4 regulates gene programs associated with multidrug-resistant fibroblast subsets and functions as a molecular bridge integrating IFN- γ -driven inflammatory signaling with TGF- β -dependent fibroblast activation. Comparative knockdown studies revealed that TEAD family members exhibit both redundant and distinct functions and partially operate independently of YAP/TAZ. Pharmacologic inhibition of TEAD activity suppressed fibroblast proliferation and migration in vitro and attenuated arthritis severity in the collagen-induced arthritis model without impairing antigen-specific immune responses. These findings identify TEAD signaling as a therapeutically targetable axis linking inflammatory and profibrotic fibroblast programs in rheumatoid arthritis.

INTRODUCTIONS

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation and destructive joint remodeling, driven by complex interactions between immune and stromal cells, including synovial fibroblasts (SFs)^{1–3}. Molecularly targeted DMARDs, such as TNF- α and IL-6 inhibitors, as well as Janus kinase (JAK) inhibitors, has substantially improved clinical outcomes. However, approximately 20% of patients remain refractory to multiple b/tsDMARDs, a condition defined as difficult-to-treat RA (D2T RA)⁴. Identifying mechanisms underlying D2T RA and developing new therapeutic strategies remain major unmet needs.

Single-cell RNA sequencing (scRNA-seq) and spatial transcriptomic analyses have revealed marked heterogeneity among synovial fibroblasts (RASFs), which exhibit distinct anatomical and functional states^{5–10}. RASFs are broadly classified into THY1[–] lining and THY1⁺ sublining populations, with the latter comprising multiple transcriptionally defined subsets, including THY1⁺CD34⁺, THY1⁺DKK3⁺, THY1⁺HLA-DR^{high}, THY1^{high}, THY1⁺GAS6⁺, THY1⁺ITGA5⁺, and THY1⁺COMP^{hi} subsets. These subsets are shaped by inflammatory cytokines such as TNF- α , IL-1 β , IFN- γ , and TGF- β , as well as developmental pathways including Notch and Wnt^{8,11,12}.

Recent studies, including the R4RA trial, have highlighted the role of synovial fibroblast subsets in treatment resistance^{13,14}. Gene signatures associated with THY1⁺DKK3⁺ and related fibroblast subset are enriched in non-responders to multiple bDMARDs. In addition, COMP^{hi} fibroblasts identified by spatial transcriptomics localize to perivascular niches, exhibit persistent TGF- β activation, and remain despite immune cell depletion¹⁰. These findings support a fibroblast-intrinsic mechanism of disease persistence in D2T RA. RASFs possess tumor-like characteristics, including proliferation, migration, extracellular matrix remodeling, and sustained production of cytokines and chemokines³. Experimental models have confirmed distinct roles of lining and sublining fibroblast subsets in tissue destruction and inflammation¹⁵. While current therapies primarily target immune cells, fibroblast-targeted approaches such as CDK inhibition are emerging^{16–18}. However, additional strategies are required to selectively target pathogenic fibroblast states.

The Hippo pathway regulates cell proliferation and survival through Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), which interact with transcriptional enhancer activator domain (TEAD) family proteins to drive expression of genes such as ANKRD1, CCN1, and CTGF^{19,20}. In fibroblasts, YAP/TAZ act as mechanotransducers integrating extracellular matrix signals and TGF- β pathways^{20,21}. YAP/TAZ signaling has been implicated in fibroblast activation in RA, and pharmacologic YAP inhibition can ameliorate arthritis in murine models^{22–24}. However, the role of TEAD transcription factors in RA synovium remain incompletely defined.

Master regulators that drive the activation and maintenance of pathogenic RASF subsets represent attractive therapeutic targets in D2T RA. To identify such regulators, we previously performed transcriptional profiling of major RASF populations, revealing ARID5B and GLI3 as negative regulators of fibroblast aggressiveness^{5,25}. However, identifying transcriptional regulators of pathogenic RASF subsets remains challenging, as transcriptome-based inference methods such as SCENIC, VIPER, and wPGSA are limited by context-dependent transcriptional regulation^{26,27}. Data-independent acquisition mass spectrometry (DIA-MS) enables direct quantification of transcription factors in nuclear proteomes, providing a complementary approach to transcriptomic analyses^{28,29}.

In this study, we applied nuclear DIA-MS to RASFs stimulated with a cytokine cocktail (TNF- α , IL-1 β , IFN- γ , and TGF- β 1) that mimics the inflammatory and fibrotic milieu of the RA synovium^{8,9,30–32} to identify transcriptional regulators of pathogenic fibroblast states. We identified TEAD4 as an IFN- γ -inducible factor associated with multidrug-resistant RASF programs. Functional analyses revealed that TEAD activity regulates pathogenic fibroblast states and that pharmacologic TEAD inhibition suppresses fibroblast-driven pathology and arthritis. These findings establish TEAD signaling as a potential therapeutic target in D2T RA.

METHODS

Study approval

This study was approved by the Ethics Committee of the Institute of Science Tokyo for human research (Approval No. M2000-979 and G2018-028) and by the Experimental Animal Ethics Committee of the Institute of Science Tokyo for animal experiments (Approval No. A2025034C2).

Patient Recruitment and Synovial Fibroblast Isolation

Synovial tissues were obtained from 8 patients with RA and 3 with OA undergoing arthroplasty after informed consent. Clinical characteristics are summarized in Supplementary Table 1. Synovial tissues from RA patients were enzymatically digested with collagenase type IV, dispase II, and DNase I, followed by erythrocytes lysis. Isolated synovial fibroblasts (RASFs) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin, and used at passages < 5–10.

DIA-MS Sample Preparation

RASFs ($n = 3$) were treated with TNF- α (1 ng/mL), IL-1 β (0.1 ng/mL), IFN- γ (10 ng/mL), and TGF- β (1 ng/mL) (4mix) for 24 hours. Nuclear and cytoplasmic extracts were prepared (PIPER nuclear extraction kit, Invitrogen). Nuclear proteins were solubilized in 100 mM Tris-HCl (pH 8.0) containing 4% sodium dodecyl sulfate and 20 mM NaCl using a BIORUPTOR BR-II (SONIC BIO Co., Kanagawa, Japan). Extracts were reduced with 20 mM tris(2-carboxyethyl)phosphine at 80°C for 10 minutes, followed by alkylation with 30 mM iodoacetamide at room temperature for 30 minutes in the dark. Protein purification and digestion were performed using the SP3 method, and tryptic digestion was conducted using 500 ng/ μ L Trypsin/Lys-C Mix (Promega, Madison, WI, USA) at 37°C overnight. The digested peptides were purified using GL-Tip SDB (GL Sciences, Tokyo, Japan), dissolved in 2% ACN containing 0.1% TFA, and quantified using a bicinchoninic acid (BCA) assay at 200 ng/ μ L.

LC-MS/MS and Data Processing

The digested peptides (500 ng) were loaded onto a 75 μ m $\times$ 12 cm nanoLC capillary column (Nikkyo Technos, Tokyo, Japan) at 60°C and separated using a 100-minute gradient with mobile phase A (0.1% FA in water) and mobile phase B (0.1% FA in 80% ACN). The gradient was set as follows: 0 min (7% B), 88 min (37% B), 94 min (70% B), and 100 min (70% B), with a flow rate of 150 nL/min using an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific). The eluted peptides were detected using a quadrupole Orbitrap Exploris 480 hybrid mass spectrometer (Thermo Fisher Scientific) operated in data-independent acquisition (DIA) mode. The MS1 scan range was set at m/z 490–745 with a resolution of 15,000, and the Auto Gain Control (AGC) target was set at 3×10^6 with a maximum injection time of auto. MS2 spectra were collected at m/z 200–1,800 with a resolution of 45,000, an AGC target of 3×10^6 , a fixed normalized collision energy of 26%, and an isolation width of 4 m/z . An optimized window arrangement for the 500–740 m/z window pattern was used in Scaffold DIA (Proteome Software, Inc., Portland, OR, USA). The raw data were searched against an in silico-predicted spectral library using DIA-NN (v1.8.1,

<https://github.com/vdemichev/DiaNN>), which was generated from the human protein sequence database (UniProt ID UP000005640, reviewed, canonical, 20,588 entries).

Western Blotting

Cells were lysed in 2% SDS sample buffer containing complete mini protease inhibitor (Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich) and subjected to SDS-PAGE and immunoblotting. Primary antibodies included pan-TEAD (Cell Signaling Technology, #13295, 1:1,000), TEAD4 (Abcam, ab58310, 1:1,000), YAP/TAZ (Cell Signaling Technology, #8418, 1:1,000; Santa Cruz, sc-101199, 1:200), Lamin A/C (Active Motif, 39287, 1:1,000), GAPDH (Cell Signaling Technology, #97166, 1:1,000), phospho-Rb (S807/811) (Cell Signaling Technology, #9308, 1:1,000), Rb (Cell Signaling Technology, #9302, 1:1,000), and β -actin (Sigma-Aldrich, A2228, 1:5,000). Signals were detected using ECL Prime Western Blotting Detection Reagent (Amersham) and analyzed using ImageQuant LAS-4000 (FujiFilm).

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted, reverse-transcribed, and analyzed by qPCR using the LightCycler 96 (Roche, Basel, Switzerland) with SYBR Green PCR Master Mix (Qiagen, Hilden, Germany). Relative expression levels of target genes were determined using the $\Delta\Delta C_t$ method, normalized to GAPDH. Primer sequences are listed in Supplementary Table 2.

Immunohistochemistry

For histological analysis, synovial tissues from RA and OA patients and hind paws from CIA mice were fixed in 10% formalin neutral buffer solution. Decalcification was performed using 0.5 M EDTA for hind paws from CIA mice, after which tissues were embedded in paraffin. Sections were stained with antibodies against TEAD4 (Abcam, ab58310, 1:200) and FAP (Abcam, ab207178, 1:100). Sections were visualized using DAB substrates, and images were acquired using a BX-50 microscope (Olympus Optical, Tokyo, Japan).

siRNA Knockdown

RASFs were transfected with siRNAs targeting TEAD1 (Thermo Fisher Scientific, Silencer Select: S13961), TEAD3 (S13967), TEAD4 (S13964 and S13966), YAP (S20366), TAZ (WWTR1) (S24787), and a negative control siRNA (Silencer Select-negative control siRNA no. 2) using Lipofectamine RNAiMax (Thermo Fisher Scientific). After 72 hours, cells were stimulated with TNF- α (1 ng/mL), IL-1 β (0.1 ng/mL), IFN- γ (10 ng/mL), and TGF- β 1 (1 ng/mL) (4mix) for 24 hours.

RNA Sequencing

RNA-seq was performed on RASFs transfected siRNAs and treated with 4mix with the assistance of BGI using their DNBSEQ™ platform (Hong Kong, China). Total RNA (RIN ≥ 7) was subjected to poly(A) enrichment or rRNA depletion, followed by fragmentation and reverse transcription. Strand-specific cDNA libraries were constructed, adaptor-ligated, PCR-amplified, circularized into DNA nanoballs, and sequenced as 100 bp paired-end reads. Differentially expressed genes (DEGs) were identified using DESeq2 ($q \leq 0.05$). KEGG pathway enrichment analysis and weighted parametric gene set analysis (wPGSA)²⁶ was performed to assess pathway activity and transcription factor regulation.

wPGSA analysis of scRNA-seq data from RASFs

Published scRNA-seq data from human RASFs were re-analyzed to perform wPGSA. The original dataset had undergone preprocessing by the authors, including sample integration, dimensionality reduction, clustering, and cell-type annotation⁷. The dataset included RASFs isolated from patients with either active or remission-state RA, and clustering analysis identified five distinct subsets. To infer transcription factor activity in active RASF relative to remission RASF subsets, $\log_2$ fold changes were computed using the FindMarkers function in the Seurat package. These values served as input for wPGSA. Transcription factors with adjusted q-values < 0.05 were considered significantly altered. Additionally, TF activity scores were calculated based on normalized expression values within each subset of active or remission-phase RASFs, allowing direct comparison of TF activities between active and remission RASFs.

EdU Incorporation Assay

RASFs were incubated with 1 μM 5-ethynyl-2'-deoxyuridine (EdU) for 24 hours and subjected to Click-iT EdU Flow Cytometry Cell Proliferation Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. After labeling with Alexa Fluor 655, cells were analyzed using flow cytometry (BD, FACSLytic™), and data were processed using FlowJo software.

Cell Migration Assay

RASFs were cultured in 10% FBS DMEM to form a monolayer. When the cells reached 80–90% confluence, a scratch was made using a sterile pipette tip, creating a wound of approximately 1 mm in width. The cells were incubated for 24 hours, and wound closure was monitored. Images were captured using BioZero (Keyence, Osaka, Japan) and analyzed using ImageJ software.

Collagen-Induced Arthritis (CIA) Model

CIA was induced in six-week-old male DBA/1J mice using bovine type II collagen (CII) emulsified in complete Freund's adjuvant (CFA, Chondrex, Inc.) as previously described³³. Briefly, mice were immunized with via intradermal injection at the base of the tail on day 0. A booster immunization with CII in incomplete Freund's adjuvant (IFA, Chondrex, Inc.) was administered on day 21. Arthritis severity was scored using a 0–4 scale per limb based on joint swelling and erythema: 0, no visible swelling; 1, mild swelling in one joint; 2, moderate swelling in multiple joints; 3, severe swelling in two or more joints; and 4, severe swelling with ankylosis in multiple joints. The total arthritis score was calculated as the sum of

all four limbs, with a maximum score of 16. Histological evaluation of synovial inflammation, bone erosion, and cartilage damage was performed as previously described³⁴.

Treatment Protocol

K975 (Accela) was suspended in 0.5% methylcellulose 400 (MC) and administered orally twice daily at doses of 100 mg/kg and 300 mg/kg, either alone or in combination with etanercept (ETN, 3 mg/kg, intraperitoneally injected once every 3 days from day 21 to day 35 for preventive treatment and from day 31 to day 42 for therapeutic treatment) as previously described^{33,35}.

Splenocyte Re-stimulation Assay

On day 35, splenocytes were harvested and re-stimulated with 30 µg/mL heat-denatured bovine type II collagen for 3 days. The concentration of IFN-γ in the culture supernatants was measured using a mouse IFN-γ ELISA kit (R&D Systems, Minneapolis, MN, USA).

Anti-Type II Collagen (CII) IgG ELISA

On day 35, serum was collected, and CII-specific IgG1 and IgG2b titers were measured using enzyme-linked immunosorbent assay (ELISA), as previously described³⁶. ELISA plate was coated with 1 µg/mL heat-denatured bovine CII, added with serially diluted mouse serum, and incubated with HRP-labeled anti-mouse IgG1 (ZYMED, 61-0120) or IgG2b (Invitrogen, #61-0320) antibodies. Signals were detected using TMB substrate, and absorbance was measured.

Statistical Analysis

Data are presented as mean ± SD or SEM. For two-group comparisons, Student's t-test or two-sided Wilcoxon signed-rank test was performed, while multiple group comparisons were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. A P value < 0.05 was considered statistically significant. Statistical analyses were conducted using R package limma and Prism 9 (GraphPad Software, San Diego, CA, USA).

RESULTS

Proteomic identification of TEAD4 as a transcription factor activated by 4mix cytokines in RA synovial fibroblasts

To identify transcriptional regulators associated with pathogenic rheumatoid arthritis synovial fibroblast (RASf) states, we performed quantitative proteomic analysis of nuclear extracts from bulk RASfs treated with a combination of four cytokines (referred to as "4mix") previously implicated in pathogenic RASf activation⁸⁻¹⁰: TNF-α, IL-1β, IFN-γ, and TGF-β1 (Fig. 1a). By analyzing nuclear extracts rather than whole-cell lysates, we sought to detect transcription factors (TFs) regulated not only at the transcriptional level but also by post-translational mechanisms, including nuclear translocation and proteasomal turnover. Quantitative data-independent acquisition mass spectrometry (DIA-MS) identified

208 TFs significantly altered by 4mix, including 87 upregulated and 111 downregulated factors (Fig. 1b, Supplementary Fig. 1a, and supplementary Table 3). Among the upregulated TFs, we detected canonical regulators associated with 4mix component signaling: NFKB1/2 and REL (TNF- α), FOSL1, JUNB, BATF2/3, and CEBPB (IL-1 β), IRF1 (IFN- γ), and SMAD1 (TGF- β). In addition, TFs previously implicated in RASF subset regulation, such as MTF1³², RUNX1^{32,37}, ETS1, and BACH1³⁸, were also identified (Fig. 1b), supporting the robustness of our nuclear proteomic approach.

We next focused on TEAD4, a member of the TEAD family (TEAD1-4), which functions together with YAP/TAZ, key mediators of the Hippo pathway (Fig. 1c)^{19,39}. In fibroblasts, the YAP/TAZ-TEAD complex mediates the mechanotransduction in response to extracellular matrix stiffness and cellular morphology²⁰, induced by TGF- β , an important cytokine for the differentiation of D2T RA-associated RASF subsets^{9,10}. Re-analysis of the DIA-MS data revealed that TEAD1, TEAD3, and TEAD4 were detectable in RASFs, while TEAD2 was not (Fig. 1d). Among these, only TEAD4 was consistently upregulated by 4mix. In contrast, YAP, TAZ, LATS1, and LATS2 were not substantially altered (Fig. 1d). Induction of TEAD4 by 4mix was further validated by Western blotting (Fig. 1e).

To further evaluate TEAD4 activity in activated RASFs, we performed RNA-seq followed by weighted parametric gene set analysis (wPGSA) in RASFs treated with 4mix or vehicle for 24 hours (Fig. 1f, Supplementary Fig. 1a,b, and Supplementary Table 4). wPGSA estimates the activity of TFs based on transcriptomic data and publicly available ChIP-seq datasets²⁶. 4mix induced genes linked to RA-relevant inflammatory pathways, including TNF, JAK-STAT, and NF- κ B signaling (Supplementary Fig. 2a,b). wPGSA of the RNA-seq data identified increased activity of TEAD4 and TEAD1, as well as IRFs, NF- κ B, AP-1, and STATs, some of which overlapped with the DIA-MS results (Fig. 1f). We then applied wPGSA to published scRNA-seq data from RA synovial tissues in active and remission states (Fig. 1g and Supplementary Table 5). This dataset included five RASF subsets: Lining, HLA^{high}, THY1^{high}, GAS6⁺, and CD34⁺ fibroblasts⁷. While GAS6⁺ cells have been proposed to represent anti-inflammatory RASFs, the THY1^{high} subset is considered pathogenic and overlaps with previously reported DKK3⁺ and COMP^{hi} populations^{7,10,13}. In Fig. 1g, we showed top 50 TFs whose activities are estimated to be high in the active THY1^{high} subsets. Comparing TF activities between active and remission samples (t-scores: Act/Rem) revealed significantly increased TEAD4 and TEAD1 activities ($q < 0.05$) in the Lining (t-score: -2.2/-5.7 and -2.2/-6.5), THY1^{high} (t-score: 3.7/-0.5 and 5.1/-1.1), and GAS6⁺ (t-score: -2.1/-4.7 and -0.8/-5.5) subsets from active RA patients. Notably, the highest TEAD4 activity was observed in the active THY1^{high} subset, which has been implicated in multidrug resistance (Fig. 1g). In addition, SMAD and AP-1 activities were also enriched in the active THY1^{high} subset (Fig. 1g). As SMADs are canonical downstream mediators of TGF- β signaling, whereas AP-1 factors can cooperate with YAP/TAZ-TEAD complex in genome-wide transcriptional programs^{20,40}, these findings suggest coordinated regulation within pathogenic THY1^{high} subsets. Collectively, these findings suggest that TEAD4, in cooperation with SMADs and AP-1, is a candidate master regulator driving the activation of pathogenic RASF subsets associated with D2T RA.

TEAD4 is inducible by IFN- γ via JAK/STAT pathway

Given that TEAD4 protein levels were increased upon 4mix stimulation, we next sought to identify which specific cytokines were responsible for this induction. Treatment of RASFs with TNF- α , IL-1 β , or TGF- β 1 alone had no significant effect, whereas IFN- γ alone, as well as in combination with other cytokines, markedly upregulated TEAD4 protein expression (Fig. 2a). To further investigate the regulation of TEAD4 at the transcriptional level, we performed qPCR at early (4 hours) and late (24 hours) time points. Consistent with the Western blot data, TEAD4 mRNA was rapidly and robustly induced by IFN- γ alone with or without other cytokines (Fig. 2b), suggesting that TEAD4 is transcriptionally regulated by IFN- γ signaling. To support the involvement of IFN- γ downstream effectors in TEAD4 regulation, we examined publicly available ChIP-seq data (<https://chip-atlas.org>)⁴¹. These analyses revealed clear STAT1 binding to the TEAD4 promoter in IFN- γ -stimulated cells, including SET-2, Huh-7, and HeLa cells (Supplementary Fig. 2).

We next tested the effects of clinically approved JAK inhibitors used in RA treatment to assess whether TEAD4 induction is dependent on the canonical IFN- γ receptor (IFNGR)-JAK1/2-STAT1 pathway (Fig. 2c). TEAD4 mRNA expression induced by 4mix was suppressed by pan-JAK and/or JAK1/2-selective inhibitors such as tofacitinib, baricitinib, and upadacitinib in a dose-dependent manner⁴². In contrast, the JAK1-selective inhibitor filgotinib had only minor effects⁴². This suppression pattern closely resembled that of IRF1, a well-known target of STAT1, and was distinct from that of IL-6, whose mRNA levels were largely unaffected. These findings collectively support that TEAD4 expression is regulated via the IFNGR-JAK1/2-STAT1 axis.

We next examined TEAD4 expression in synovial tissue. According to recent spatial transcriptomics studies of RA synovial tissues, nuclear pSTAT1 expression has been observed in both THY1⁻ lining and THY1⁺ sublining fibroblasts, located in proximity to IFN- γ -producing CD8⁺ T cells and NK cells⁸. Consistent with this, we observed nuclear TEAD4 staining in spindle-shaped cells within both the lining and sublining layers of RA synovial tissues, whereas TEAD4 was minimally expressed in osteoarthritis (OA) tissues (Fig. 2d). Notably, TEAD4 was also detected in endothelial and/or mural cells but was absent in lymphocytes. Together, these results demonstrate that TEAD4 is a transcription factor induced by IFN- γ and highly expressed in active RASF subsets within RA synovial tissue.

TEAD4 contributes to the active state of pathogenic RASF subsets

To investigate the functional significance of TEAD4 in the active state of RASF subsets, we performed RNA-seq analysis of RASFs transfected with siRNA targeting TEAD4 (siTEAD4) or control siRNA (siCtrl), followed by stimulation with 4mix for 24 hours. A total of 585 genes were significantly downregulated and 241 genes were upregulated by TEAD4 knockdown ($|\log_2FC| > 1.0$, $q < 0.05$) (Fig. 3a and Supplementary Table 6). Among known canonical targets of the YAP/TAZ/TEAD pathway, ANKRD1 was significantly downregulated upon TEAD4 knockdown, whereas CCN and CTGF were less affected,

suggesting that TEAD4 selectively regulates specific YAP/TAZ/TEAD-responsive genes in RASFs. KEGG pathway enrichment analysis of the DEGs revealed downregulation of key signaling pathways associated with fibroblast pathogenicity, including focal adhesion, PI3K-Akt, mTOR, MAPK, and TGF- β signaling (Fig. 3b). In contrast, genes related to cytosolic DNA-sensing and metabolic pathways were slightly upregulated (Fig. 3b).

Given the elevated transcriptional activity of TEAD4 in pathogenic RASF subsets associated with D2T RA (as shown in Fig. 1g), we next evaluated the effect of TEAD4 knockdown on gene signatures associated with multidrug resistance (Fig. 3c). Genes previously identified in the R4RA trial as resistance-associated signatures¹³, as well as marker genes of the DKK3⁺ and THY1^{high} subsets^{6,7}, were all significantly suppressed by TEAD4 knockdown compared to control (Fig. 3c). To confirm the specificity of these findings, we conducted qPCR using additional independent siRNAs targeting TEAD4. The results validated the suppression of 4mix-induced ANKRD1 expression (Fig. 3d). Notably, knockdown of TEAD4 also significantly downregulated COMP and PRELP, which are associated with multidrug-refractory RASF subsets^{10,13}. Furthermore, TEAD4 knockdown led to reduced expression of additional genes implicated in RASF pathogenicity, such as TGFBR2^{9,10} and IL1R1⁴³. Taken together, these results indicate that IFN- γ -inducible TEAD4 governs a transcriptional program associated with aggressive, drug-resistant RASF subsets, highlighting its potential role as a master regulator in the pathogenesis of D2T RA.

TEAD1, TEAD3, and TEAD4 exhibit redundant and distinct functions and regulate transcriptional programs partially independent of YAP/TAZ

RNA-seq analysis after siTEAD4 suggested that TEAD4 alone is not sufficient to fully mediate the Hippo-associated transcriptional program in RASFs. We therefore examined the roles of TEAD1, TEAD3, and TEAD4 individually and in combination. Western blotting using a pan-TEAD antibody revealed two major bands around 50 kDa in RASFs, the upper and lower bands were selectively lost following siTEAD1 and siTEAD3, respectively, while a TEAD4-specific antibody confirmed efficient depletion of TEAD4 (Fig. 4a).

We next examined the impact of these individual and combinatorial knockdowns on the expression of canonical YAP/TAZ/TEAD target genes (Fig. 4b). Interestingly, even among well-established targets, the contribution of each TEAD isoform was gene-specific. For example, ANKRD1 expression was almost completely abrogated by siTEAD4 alone, with siTEAD1 having a partial additive effect. In contrast, CCN1 required the combined knockdown of all TEADs for effective suppression, indicating strong redundancy. For CTGF, TEAD1 and TEAD3 played predominant roles, although TEAD4 contributed modestly to its regulation. We also investigated whether TEAD isoforms influenced the expression of inflammatory mediators. Prior studies in cancer have implicated the YAP/TAZ-TEAD complex in regulating proinflammatory cytokines, including IL-6 and CCL2^{44,45}. In our system, knockdown of TEAD3 or TEAD4 moderately reduced 4mix-induced expression of CCL2, while IL6 expression was largely unaffected, indicating that certain inflammatory genes may be selectively regulated by specific TEAD isoforms in RASFs.

To compare TEAD-dependent and YAP/TAZ-dependent transcriptional programs more directly, we performed RNA-seq in 4mix-treated RASFs transfected with siTEAD1/3/4 (Fig. 4c and Supplementary Table 7) or siYAP/TAZ (Fig. 4d and Supplementary Table 8). As shown in the Venn diagram (Fig. 4e), differentially expressed genes partially overlapped between the two conditions. Canonical YAP/TAZ-TEAD targets, including ANKRD1, CTGF, and DKK1, were commonly downregulated. On the other hand, inflammation-related genes such as CCL7, IL1R1, and LIF were preferentially suppressed by siTEAD1/3/4, whereas cell cycle-associated genes including E2F1, FOXM1, and STMN1 were more prominently reduced by siYAP/TAZ. Consistent with this distinction, phosphorylation of Rb was specifically suppressed by siYAP/TAZ but not by siTEAD1/3/4 (Fig. 4f), although KEGG pathway enrichment analysis suggested potential involvement of Hippo and MAPK signaling in the both conditions (Supplementary Fig. 3a,b). Together, these data indicate that TEAD isoforms exhibit both redundancy and specificity and that TEAD-dependent transcription in RASFs is only partially overlapping with YAP/TAZ-dependent programs.

Pan-TEAD inhibitor suppresses fibroblast pathogenicity and attenuates arthritis without impairment of collagen-specific immune responses

Given the functional redundancy observed among TEAD isoforms (Fig. 4), we next examined whether pharmacologic inhibition of TEAD activity at the family level could suppress pathogenic fibroblast phenotypes with K975, a covalent pan-TEAD inhibitor targeting the palmitate-binding pocket of TEAD proteins, thereby disrupting the YAP/TAZ-TEAD complex and blocking downstream transcriptional activation³⁵. K975 significantly reduced RASF proliferation, as assessed by EdU incorporation, and mildly impaired migratory capacity in scratch assays (Fig. 5a,b). These results indicate that the inhibition of TEAD activity at the family level effectively suppresses key features of RASF activation in vitro.

We then evaluated the in vivo relevance of TEAD inhibition using the collagen-induced arthritis (CIA) mouse model. Based on previous studies in mesothelioma models³⁵, we first performed a dose titration of K975. In a preventive setting, daily oral administration of K975 dose-dependently ameliorated clinical arthritis scores in CIA mice (Fig. 5c), demonstrating its anti-arthritic efficacy. We next investigated the therapeutic synergy of K975 in combination with etanercept (ETN) under a suboptimal concentration suppressing CIA³³, a TNF inhibitor widely used in RA treatment (Fig. 5d). When combined with suboptimal TNF blockade, K975 further enhanced disease suppression compared with either treatment alone (Fig. 5d). Histological analyses demonstrated reduced synovial inflammation, bone erosion, and cartilage damage, with the most pronounced effects observed in the combination group (Fig. 5e and Supplementary Fig. 4a,b). Consistently, fibroblast activation, assessed by fibroblast activation protein (FAP) staining, was reduced following TEAD inhibition (Fig. 5e and Supplementary Fig. 4c), supporting suppression of fibroblast-driven pathology in vivo. In a therapeutic setting initiated after disease onset, all treatments attenuated further disease progression, although reversal of established arthritis was not observed (Supplementary Fig. 4d). The absence of clear separation between treatment groups likely

reflects the limited reversibility of established inflammation at this stage rather than insufficient pathway engagement.

To determine whether TEAD inhibition affected adaptive immune responses, we assessed antigen-specific readouts in the CIA model. We analyzed IFN- γ secretion by splenocytes restimulated *ex vivo* with type II collagen (CII), as well as serum anti-CII IgG responses on day 35. K975 monotherapy or combination therapy with ETN did not significantly reduce IFN- γ secretion from CII-restimulated splenocytes compared with vehicle-treated controls (Fig. 5f). In addition, serum levels of anti-CII IgG1 and IgG2a, reflecting Th2- and Th1-type humoral responses, respectively, were unaffected by K975 treatment (Fig. 5g). These data suggest that pan-TEAD inhibition does not impair systemic adaptive immune responses in this model.

Taken together, these findings demonstrate that the pan-TEAD inhibitor K975, especially in combination with TNF blockade, effectively suppresses arthritis in the CIA model without dampening collagen-specific adaptive immunity. These results support the therapeutic potential of TEAD inhibition as a fibroblast-targeted approach in RA.

DISCUSSION

Recent single-cell transcriptomic and spatial analyses of RA synovial tissues have revealed substantial heterogeneity among synovial fibroblasts in RA, with specific subsets contributing to treatment resistance. The pauci-immune pathotype, characterized by fibroblast dominance, has been associated with multidrug-resistant RA^{13,14}. Several RASF subsets, such as THY1⁺DKK3⁺, PDPN⁺ITGA5⁺, and more recently, THY1⁺COMP^{hi} fibroblasts, have emerged as key populations linked to D2T RA^{10,13}. COMP^{hi} fibroblasts, characterized by high expression of cartilage oligomeric matrix protein (COMP), localize to perivascular niches and display persistent activation of TGF- β signaling and remain despite immune cell depletion¹⁰, supporting a fibroblast-intrinsic mechanism of disease persistence. Using nuclear proteomics with DIA-MS, we identified TEAD4 as an IFN- γ -inducible transcription factor associated with these pathogenic fibroblast states (Fig. 6). In parallel, pharmacologic inhibition of TEAD activity suppressed arthritis *in vivo* in the CIA model. Together, these findings position TEAD signaling as a candidate regulatory axis in D2T RA.

Our results identify TEAD4 as an IFN- γ -inducible node linking inflammatory signaling with TGF- β -dependent mechanotransduction (Fig. 6). Importantly, TEAD4 activity was enriched in pathogenic fibroblast subsets associated with D2T RA, suggesting that IFN- γ -driven TEAD4 induction contributes to the establishment and maintenance of these disease-relevant fibroblast states. Although crosstalk between IFN- γ and Hippo signaling has not been well defined, recent studies in cancer biology have shown that IFN- γ can induce YAP nuclear condensation and promotes TEAD target gene expression, contributing to resistance to immune checkpoint inhibitors⁴⁶. The increased TEAD4 staining observed in RA synovial tissues compared with OA tissues, in both lining and sublining layers, may reflect enhanced STAT1 activation driven by IFN- γ , as suggested by recent spatial transcriptomic analyses⁸. While TGF- β -

driven fibroblast activation has been implicated in pathogenic RASF subsets, RA is primarily an inflammatory disease, raising the question of how inflammatory cytokines are linked to fibrogenic transcriptional programs. In this context, our findings suggest that TEAD4 may serve as a molecular bridge between IFN- γ -mediated inflammatory signaling and TGF- β -associated fibroblast activation. Consistent with this, a recent scRNA-seq study by Zheng et al. identified a pathogenic ITGA5⁺ fibroblast subsets uniquely activated by both IFN- γ and TGF- β ⁹. In addition, JAK inhibitors suppressed IFN- γ -induced TEAD4 expression, raising the possibility that part of their clinical efficacy in D2T RA may involve modulation of TEAD-dependent transcriptional programs.

Our data also support a role for TEAD4 in regulating transcriptional programs associated with drug resistance in D2T RA. Knockdown of TEAD4 suppressed a number of genes previously identified in non-responder RA tissues from the R4RA trial, as well as signature genes of DKK3⁺ and THY1^{high} subsets (Fig. 3c). Importantly, TEAD4 knockdown significantly reduced the expression of COMP and other extracellular matrix-associated genes, including PRELP, which are hallmarks of the recently defined COMP^{hi} RASF subsets^{10,13}. This population, localized to perivascular regions and associated with non-remission status, displays constitutive activation of the TGF- β pathway and is thought to underlie fibroblast-intrinsic treatment resistance. Collectively, our results suggest that TEAD4 links inflammatory signaling to fibrogenic, treatment-refractory gene programs and contributes to the persistence of pathogenic fibroblast states in D2T RA.

While TEAD4 was identified as an IFN- γ -inducible regulator, our functional analyses indicate that regulation of TEAD-dependent transcriptional programs is not restricted to TEAD4 alone but involves multiple TEAD isoforms. Consistent with this, siRNA experiments demonstrated both redundancy and isoform-specific functions among TEAD1, TEAD3, and TEAD4 in controlling canonical and subset-associated gene expression. This redundancy provides a rationale for targeting TEAD activity at the family level rather than focusing on a single isoform. Most currently available TEAD inhibitors are designed to disrupt YAP/TAZ-TEAD interactions and therefore may share partially overlapping effects with YAP/TAZ inhibition. A previous study using conditional YAP1 knockout mice demonstrated that YAP1 contributes to RA pathogenesis, and that verteporfin, a pharmacologic YAP inhibitor of the YAP-TEAD interaction, ameliorates arthritis²². However, our comparative analyses indicate that TEAD and YAP/TAZ perturbations are not functionally equivalent. While canonical targets are shared, distinct gene sets were differentially regulated by TEAD and YAP/TAZ knockdown, suggesting partially independent TEAD functions. This distinction may be explained by the broader interaction network of TEAD proteins. In addition to YAP/TAZ, TEADs interact with other cofactors, including VGLL family proteins, which can modulate transcriptional output. Recent studies have shown that certain TEAD inhibitors alter the balance between YAP/TAZ and VGLL4 interactions rather than simply blocking TEAD activity⁴⁷. Accordingly, genetic knockdown and pharmacologic inhibition may produce distinct transcriptional consequences. Taken together, these findings suggest that TEAD inhibition represents a conceptually distinct approach within the Hippo pathway, potentially enabling more selective modulation of fibroblast-specific transcriptional programs compared with upstream YAP inhibition. Although pan-TEAD inhibitors

are currently under development, they may be associated with class-specific toxicities, including reversible renal effects⁴⁸. Isoform-selective targeting of TEADs may therefore offer improved specificity. Our data, together with emerging TEAD degraders⁴⁹, support further exploration of TEAD-targeted strategies in fibroblast-driven diseases.

This study has several limitations. First, the number of RA and OA synovial tissue samples was limited, and disease duration varied among patients. These factors may have influenced TEAD4 expression and YAP/TAZ-TEAD pathway activity. Second, the role of TEAD4 in the ontogeny and fate decisions of RASF subsets was not directly assessed. Fibroblast-specific TEAD4 knockout mice coupled with scRNA-seq will be needed to elucidate its role in subset formation and plasticity. Third, the fibroblast-specific contribution of TEADs to arthritis amelioration following pharmacologic inhibition was not fully addressed in vivo. Fibroblast-specific TEAD knockouts may help distinguish cell-intrinsic versus systemic effects. Cytokine profiles in synovial tissues following TEAD inhibition should be further evaluated. Finally, the mechanisms underlying TEAD isoform-specific target gene selectivity remain incompletely understood. Integrated epigenomic and proteomic approaches, including ATAC-seq and ChIP-seq, will be required to map TEAD-regulated enhancer landscapes in RASFs.

In summary, we identify TEAD4 as an IFN- γ -inducible transcription factor that bridges inflammatory and mechanotransduction signaling in synovial fibroblasts via the YAP/TAZ/TEAD pathway. TEAD4 promotes transcriptional programs associated with multidrug-resistant RASF subsets, including the THY1⁺DKK3⁺ and COMP^{hi} populations. Pan-TEAD inhibition effectively suppressed RASF pathogenicity and arthritis in vivo, suggesting that TEAD blockade may serve as a novel fibroblast-targeted strategy for D2T RA.

Declarations

Written informed consent for publication was obtained from all the patients.

We have confirmed the ethics approval from the medical research ethics committee of Institute of Science Tokyo (Approval No. M2000-979 and G2018-028).

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CONTRIBUTIONS

TS, EK and SY designated the research. TS, MT, KH, TN, RD and ST performed experiments. KH and EK performed statistical analyses. TS, KH and SY analyzed and interpreted the data. TS, TH and SY drafted the manuscript. AY, SN, YT, KS, KN, HK and IK provided synovial tissues from patients. All authors contributed to the discussion and had opportunities to revise the manuscript.

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DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request. The RNA-seq data used in the current study are available at NCBI GEO under accession number GSE305532, GSE306632, GSE 328455, and GSE328455.

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Figures

Fig. 1

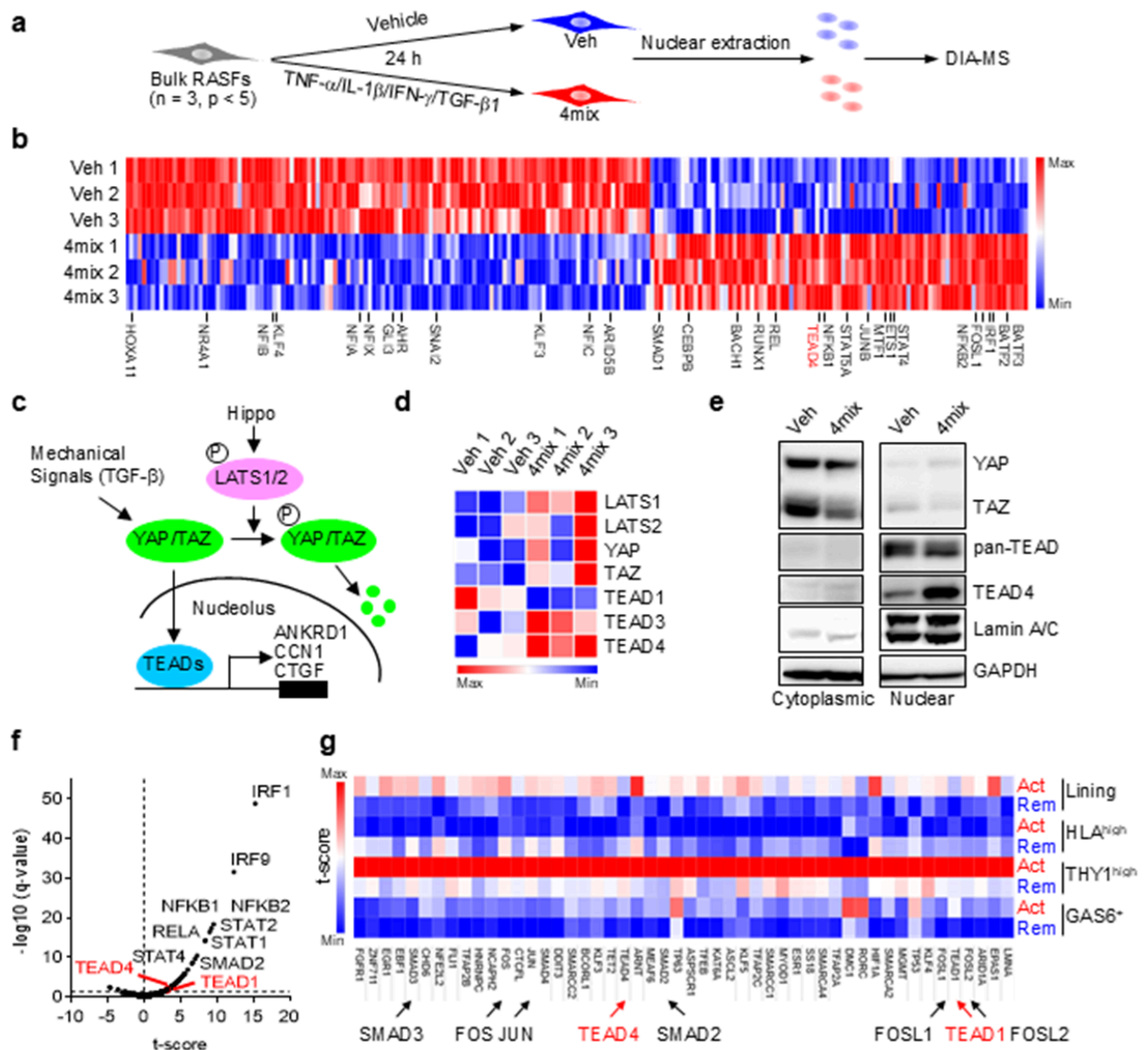


Figure 1

Nuclear proteomics identifies TEAD4 as a cytokine-regulated transcription factor enriched in pathogenic RASF subsets. **a** Experimental scheme. RASFs (n = 3, passages < 5) were treated with either vehicle (DMSO) or a cytokine combination (4mix) consisting of TNF- α (1 ng/mL), IL-1 β (0.1 ng/mL), IFN- γ (10 ng/mL), and TGF- β 1 (1 ng/mL) for 24 hours. Nuclear extracts were prepared and analyzed by data-independent acquisition mass spectrometry (DIA-MS). **b** Heatmap of nuclear transcription factors differentially expressed in 4mix-treated RASFs compared with Veh controls ($|\log_2FC| > 0.65$ and $P < 0.05$). **c** Schematic of Hippo and TGF- β -mediated mechanotransduction pathways. **d** Heatmap of YAP/TAZ/TEAD pathway-related proteins identified by DIA-MS. **e** Western blot analysis of YAP, TAZ, and

TEAD proteins in nuclear and cytoplasmic fractions. Lamin A/C and GAPDH served as controls. **f** Weighted parametric gene set analysis (wPGSA) of RNA-seq data from RASFs treated with 4mix or Veh for 24 hours. Transcription factors identified by both DIA-MS and wPGSA are highlighted ($n = 3$, $q < 0.05$). **g** Heatmap of transcription factor activity scores from wPGSA applied to scRNA-seq data of four RASF subsets, showing the top 50 factors differentially activated in active versus remission conditions (t-score and $q < 0.05$) of $\text{THY1}^{\text{high}}$ subset.

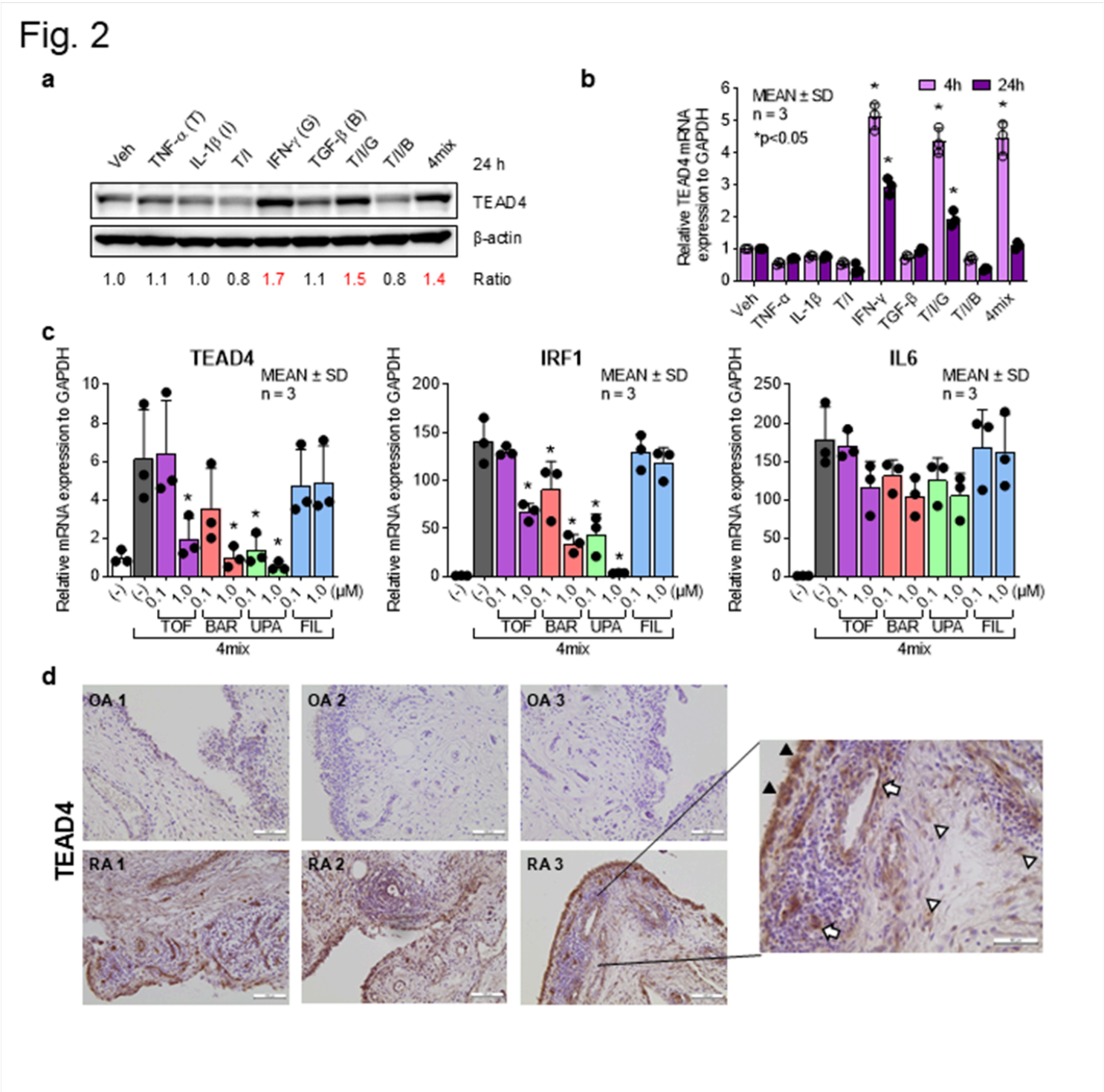


Figure 2

TEAD4 is induced by IFN- γ -JAK signaling and enriched in RA synovial fibroblasts. **a** Western blot analysis of TEAD4 protein in RASFs treated with TNF- α , IL-1 β , IFN- γ , TGF- β 1, or their combinations for 24 hours. Band intensities are shown as ratios to β -actin. **b** qPCR analysis of TEAD4 mRNA expression at 4 or 24 hours following cytokine stimulation. **c** Effects of JAK inhibitors, tofacitinib (TOF), baricitinib (BAR), upadacitinib (UPA), or filgotinib (FIL), on TEAD4 expression in 4mix-stimulated RASFs. **d** Immunohistochemical staining of TEAD4 in RA and OA synovial tissues. Scale bars, 100 μ m. Black arrowheads: lining cells; white arrowheads: sublining fibroblasts; white arrows: endothelial and mural cells. n = 3; mean $\pm$ SD; one-way ANOVA with Dunnett's test. *P < 0.05.

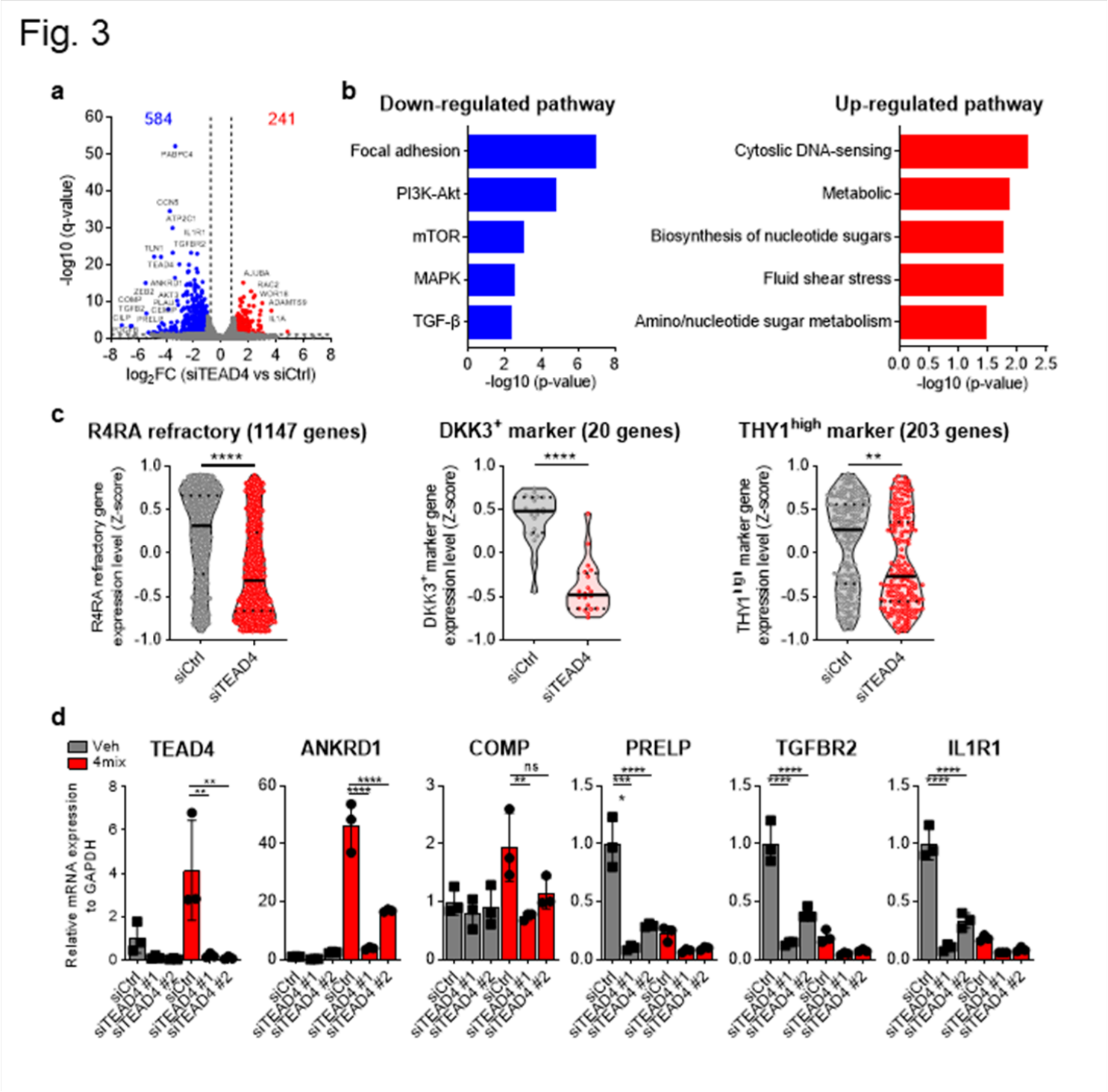


Figure 3

TEAD4 regulates transcriptional programs associated with pathogenic RASF subsets. **a** Volcano plot showing differentially expressed genes (DEGs) in 4mix-treated RASFs transfected with siTEAD4 versus siControl (n = 3, $|\log_2FC| > 1.0$, $q < 0.05$). **b** KEGG pathway enrichment analysis of DEGs following TEAD4 knockdown. Top 5 enriched pathways ($P < 0.05$) are shown. **c** Expression of genes associated with D2T RA and pathogenic RASF subsets in siTEAD4 versus siControl cells stimulated with 4mix. n = 3; ** $P < 0.01$, **** $P < 0.0001$; two-sided Wilcoxon signed-rank test. **d** qPCR validation of selected YAP/TAZ/TEAD target and multidrug resistance genes in RASFs transfected with two independent siRNAs against TEAD4. n = 3; mean $\pm$ SD; one-way ANOVA with Dunnett's test. ** $P < 0.01$, *** $P < 0.0001$, ns = not significant.

Fig. 4

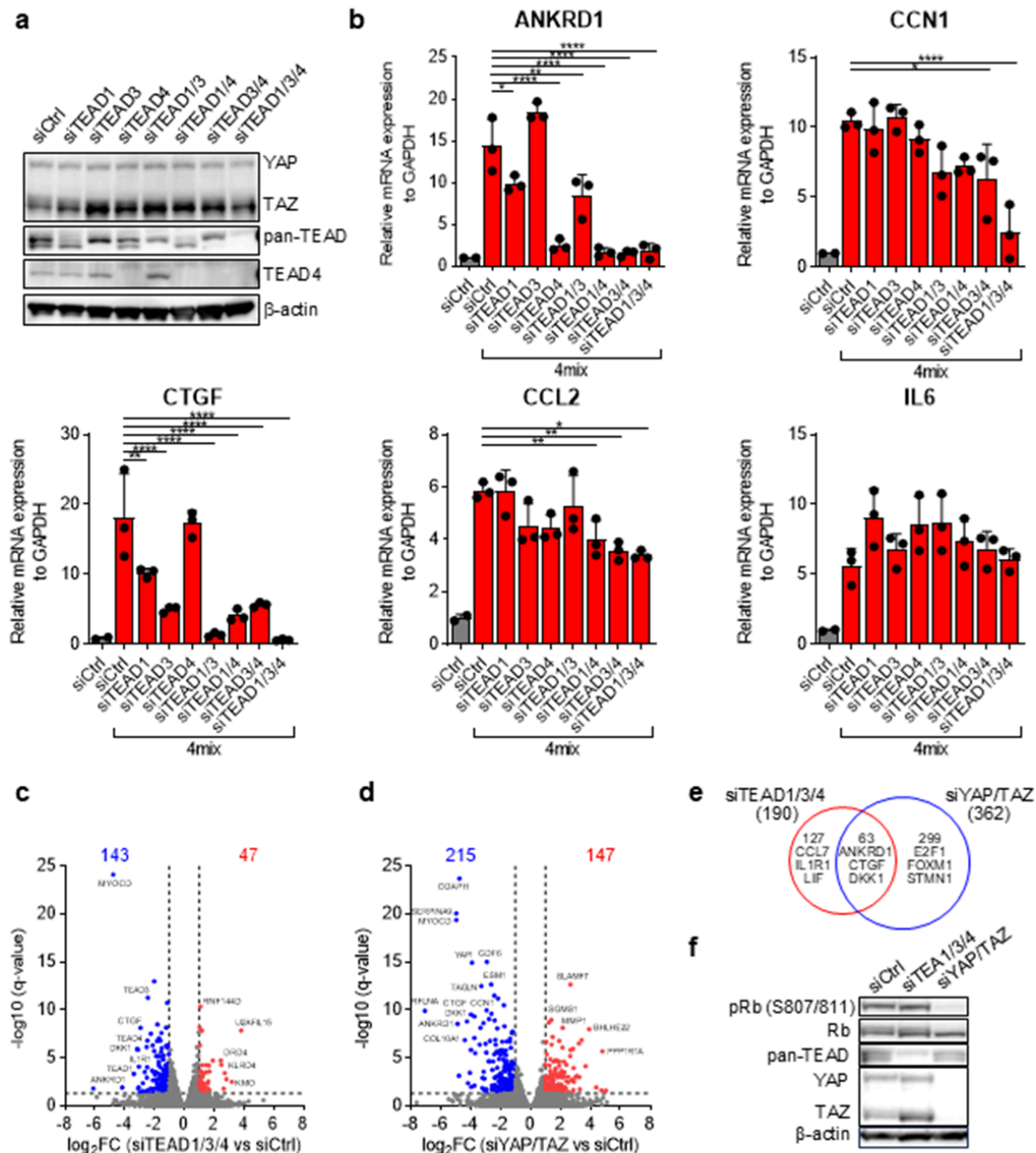


Figure 4

TEAD isoforms exhibit redundant and distinct roles and regulate transcriptional programs partially independent of YAP/TAZ in RASFs. **a** Western blot analysis of TEAD isoforms in RASFs transfected with individual or combinatorial TEAD siRNAs. **b** qPCR analysis of canonical YAP/TAZ/TEAD target genes in RASFs transfected with TEAD siRNAs and treated with 4mix. $n = 3$; mean $\pm$ SD; one-way ANOVA with Dunnett's test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. **c,d** Volcano plots of DEGs in RASFs transfected with

siTEAD1/3/4 (c) and siYAP/TAZ (d) compared with siControl (n = 3, |log2FC| > 1.0, q < 0.05). e Overlap of DEGs between siTEAD1/3/4 and siYAP/TAZ conditions. f Western blot analysis of phospho-Rb in RASFs transfected with siTEAD1/3/4 or siYAP/TAZ.

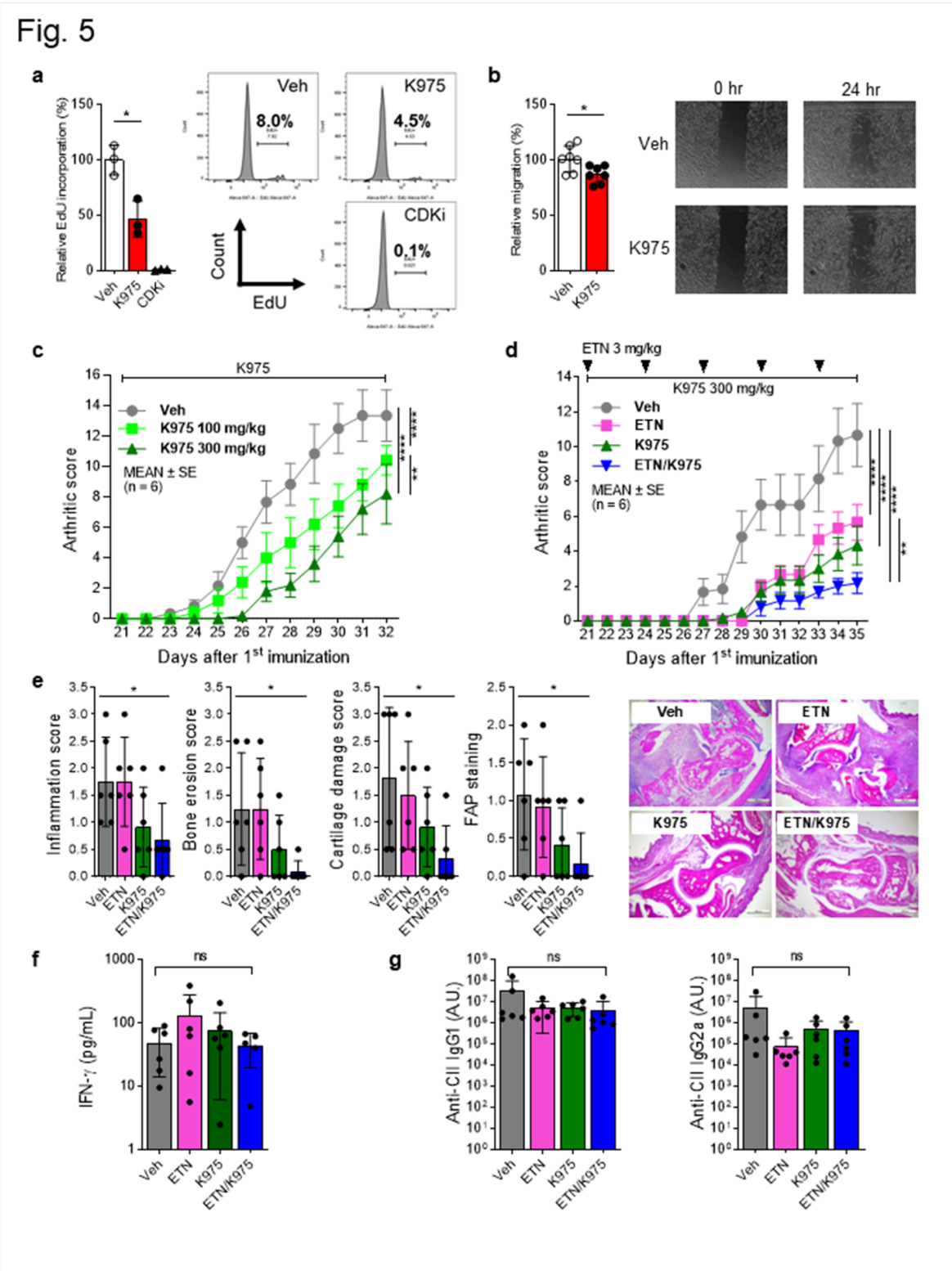


Figure 5

Pan-TEAD inhibition suppresses fibroblast pathogenicity and attenuates arthritis without impairing collagen-specific immune responses. **a** EdU incorporation assay showing reduced proliferation of RASFs following treatment with K975 (0.5 μ M) compared with vehicle control; CDK inhibitor (CDKi) was used as a positive control. **b** Scratch migration assay demonstrating reduced migratory capacity of RASFs after K975 treatment. Representative images and quantification are shown. $n = 3-7$; mean $\pm$ SD; Mann–Whitney U test. $*P < 0.05$. **c** Preventive CIA model showing dose-dependent effects of K975 (100 or 300 mg/kg, twice daily) on arthritis scores. **d** Combination treatment with K975 (300 mg/kg) and etanercept (ETN, 3 mg/kg, intraperitoneally on days 21, 24, 27, 30, and 33) showing enhanced suppression of arthritis. $n = 6$ per group. Mean $\pm$ SEM. $**P < 0.01$, $****P < 0.0001$; two-way ANOVA with Bonferroni’s test. **e** Histological evaluation of inflammation, bone erosion, and cartilage damage with representative H&E images of hind paws. **f** IFN- γ production by splenocytes restimulated with type II collagen. **g** Serum anti-CII IgG1 and IgG2a levels on day 35. $n = 6$ per group. Mean $\pm$ SEM; one-way ANOVA with Dunnett’s test. ns = not significant, $*P < 0.05$.

Fig. 6

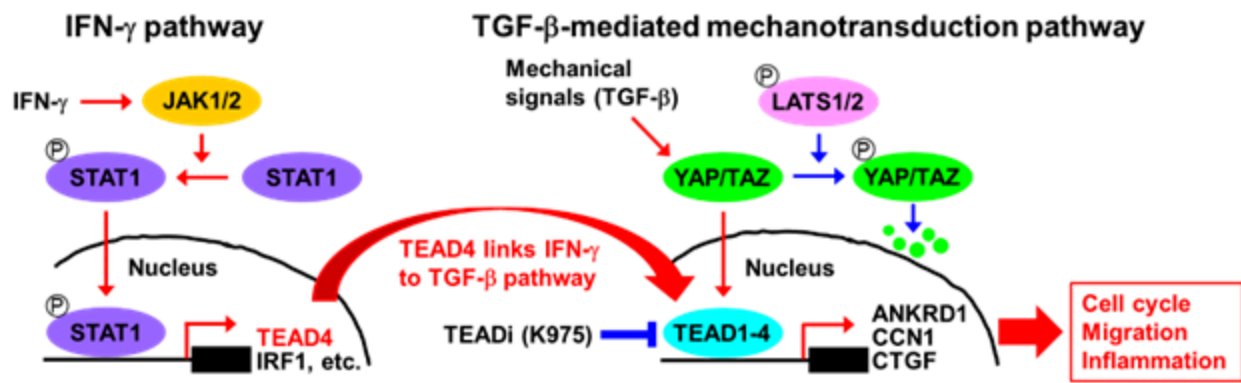


Figure 6

Proposed model of TEAD4-mediated regulation of pathogenic fibroblast programs in RA. IFN- γ induces TEAD4 expression via JAK/STAT signaling in synovial fibroblasts. TEAD4, together with other TEAD isoforms, contributes to transcriptional programs associated with TGF- β -related mechanotransduction and pathogenic fibroblast activation. These programs include genes involved in extracellular matrix remodeling, proliferation, and treatment resistance. Pharmacologic TEAD inhibition suppresses these fibroblast-driven processes and attenuates arthritis, supporting TEAD signaling as a potential therapeutic target in D2T RA.

Supplementary Files

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