JAB1 Determines the Response of Rheumatoid Arthritis Synovial Fibroblasts to Tumor Necrosis Factor- α

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Fibroblast-like synoviocytes (FLSs) of patients with rheumatoid arthritis (RA FLSs) exhibit prosurvival, rather than apoptotic, response to tumor necrosis factor (TNF)- α stimulation. Here, we show that JAB1 is a critical regulator of the TNF- α -mediated anti-apoptosis pathways in RA FLSs. We found that knock-down of JAB1 using small interfering (si)RNA led to restoration of the TNF- α -induced apoptosis response, reduction of nuclear factor- κ B activity, delayed degradation of I κ B- α , and inhibited phosphorylation of JNK. Analysis of the interactions of JAB1 by reciprocal co-immunoprecipitations and confocal microscopy revealed that JAB1 interacts with TNF receptor-associated-factor 2 (TRAF2). The generation of the anti-apoptotic signal on binding of TNF- α to the TNF receptor (TNFR)1 has been shown to be associated with the recruitment of TRAF2 to the TNFR1 in a process that requires ubiquitination of TRAF2 with lysine-63-linked polyubiquitin chains. We found that TNF- α stimulation of JAB1 siRNA-transfected RA FLSs failed to stimulate ubiquitination of TRAF2. Thus, we conclude that JAB1-regulated ubiquitination of TRAF2 is a novel mechanism whereby TNF- α can induce anti-apoptosis signaling and production of matrix metalloproteinases through activation of nuclear factor- κ B and JNK in RA FLSs. (*Am J Pathol* 2006, 169:889–902; DOI: 10.2353/ajpath.2006.051161)

JAB1 is the fifth component (CSN5) of the COP9 signalosome (CSN) complex. The CSN complex, which is

composed of eight subunits (CSN1 to CSN8), is well conserved from yeast to human, and is involved in a variety of biological responses.^{1,2} JAB1 is a multifunctional protein that interacts with numerous molecules involved in intracellular signaling and cell-cycle control, including c-Jun,³ p27,⁴ HIF1 α ,⁵ Smad4,⁶ p53,⁷ and CUL1.⁸ It regulates the activity of these molecules through the JAB1-associated kinases that mediate phosphorylation of the interacting proteins thereby altering their stability. JAB1 also interacts with the Skp1-Cullin-F box protein (SCF) ubiquitin ligases^{8–13} and regulates their ligase activity by catalyzing removal of a ubiquitin-like polypeptide, Nedd8, from the Cullin subunit (deneddylation).⁸

The binding of tumor necrosis factor (TNF)- α to its cell surface receptor, TNF-R1, causes the recruitment of the adapter protein TRADD to the cytoplasmic domain of the receptor. TRADD then serves to recruit additional signaling molecules, including FADD, TRAF2, and RIP1.¹⁴ The apoptotic responses to TNF- α are mediated through recruitment of FADD, which leads to activation of caspase-8,¹⁵ whereas prosurvival responses are mediated by recruitment of TRAF2 and RIP1, both of which lead to activation of the nuclear factor (NF)- κ B pathway.^{16,17} The cellular response to TNF- α , in part, represents a balance between these opposing pathways. TNF- α also can activate JNK^{14,17} through a TRAF2-dependent mechanism.¹⁷ It has been established that JNK activation is critical for TNF-stimulated AP-1-dependent gene expression,¹⁸ which results in the up-regulation of a number of matrix metalloproteinases (MMPs) that degrade cartilage in the joints of patients with rheumatoid arthritis (RA). Therefore, TRAF2 plays a central role in the TNF- α -mediated prosurvival pathway.

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The ubiquitination of TRAF2 by noncanonical polyubiquitin chains that are formed by linkage through lysine-63 residues has been implicated in the regulation of both the NF- κ B pathway and the JNK pathway.^{19,20} TRAF2 exhibits E3 ligase activity that results in its own ubiquitination.²¹ The TRAF2 E3 ligase activity also has been implicated in the global activation of TRAF2 downstream effector molecules, including the ubiquitination of RIP1, which is required for the activation of the NF- κ B and JNK downstream effector pathways.^{16,17,21} Thus, ubiquitination appears to play a central role in the regulation of TRAF2 activities. However, molecular basis underlying regulation of the ubiquitination of TRAF2 remains unknown.

Ubiquitin is a 76-amino acid protein that is highly conserved and is essential for the degradation of proteins that are involved in regulating cellular responses to changes or stresses in the microenvironment.^{22,23} It has been shown *in vivo* that ubiquitination of proteins with polyubiquitin in which linkages are formed through interactions of the lysine 48 residues leads to the recognition and degradation of the proteins by proteasomes.²⁴ In contrast, ubiquitination of proteins with polyubiquitin in which the linkages are formed through interactions of the lysine 63 residues is not associated with proteasomal degradation but has been implicated in other biological processes,^{24–27} including the activation of RIP1 kinase by the E3 ligase activity of TRAF2 and autoactivation of TRAF2.^{24–27}

During the course of the inflammatory process in RA, activated macrophages and synovial fibroblasts produce TNF- α and other cytokines. These cytokines, in turn, stimulate the overgrowth of synovial fibroblasts to form a mass of synovial tissue called pannus, which invades the bone and cartilage through the actions of the MMPs it produces. Early analyses of cytokine gene regulation at the local site of the disease, the synovium, identified the potential importance of TNF- α . This has since been verified in extensive studies of animal models and in human clinical studies of anti-TNF- α therapeutic strategies. It is well established that TNF- α acts to mediate anti-apoptosis responses in RA fibroblast-like synoviocytes (FLSs) and that these responses are pathogenic in RA. Although the ability to induce TNF- α -mediated apoptosis responses in RA FLSs is considered as a potentially beneficial strategy in RA,^{28–33} a molecule(s) that can be targeted to enhance such TNF- α -mediated apoptosis pathways in RA FLSs has not been identified.

Here, we present evidence that JAB1 is required for the prosurvival response of RA FLSs to TNF- α and the production of MMPs and that its knockdown results in restoration of an apoptotic response and a reduction in production of MMPs, suggesting that JAB1 may be a potential target for development of therapeutic strategies for treatment of RA. We further show that JAB1 interacts with TRAF2 in RA FLSs and that this interaction is required for ubiquitination of TRAF2 with lysine 63-based polyubiquitin, and subsequent activation of NF- κ B and JNK that mediate the prosurvival response. siRNA knockdown of JAB1 resulted in the prevention of

TRAF2 recruitment to the TNFR1 complex thereby abrogating the activation of NF- κ B and JNK in RA FLSs.

Materials and Methods

Cells

For primary culture, rheumatoid or osteoarthritis synovial fibroblasts were obtained from 12 patients with RA and 5 patients with osteoarthritis (OA) at the time of total joint replacement as described previously.³⁴ The diagnosis of RA conformed to the ACR 1987 revised criteria.³⁵ Synovial tissue was chopped into fragments of less than 1 mm, washed extensively in sterile phosphate-buffered saline (PBS), and digested with 1 mg/ml collagenase 1 (Sigma, St. Louis, MO) in PBS for 2 hour at 37°C under continuous agitation. The resulting cell suspension was seeded into tissue culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum at 37°C in an atmosphere of 5% CO₂. Adherent fibroblasts from passages 3 to 6 were used for all experiments. These are referred to as FLSs throughout this article. WI38 cells were obtained from Dr. Trygve Tollefsbol (University of Alabama at Birmingham). Human foreskin fibroblast cells (FS) were purchased from American Type Culture Collection (Rockville, MD). Both types of cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS).

Reagents

TNF- α and biotinylated TNF- α were purchased from R&D Systems, Minneapolis, MN. Mammalian expression vectors, including pcDNA3-TRAF2-Flag, were kindly provided by Dr. Ze'ev Ronai, Mount Sinai School of Medicine, New York, NY. pEF-ub63-HA and pEF-HA (kindly provided by Dr. Jian Chen, University of Texas Southwestern Medical Center, Dallas, TX)³⁶ and pcDNA3-JAB1-HA, as described previously,⁶ were used for transfection of RA FLSs with LipofectAmine 2000 (Invitrogen) according to the instruction manual. Affinity-purified mouse anti-HA was purchased from Covance Research Products, Inc., Denver, CO, and mouse anti-Flag antibody and anti- β -actin were purchased from Sigma. A mouse anti-JAB1, mouse anti-p53, rabbit anti-human TRAF2, and rabbit anti-human TRAF1 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Mouse anti-I κ B- α , mouse anti-FADD, and mouse anti-RIP1 were purchased from BD Pharmingen, San Diego, CA. Rabbit anti-JNK and phosphorylated JNK were purchased from Cell Signaling Technology, Beverly, MA.

Induction of Apoptosis of RA FLSs

To determine whether knockdown of JAB1 can enhance the apoptosis of RA FLSs treated with human TNF- α , the RA FLSs were grown to 80% confluence in 96-well plates and treated with TNF- α at the concentrations described for 15 hours at 37°C, 5% CO₂. Hoechst 33342 staining was then performed to determine the percentage of

apoptotic cells as described previously.³⁴ Independently, the induction of apoptosis was determined by flow cytometry analysis of propidium iodide and annexin V-fluorescein isothiocyanate staining.³⁴ Briefly, RA FLS cells were detached from the six-well plate with cell-disassociation buffer (Cellgrowth, Mediatech Inc., Herndon, VA). The cells were then washed three times with PBS. Apoptotic cells were defined as single-positive (annexin V⁺ PI⁻) cells plus the dead, double-positive (annexin V⁺ PI⁺) cells. The percentages of apoptotic cells were determined by flow cytometry using a FACS Calibur flow cytometer and Cellquest software (Becton Dickinson, San Jose, CA).

Transfection of RA FLSs with siRNA

siRNAs including JAB1 and scrambled siRNA (Dharmacon Research, Inc., Lafayette, CO) were purchased as single strands, deprotected, and annealed according to the manufacturer's instructions. A mixture of four human JAB1 siRNAs (Dharmacon), which bind to different regions of JAB1 mRNA, was supplied as duplexes and resuspended in siRNA buffer according to the manufacturer's instructions before use. siRNA transfection of RA FLSs was performed using a previously described method³⁷ with a slight modification. In brief, RA FLS cells were plated onto 24-well plates at a density of 3×10^4 cells/well in antibiotic-free media. The next day, the cells were transfected with one-tenth of the full dose of LipofectAmine2000 as recommended in the instruction manual (Invitrogen) for 1 hour at 37°C, and then retransfected with siRNA JAB1 or scrambled siRNA as a control with full-dose LipofectAmine2000 (Invitrogen) for 5 hours. The transfected cells were washed gently with DMEM and cultured in DMEM supplemented with 10% FBS for an additional 36 hours. To determine the transfection efficiency, RA FLS cells also were transfected with fluorescent-labeled siGLO siRNA (Dharmacon). The transfection efficiency was determined by calculation of the percentage of fluorescent-positive cells among the total RA FLSs counted (80 to 90% transfection efficiency was achieved reproducibly). Similar protocols were used for transfection of plasmid DNAs.

Confocal Microscopic Analysis of JAB1 and TRAF2

RA FLSs were fixed with 3% formaldehyde (Tousimis Corp., Rockville, MD) in PBS for 45 minutes at room temperature, permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 3 minutes at room temperature, and then incubated for 1 hour in 1% bovine serum albumin in PBS to prevent nonspecific staining. Cells were then incubated overnight at 4°C with either primary mouse monoclonal, anti-JAB1 antibody (1:300; Santa Cruz Biotechnology) labeled with Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR) or rabbit anti-TRAF2 antibody (1:300; Santa Cruz Biotechnology) labeled with Alexa Fluor 594 (Molecular Probes). Stained cells were then washed and assessed using a Zeiss LSM 510 confocal

microscope with a digital image analysis system (Pixera, San Diego, CA).

Preparation of Cell Nuclear Extracts and Colorimetric NF- κ B Assay

The indicated numbers of RA FLS cells were stimulated with TNF- α (10 ng/ml) for the indicated time periods. Nuclear extracts were prepared from the harvested cells using a nuclear extraction kit (Active Motif, Carlsbad, CA) and NF- κ B DNA binding activity was detected using the TransAM NF- κ B family transcription factor assay kit (Active Motif) according to the manufacturer's instruction. Briefly, microwells coated with a double-stranded oligonucleotide containing the NF- κ B consensus sequence were incubated with the nuclear extract for 1 hour at room temperature and then washed three times. The captured active transcription factor was incubated for 1 hour with antibodies specific for the p65 or p50 NF- κ B subunits, and then for 1 hour with horseradish peroxidase-coupled anti-rabbit IgG-. After incubation with developing solution for 10 minutes, the optical density was measured at 450 nm using a microtiter plate spectrophotometer.

Evaluation of the Effect of JAB1 on the Induction of Collagenase (MMP-1) Production by TNF- α

RA FLS cells grown to 80% confluence in DMEM plus 10% FBS were transfected with either siRNA JAB1 or scrambled siRNA as a control as described above. Cells were then washed with PBS and cultured for 24 hours in DMEM supplemented with 2% FBS before the addition of TNF- α (10 ng/ml) as described above. Supernatants were then collected throughout a period of 24 hours and subjected to enzyme-linked immunosorbent assays (ELISA) to determine the levels of collagenase (Amersham Pharmaceutical, Princeton, NJ). This assay uses a two-site ELISA sandwich format. Standards and samples were incubated in microtiter wells precoated with rabbit anti-MMP-1 antibody. The sample RA FLS supernatant was added at different dilutions, and incubated for 1 hour before second polyclonal antibody to MMP-1 was added. The binding of the second antibody bound to the wells was detected with donkey anti-rabbit conjugated with horseradish peroxidase using tetramethylbenzidine-hydrogen peroxide, in dimethylformamide as the developing agent. Reaction was stopped by addition of an acid solution, and absorbance of the reaction mixture was measured at 450 nm using a microtiter plate spectrophotometer. Concentration of MMP-1 in a sample was determined by interpolation from a standard curve. The sensitivity and linear range of this standard curve was 6.25 to 100 ng/ml.

Western Blot Analysis

Western blot analyses were performed as described previously.³⁴ RA FLS cells were cultured in DMEM with 10%

fetal calf serum in 100-mm dishes at 80% confluence and starved in DMEM with 0.1% fetal calf serum for 24 hours. The cells were then incubated with medium alone or TNF- α (10 ng/ml) for 5 to 60 minutes. Cells were washed with PBS, and protein was extracted using lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1 mmol/L MgCl₂, 1.5 mmol/L ethylenediaminetetraacetic acid, pH 8.0, 20 mmol/L β -glycerophosphate, 50 mmol/L NaF, 1 mmol/L Na₃VO₄, 10 μ g/ml aprotinin, 1 μ mol/L pepstatin A, and 1 mmol/L phenylmethyl sulfonyl fluoride). Concentrations of protein in RA FLS lysates were determined using the DC protein assay kit (Bio-Rad, Hercules, CA). Fifty μ g of protein from treated RA FLS cells were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were blocked with 5% dry milk in PBS for 1 hour at room temperature, followed by incubation with the appropriate antibody at 4°C overnight. The membranes were washed three times and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour. Proteins were visualized with chemiluminescence using Kodak X-AR film (Eastman Kodak, Rochester, NY). The intensity of each band was scanned and quantified by the Quality One software program (Bio-Rad).

Immunoprecipitation Assays

Lysates of RA FLS cells were prepared as described above and precleared with 150 μ l of protein G-agarose slurry and 10 μ g of isotype control antibody (Ab) overnight at 4°C. One mg of total protein lysate was diluted in 1 ml of immunoprecipitation buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L NaF, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L EGTA, 1 mmol/L NaVO₄, 1 mmol/L phenylmethyl sulfonyl fluoride, and 1% Triton X-100) and incubated overnight at 4°C with 100 μ l of diluted primary antibody or a control nonimmune IgG coupled to protein G-agarose beads. After 4 $\times$ washes with washing buffer (Pierce, Rockford, IL), the protein complexes were eluted with 100 μ l of 0.1 mol/L glycine (pH 2.8), mixed with sample buffer with 2% β -mercaptoethanol, heated to 95°C, centrifuged, and supernatant was subjected to 10% SDS-PAGE gel followed by transfer to a nitrocellulose membrane (Bio-Rad) followed by Western blot analysis. The following antibodies were used for immunoprecipitation and Western blots: mouse anti-JAB1 monoclonal antibody (mAb), rabbit polyclonal TRAF2 Ab, rabbit polyclonal anti-TRAF1 Ab, mouse anti-HA mAb, and anti-Flag mAb.

To immunoprecipitate the TNFR1-associated complex, RA FLS cells were incubated with biotinylated TNF (100 ng/ml) and streptavidin-coated 50-nm MACS microbeads for 0 or 10 minutes in DMEM media at 37°C. Cells were pelleted, washed with PBS, and lysed in 1 ml of lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1% Triton X-100, and 1 mmol/L ethylenediaminetetraacetic acid) and a cocktail of protease inhibitors (Roche, Indianapolis, IN) for 30 minutes on ice. Cell lysates were cleared by

centrifugation for 10 minutes at 1000 $\times$ *g*. The microbead-coupled immunoprecipitates were pelleted and washed twice in 1 ml of washing buffer by centrifugation. Finally, the beads were resuspended in 50 μ l of protein sample loading buffer before Western blot analysis.

Reporter Assay of AP-1 Activity

RA FLS cells were seeded on six-well dishes and transfected with 0.1 μ g of luciferase reporter vector and 0.01 μ g of pRL (a plasmid expressing the enzyme *Renilla* luciferase from *Renilla reniformis*; Promega, Madison, WI) for 24 hours. Cells were then stimulated with TNF- α (10 ng/ml) for 24 hours, harvested, and lysed in passive lysis buffer (Promega). Cell lysates (50 μ l/well) were transferred to a 96-well luminometer plate, and firefly and *Renilla* luciferase activities were assayed using the Dual-Luciferase Reporter System (Promega). Light emission was quantitated using the Monolight 2010 luminometer as specified by the manufacturer (Analytical Luminescence Laboratory, San Diego, CA).

Yeast Two-Hybrid System

For yeast two-hybrid studies, the coding region of human TRAF2 protein was fused in-frame with the GAL4 DNA-binding domain of the pGBKT7 vector (Clontech, Palo Alto, CA). The resulting bait plasmid (pGBKT7-TRAF2) was used to screen a RA synovial fibroblasts cDNA library (mixture of four RA FLSs RNA obtained from four individuals of RA patients) constructed by using the Matchmaker Two-Hybrid System 3 according to manual instruction (Clontech). Isolated clones that grew on Trp⁻ Leu⁻ His⁻ medium did not autoactivate the β -galactosidase reporter gene, and demonstrated specificity for their interaction with JAB1. TRAF2-JAB1 interaction was further confirmed by *in vitro* co-immunoprecipitation using the Matchmaker Co-IP kit (Clontech).

5-Bromo-2-Deoxy-Uridine (BrdU) Incorporation Assay

Cells were starved for 24 hours and then incubated with bromodeoxyuridine (BrdU) for 18 hours and stained with anti-BrdUrd mAb and fluorescein isothiocyanate-conjugated anti-mouse IgG using a BrdU labeling and detection kit according to the manufacturer's instruction (Boehringer-Mannheim, Indianapolis, IN). Cell samples were analyzed by phase-contrast or fluorescence microscopy. More than 500 cells were counted for each type of cells.

Statistical Analyses

Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) to calculate the means and SEMs. Group means were compared by using one-way analysis of variance, followed by Bonferroni's multiple comparison post test to identify statistically significant differences (ie, $P < 0.05$).

Figure 1. JAB1 interacts with TRAF2. RA FLS cells were transfected with a pcDNA3-TRAF2-Flag (**A**, bottom), a pcDNA3JAB1-HA (**B**, bottom), or an empty pcDNA3 empty vector (**A** and **B**, top) or untransfected (**C**) using a protocol as described in the Materials and Methods section. The cells were then lysed using the immunoprecipitation lysis buffer, and 1.0-mg aliquots of the total protein lysates were immunoprecipitated with antibodies (as labeled in the figures) coupled to protein-G agarose beads or beads alone (**A** and **B**, lanes 5) and Western blotted with anti-TRAF2 (**A**, top; **C**, left), anti-JAB1 (**B**, top; **C**, right), anti-Flag (**A**, bottom), or anti-HA antibody (**B**, bottom). For confocal microscopic analysis, RA FLS cells at 80% confluency were fixed with 3% formaldehyde, immunostained with anti-JAB1 (green fluorescence) and anti-TRAF2 (red fluorescence), and analyzed by confocal microscopy to determine the intracellular co-localization of JAB1 with TRAF2. Representative photos are shown. **D:** Each photograph is representative of five different independent experiments. RA FLS cells isolated from five patients were analyzed individually. Original magnifications, $\times 640$.

Results

In Vitro Interaction between TRAF2 and JAB1

Because TRAF2 is a critical adaptor molecule in the TNF- α signaling pathway, we are interested in proteins that may interact with TRAF2 expressed in the human rheumatoid synovial fibroblast cDNA library constructed in a pGADT7 plasmid (Clontech). To screen for such proteins, a TRAF2 cDNA fused with the Gal4 DNA-binding domain was used as bait. We found that one of the clones interacted with TRAF2 and that this clone encoded JAB1 (data not shown).

To further explore this interaction, reciprocal co-immunoprecipitation experiments were performed. Western blot analysis of proteins immunoprecipitated from lysates of RA FLSs by anti-TRAF2 confirmed that JAB1 co-immunoprecipitates with TRAF2 (Figure 1A, top, lane 2) and Western blot analysis of proteins immunoprecipitated from lysates of RA FLSs by anti-JAB1 indicated that TRAF2 also co-immunoprecipitates with JAB1 (Figure 1B, top, lane 3). The co-precipitation appears to be specific because neither TRAF2 nor JAB1 was detected in proteins precipitated by anti-TRAF1 antibody (Figure 1, A and B, lanes 4), FADD antibody (Figure 1A, lane 3),

mouse IgG (Figure 1, A and B, lanes 1), or beads alone (Figure 1, A and B, lane 5). An endogenous TRAF2-JAB1 interaction also was observed (Figure 1C) in cell lysates harvested from untransfected RA FLSs when an immunoprecipitation protocol identical to that described above was used, suggesting that a TRAF2-JAB1 interaction occurs in RA FLS cells.

The JAB1-TRAF2 interaction was then demonstrated independently by anti-hemagglutinin (HA) immunoprecipitation followed by Flag Western blot analysis using protein lysates harvested from TNF- α -stimulated RA FLS cells that had been co-transfected with pcDNA3-JAB1-HA and pcDNA3-TRAF2-Flag (Figure 1A, bottom). Similarly, the JAB1-TRAF2 interaction was confirmed by HA/Flag reciprocal immunoprecipitation followed by Western blot analysis using protein lysates harvested from RA FLS cells co-transfected with pcDNA-JAB1-HA and pcDNA3-TRAF2-Flag (Figure 1B, bottom). The interaction of JAB1 with TRAF2 was furthermore demonstrated by confocal microscopic analysis of RA FLS cells, in which co-localization of JAB1 with TRAF2 was observed in the cytoplasm (Figure 1D).

Knockdown of JAB1 Enhances TNF- α -Mediated Apoptosis

To identify the role of JAB1-TRAF2 interaction in TNF- α signaling in RA FLSs, we first determined whether JAB1 is required for TNF- α -mediated signaling in the RA FLSs by using a siRNA approach. Primary RA FLS cells were transfected with either scrambled siRNA or JAB1 siRNA for 36 hours. It was found by Western blot analysis that at a concentration of 100 nmol/L, siRNA JAB1 reduced the expression of JAB1 protein by more than 90% (Figure 2A, top), whereas the control scrambled siRNA did not affect the levels of endogenous JAB1 (Figure 2A, middle) or β -actin (Figure 2A, bottom). On treatment of the transfected RA FLSs with TNF- α (10 ng/ml) for 48 hours, apoptosis was induced in 80 to 90% of synovial cells as indicated by Hoechst staining (Figure 2B, inset), whereas the same treatment failed to induce apoptosis in RA FLS cells that were transfected with the control scrambled siRNA (Figure 2B, inset), and in untransfected RA FLS cells (data not shown). Transfection with siJAB1 resulted in a statistically significant enhancement of apoptosis in primary RA FLS cells obtained from 12 patients (Figure 2B). To further confirm our results, the RA FLS cells were stained with annexin V and percentages of apoptotic cells were determined by fluorescence-activated cell sorting after TNF- α stimulation with or without siRNA JAB1 knockdown (100 nmol/L). Similar results were obtained using this approach as those obtained using Hoechst staining (Figure 2C) in that significant apoptosis reached a peak at 48 hours (80%).

Knockdown of JAB1 Results in the Attenuation of TNF- α -Mediated Activation of NF- κ B

Activation of NF- κ B has been shown to play an essential role in the TNF- α -mediated anti-apoptosis pathway.¹⁴ To

Figure 2. Transfection with JAB1 siRNA enhances TNF- α -mediated apoptosis of RA FLSs. RA FLS cells (1×10^6) were transfected with siRNA JAB1, or scrambled siRNA using LipofectAmine 2000 for 36 hours. **A:** Fifty μ g of total cell lysates were used for Western blot analysis of JAB1 expression. Primary RA FLS cells between passages 3 and 6 were plated onto 96-well plates (1×10^3 cells per well) and transfected with different concentrations of either JAB1 siRNA or control scrambled siRNA for 36 hours. The cells were then treated with TNF- α (10 ng/ml) for 48 hours at 37°C, fixed with 10% buffered formalin and stained with Hoechst 33342 dye, which is taken up by apoptotic cells. After treatment with TNF- α (10 ng/ml), apoptotic cells with typical nuclear condensation and extensive blue nuclear staining were apparent on treatment of the RA FLSs transfected with JAB1 siRNA but not in the RA FLSs transfected with scrambled siRNA. A representative photomicrograph is shown (Figure 1B, inset). **B:** The percentage of apoptotic cells was calculated by counting the total number of cells and the number of Hoechst-stained cells in at least five randomly selected fields and is expressed as the mean of the results of assays performed in triplicate using RA FLSs derived from each of 12 individuals. The proportion of early apoptotic (annexin V⁺ PI⁻) plus dead cells (annexin V⁺ PI⁺) (percentage) also was determined by flow cytometric analysis after annexin V and propidium iodide (PI) staining after RA FLS cells were transfected with siRNAs (100 nmol/L) as indicated in the figure. One representative of three independent experiments is shown (C, inset). **C:** Results obtained from RA FLSs isolated from each of six individuals, in three independent experiments, were pooled and expressed as mean $\pm$ SD. The asterisk above the bar indicates exosome treatment groups that were significantly different from the control group. * $P < 0.05$; ** $P < 0.01$.

Figure 3. Transfection with JAB1 siRNA prevents activation of NF- κ B in RA FLS cells. Cells (1×10^7) of RA FLSs were transfected with either siRNA JAB1 (100 nmol/L) or control, scrambled siRNA (100 nmol/L) for 36 hours and then stimulated with TNF- α (10 ng/ml) throughout a period of 45 minutes. The nuclei (**A**) or protein lysates (**B**) were prepared at the time points indicated. The nuclei were used to measure NF- κ B DNA binding activity using a colorimetric NF- κ B assay as described in the Materials and Methods section. **A:** Values represent the means of triplicate determinations ($n = 5$). Data are presented as the means $\pm$ SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$. **B:** Aliquots (50 μ g) of the protein lysates were separated by 10% SDS-PAGE, and I κ B- α and β -actin Western blot analyses were performed. Representative gels are shown. The data presented in Figure 2, A and B, were derived from RA FLSs isolated from five patients analyzed individually.

assess the role of JAB1 in the TNF- α -induced NF- κ B signal transduction pathway, RA FLS cells were transfected with either siRNA JAB1 or control scrambled siRNA as described above. The transfected cells were then assessed for TNF- α -induced NF- κ B activation and I κ B- α degradation 45 minutes after TNF- α stimulation. As shown in Figure 3A, transfection with siRNA JAB1, but not with scrambled siRNA, blocked TNF- α -promoted NF- κ B DNA binding activity of p65 (Figure 3A, left) and p50 (Figure 3A, right). We next determined the time course of degradation of I κ B- α , which is an essential inhibitor of NF- κ B activation. In RA FLS cells transfected with scrambled siRNA, degradation of I κ B- α protein was apparent within 10 minutes after addition of TNF- α , and I κ B- α protein declined to undetectable levels within 20 minutes (Figure 3B, middle). In RA FLS cells transfected with JAB1 siRNA, the TNF- α induction of I κ B- α degradation was delayed (Figure 3B, the right columns of middle panel). The efficacy of siRNA knockdown of JAB1 was confirmed by Western blot analysis of the level of JAB1 expression (Figure 3B, top) and the specificity of its effects was confirmed by lack of effect on the expression of β -actin (Figure 3B, bottom).

Knockdown of JAB1 Leads to the Reduction of JNK Activity

TNF- α signaling also activates the JNK pathway, which has been implicated in the pathogenesis of RA and represents a potential therapeutic target.^{38,39} To assess the potential role of JAB1 in TNF- α -mediated activation of JNK, we used a Western blotting approach in which JNK was detected using polyclonal anti-JNK or anti-phosphorylated JNK. In RA FLS cells that were transfected with scrambled siRNA, JNK was expressed constitutively (Figure 4A, the third panel from the top) and TNF- α -stimulated phosphorylation of JNK was apparent within 20 minutes, with the induction of phosphorylation reaching a peak at 30 minutes after stimulation (Figure 4A, the second panel from the top). Knockdown of JAB1 by siRNA JAB1 completely inhibited the TNF- α -stimulated phosphorylation of JNK (Figure 4A, the second panel from the top), suggesting that JAB1 effectively regulates TNF- α -mediated activation of JNK. Although the level of total JNK protein appears to be reduced, analysis by densitometer indicates that such reduction fails to reach statistical significance as shown in Figure 4B.

Figure 4. Transfection with JAB1 siRNA prevents activation of JNK and induction of MMP1 in RA FLS cells. **A:** A 50-μg aliquot of the protein lysates described in Figure 2B were separated by 10% SDS-PAGE, and subjected to JAB1, pJNK, JNK, and β-actin Western blot analyses. Representative gels are shown. **B:** Bands corresponding to JNK and pJNK were scanned and quantitated. The bars show the relative fold increase as compared to the amount of β-actin. **C:** The levels of MMP1 in the culture supernatants were measured by ELISA assays after 6, 12, and 24 hours of TNF-α stimulation. The values represent the means of triplicate determinations ($n = 5$). Data are presented as the means $\pm$ SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$, calculated as untreated cells versus stimulated cells. The data presented in **A–C** were derived from RA FLSs isolated from five patients analyzed individually. RA FLS cells were co-transfected with the reporter plasmid pAP-1-LUC (100 ng/each) and pRL-CMV. After 24 hours, cells were stimulated with TNF-α (10 ng/ml) and harvested at the times indicated. Cell extracts were then prepared, and firefly and *Renilla* luciferase activities were assayed. *Renilla* luciferase activity was used for normalization of LUC activity due to transfection variables. **D:** The fold increase of LUC activity from stimulated cells compared to that of nonstimulated cells was calculated. Data are presented as the means $\pm$ SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

JNK is known to play a key role in the regulation of *MMP* gene expression, which contributes to the pathogenic process in RA.^{38,39} We therefore evaluated the effect of knock-down of JAB1 in RA FLS cells on induction of MMP1. RA FLS cells transfected with siRNA JAB1 or control scrambled siRNA as described in Figure 1A were stimulated with TNF-α for 24 hours, and the levels of MMP1 in cell lysates were quantified by ELISA assay. TNF-α-stimulated induction of MMP was significantly lower in RA FLS cells transfected with siRNA JAB1 than those transfected with scrambled siRNA. To eliminate the possibility that these reductions in the levels of MMP1 were associated with impaired cell viability, we analyzed the viability of the RA FLSs using an ATPLite assay. The results confirmed that, at that time point, viability of the RA FLS cells transfected with siRNA JAB1 was not reduced significantly (data not shown). This result is consistent with our finding that apoptosis occurs 48 hours after TNF-α stimulation shown in Figure 2C.

Induction of MMP1 was observed starting at 12 hours and reached a peak at 18 hours.

Because activity of JNK has a direct effect on AP1 transcription activity, AP1 activity was quantified to determine the effect of JAB1 on AP1 activity in RA FLSs. In RA FLS cells transfected with the luciferase reporter gene driven by a AP1 promoter, AP1 activity began to increase at 6 hours and reached a peak at 24 hours, after TNF-α stimulation (Figure 4D). Moreover, siRNA JAB1 knock-down resulted in reduction in AP1 activity induced by TNF-α stimulation (Figure 4D).

JAB1 Regulates the TRAF2 Ubiquitination Pathway

Recent studies suggest that TRAF2 undergoes rapid ubiquitination in response to TNF-α stimulation.^{40,41}

Figure 5. JAB1 is required for the ubiquitination of TRAF2 with lysine-63-conjugated polyubiquitin chains in TNF- α stimulated RA FLSs. **A:** RA FLS cells were transfected with pEF-ub63-HA (1 μ g). At 24 hours after transfection, RA FLS cells were stimulated with TNF- α (10 ng/ml) for 0, 1, and 5 minutes. The cells were then lysed, and 500 μ g of total proteins were immunoprecipitated with anti-HA antibody followed by TRAF2 Western blot analysis. RA FLS cells were transfected with either JAB1 siRNA or scrambled siRNA (100 nmol/L) for 36 hours and subsequently transfected with either pEF-ub63-HA or an empty vector pEF-HA (1 μ g). At 24 hours after transfection, RA FLS cells were stimulated with (**B**, top) or without (**B** and **C**, bottom) TNF- α (10 ng/ml) for 5 minutes and then lysed. Five hundred μ g of total protein were used for HA immunoprecipitation. The immunoprecipitated pellets were then used for Western blot analyses of TRAF2 (**B**) or RIP1 (**C**). RA FLS cells isolated from five patients were analyzed individually. Representative results are shown.

TRAF2 acts as an E3 ubiquitin ligase resulting in auto-ubiquitination with lysine-63-conjugated polyubiquitin chains. This conjugation system, in contrast to ubiquitination with lysine-48-conjugated polyubiquitin chains, is not associated with proteolysis, but instead triggers signaling through an undefined mechanism.²¹ We therefore analyzed the role of JAB1 in regulating the ubiquitination of TRAF2 under endogenous conditions. For these studies, we used pEF-ub-K63-HA construct, in which all of the lysine residues of ubiquitin are mutated except for the lysine residue at position 63. pEF-HA was used as the control. RA FLS cells were transfected with pEF-ub-K63-HA, 24 hours before being stimulated with TNF- α for 0, 1, or 5 minutes. The cells were then harvested, lysed, and subjected to HA immunoprecipitation followed by Western blot analysis of TRAF2. The results indicated that ubiquitination of TRAF2 was increased as early as 5 minutes after TNF- α stimulation (Figure 5A, lane 3). We then transfected RA FLS cells in which JAB1 had been

knocked down using siRNA as described above, with pEF-ub-K63-HA. Twenty-four hours after the transfection, the cells were stimulated with TNF- α (Figure 5B, top) or left unstimulated (Figure 5B, bottom) and the ubiquitination of endogenous TRAF2 was examined by HA immunoprecipitation followed by TRAF2 Western blot analysis. By 5 minutes after TNF- α stimulation, ubiquitination of TRAF2 through the lysine-63-based ubiquitin conjugation was greatly reduced in cells transfected with JAB1-specific siRNA (Figure 5B, top, lane 3), whereas ubiquitination of TRAF2 was markedly enhanced in RA FLS cells transfected with the control, scrambled siRNA (Figure 5B, top, lane 4). Ubiquitination of TRAF2 was not detected in RA FLS cells transfected with pEF-HA (Figure 5B, top, lanes 1 and 2) or in unstimulated RA FLS cells (Figure 5B, bottom).

Because it has been shown that ubiquitination of TRAF2 is required for subsequent ubiquitination of RIP1, which is a key step for activation of NF- κ B, we also

Figure 6. Transfection with JAB1 siRNA leads to the prevention of TRAF2 recruitment to TNFR1 in TNF- α -stimulated RA FLSs. RA FLS cells were transfected with scrambled siRNA (**A**) or JAB1 siRNA (**B**) for 36 hours and then stimulated with biotinylated TNF- α (100 ng/ml) (**A** and **B**, lane 2) or the diluent (PBS) (**A** and **B**, lane 1) for 5 minutes. The cells were then lysed using an immunoprecipitation lysis buffer and 1.0-mg aliquots of the total protein lysates were immunoprecipitated with streptavidin-conjugated agarose beads. Precipitates were separated by 10% SDS-PAGE, and Western blot analyses of TRAF2, RIP1, JAB1, TRADD, and TNFR1 were performed. Fifty μ g of total lysates were also used for Western blot analyses of TRAF2, RIP1, JAB1, and TRADD (**C**) to estimate the variability of each of them in the cell lysates.

analyzed the immunoprecipitates generated in the above experiments for the ubiquitination of RIP1. The results indicated that RIP1 is indeed ubiquitinated on TNF- α stimulation of RA FLS cells (Figure 5C, top, lane 2). Remarkably, in RA FLS cells in which JAB1 was knocked down, RIP1 became nonubiquitinated (Figure 5C, top, lane 1). RIP1 was not ubiquitinated in unstimulated RA FLS cells (Figure 5C, bottom), suggesting that RIP ubiquitination is TNF- α related. Parallel studies using HT1080 fibrosarcoma cells yielded similar results (data not shown). These findings suggest that JAB1 plays a critical role in ubiquitination of TRAF2, at least in this *in vitro* model. The effect of JAB1 on the ubiquitination of RIP1 is most likely regulated through TRAF2 because JAB1 did not co-immunoprecipitate with RIP1 (data not shown).

siRNA Knockdown of JAB1 Prevents TRAF2 Recruitment to TNFR1

To further identify the events that lead to the inhibition of ubiquitination of TRAF2 in RA FLS cells in which JAB1 has been knocked down, we incubated the cells with biotin-labeled human TNF- α for 5 minutes at 37°C. Cells were then lysed and subjected to immunoprecipitation using streptavidin-coated beads. The results from Western blot analysis of the immunoprecipitates indicate that TRAF2, JAB1, and RIP1 were co-immunoprecipitated with TNFR1 on TNF- α stimulation in the RA FLSs pretreated with control, scrambled siRNA (Figure 6A, second column). In

contrast, in the RA FLS cells in which JAB1 was knocked down, there was a dramatic reduction in the recruitment of JAB1, RIP1, and TRAF2 to the TNFR1 complex (Figure 6B, the second column) although the amounts of TRADD that were detected in the TNFR1 complex were similar to those detected in RA FLSs that had not been subjected to transfection with JAB1 siRNA. In the absence of stimulation, none of these proteins were detected in the TNFR1 complex in either RA FLS cells that had been transfected with JAB1 siRNA or control scrambled siRNA (Figure 6, A and B, lane 1). The inability to detect these proteins in the TNFR1 complex was not because of a reduction in their cellular expression because all of them were present at approximately the same levels in the whole cell lysates (Figure 6C). These experiments were repeated using primary RA FLSs isolated from four additional individuals with RA, and similar results were obtained for each of the individual primary RA FLSs (data not shown). These data in combination with the data shown in Figure 5 suggest that an interaction of JAB1 with TRAF2 may play a role in initiation for the ubiquitination of TRAF2 and recruitment of TRAF2 and RIP1 to the TNFR1 complex on TNF- α stimulation.

P53 Recruited in the JAB1-TRAF2 Complex Is Associated with Hyperproliferation of RA FLSs

To determine whether the interaction of JAB1 with TRAF2 is a characteristic of RA FLSs, reciprocal JAB1-TRAF2

Figure 7. Higher level of p53 associated with JAB1-TRAF2 complex in RA FLSs is correlated with their rapid proliferation. **A:** Cells (5×10^6) were cultured for 48 hours and lysed using immunoprecipitation lysate buffer (Roche). Aliquots of 1 mg of the total protein lysates were immunoprecipitated with a polyclonal anti-Jab1 antibody. The immunoprecipitates were then used for Western blot analysis of p53 (top) and TRAF2 (A, second panel from the top). Fifty μ g of total lysates were used for Western blot analysis of p53 (third panel from the top) and β -actin (bottom). **B:** BrdU was added 2 hours after beginning culture and percentages of BrdU-positive cells were determined by BrdU immunolabeling 18 hours later using a method as described under Materials and Methods. **B, inset:** BrdU-positive cells were counted in 10 random fields. Statistically significant differences with a P value <0.001 were assessed and are indicated by the asterisks. RA FLSs and OA FLSs isolated from five patients were analyzed individually.

co-immunoprecipitation experiments were performed using lysates of human foreskin fibroblasts, human embryonic kidney 293, the human fibroblast cell line WI38, and OA FLSs. Western blot analysis indicated that the interaction of JAB1 with TRAF2 takes place in all of the different types of cells tested (data not shown).

One characteristic of RA FLS cells is their relatively rapid proliferation as compared with OA FLSs. JAB1 was identified as a direct negative regulator of the p53^{7,42} and that plays a role in RA. Therefore, we analyzed whether there is a difference in the association of p53 with JAB1 in RA FLS and OA FLS cells. The results of Western blot analysis of JAB1 immunoprecipitates using anti-p53 indicate that more p53 was associated with JAB1 precipitates from RA FLSs than OA FLSs, WI38, or foreskin fibroblasts (Figure 7A). There did not appear to be differences among the cell types in terms of the total amounts of p53 detected, nor were there differences in the amounts of TRAF2 precipitated or the amounts of sample loaded as determined by blotting for β -actin. The results of a BrdU incorporation assay further indicated that RA FLS cells exhibit greater incorporation of BrdU than from OA FLSs, WI38 cells, or foreskin fibroblasts (Figure 7B). These data suggest that JAB1 also may regulate the proliferation of RA FLSs through an interaction with p53. Currently, the potential effects of the JAB1-p53 interaction on RA FLS proliferation is under investigation.

Discussion

The experiments reported here demonstrate that the activation of NF- κ B and JNK, which is associated with the TNF- α -mediated prosurvival response in RA FLSs, is dependent on the COP9 signalosome subunit CSN5/JAB1. They also demonstrate that interaction of JAB1 with

TRAF2 plays a role in TNF- α -mediated anti-apoptosis response in RA FLSs. Knockdown of JAB1 in RA FLSs using JAB1 siRNA resulted in high levels of apoptosis on TNF- α stimulation. In addition, knockdown of JAB1 resulted in reduced activation of NF- κ B, which was concordant with accumulation of I κ B- α , reduced activation of JNK, and subsequent reduction in the production of MMP1. Finally, knockdown of JAB1 led to loss of lysine-63-based ubiquitination of TRAF2 on TNF- α stimulation. This further suggests that activation of JNK is not associated with the TNF- α -induced apoptosis in these cells because the TNF- α induced activation of JNK has been shown to be dependent on lysine-63-based ubiquitination of TRAF2.²⁴ The lack of lysine-63-based ubiquitination of TRAF2 also was consistent with the lack of ubiquitination of RIP1, which in turn would contribute to the loss of activation of NF- κ B. Thus, we conclude that the interaction of JAB1 with TRAF2 regulates the TNF- α -mediated prosurvival response and the production of MMPs in RA FLSs. Although our yeast-two hybrid results indicate a direct interaction between JAB1 and TRAF2, the possibility of an indirect interaction should not be eliminated because of the risk of false-positive interactions in a yeast-two hybrid system. Future study will involve identifying the domains that mediate JAB1-TRAF2 interaction in RA FLS cells.

The simplest explanation for the ability of JAB1 to regulate TNF- α -mediated activation of both JNK and NF- κ B would be that JAB1 regulates the E3 ligase activity of TRAF2. Other investigators have reported that the E3 ligase activity of TRAF2 regulates TNF- α -mediated activation of both NF- κ B and JNK through ubiquitination of RIP1 and JNK.^{40,41,43–45} Although we found that TRAF2 co-precipitates with JAB1, we were unable to demonstrate co-precipitation of RIP1 with JAB1 using a tech-

Figure 8. Model for the involvement of JAB1 in TNF- α signaling pathway. Although TRAF2-JAB1 forms a complex in the cytosol, the activation of kinase(s) because of TNF- α stimulation leads to the replacement of a suppressor with activated kinase(s), which is associated with JAB1. As a result, JAB1-mediated activation of TRAF2 is initiated by a cascade of phosphorylation reactions followed by ubiquitination TRAF2 at position of K63. Eventually, TRAF2 is activated and recruited to the TNFR1 complex.

nique in which HA-tagged JAB1 was immunoprecipitated from RA FLSs and the immunoprecipitates subjected to Western blot analysis of TRAF2 or RIP1. However, siRNA knockdown of JAB1 in RA FLSs resulted in a loss of ubiquitination of RIP1 as well as TRAF2. These results suggest that the effects of JAB1 on ubiquitination of RIP1 are mediated through the effects of JAB1 on TRAF2 E3 ligase activity, and that JAB1 results in an enhancement of this activity.

The mechanism by which JAB1 enhances TRAF2 E3 ligase activity is unknown. It is known that phosphorylation of TRAF2 is required for its E3 activity.^{21,46} Although CSN5/JAB1 is not a kinase, it associates with several kinases that regulate the activities of p53, p27, and other key regulators of cellular activity.^{1,7,12} Therefore, it can be speculated that JAB1 promotes TRAF2 E3 ligase activity through the action of one of its associated kinases, which have not been identified in this study (Figure 8). Alternatively, JAB1 may modulate the deubiquitination. A preliminary analysis of CYLD protein levels suggested that siRNA knockdown of JAB1 in RA FLSs enhanced the protein levels of this deubiquitinating enzyme although it did not affect the mRNA levels (data not shown). It is well established that CYLD interacts with TRAF2, causing deubiquitination of both TRAF2 and RIP1^{44,47–50} and that activation of CYLD can block NF- κ B activation induced by TNF- α stimulation.^{41,44}

Nuclear factor- κ B (NF- κ B) is a transcription factor composed of dimeric complexes of p50 (NF- κ B1) or p52 (NF- κ B2) usually associated with members of the Rel family (p65, c-Rel, Rel B), which have potent trans-activation domains. In unstimulated cells, the I κ B proteins complex with NF- κ B subunits such as c-Rel and sequester them from the nucleus. Phosphorylation of I κ B molecules causes proteasomal degradation of I κ B proteins and release of NF- κ B molecules to the nucleus where they induce their downstream target genes, including the I κ Bs. Among the I κ B molecules, I κ B- α is

highly inducible by NF- κ B. This feedback regulation leads to oscillation of NF- κ B function when stimulation is sustained throughout a long period of time. Our results show that in RA FLSs transfected with JAB1 siRNA, the activity of NF- κ B was reduced to almost basal level yet I κ B- α degradation induced by the TNF- α pathway was not completed. The stability of I κ B- α is regulated by a number of pathways, including activation of the Ikks kinase complex or κ B- α E3 kinase- β -Trcep. Therefore, siRNA knockdown of JAB1 may affect only one of several possible pathways that may lead to the degradation of I κ B- α . Because JAB1 has been shown to shuffle proteins between nucleus and cytoplasm and to regulate protein functions, it is also possible that JAB1 may play a role in enhancing nuclear translocation of activated NF- κ B. Therefore, knockdown of JAB1 by siRNA may lead to attenuation of NF- κ B nuclear translocation. In the future, it will be of interest to determine how JAB1 affects the stability of I κ B- α and whether JAB1 regulates NF- κ B translocation directly.

In this study, the interaction of JAB1 with TRAF2 did not appear to be RA FLS-specific; however, there did appear to be differences between RA FLSs and the other types of cells tested in the amount of proteins that were associated with JAB1. In particular, the amounts of p53 associated with JAB1 appear to be greater in RA FLSs than in OA FLS or WI38 cell. p53 is one of the major regulators of inhibition of the cell cycle and promotion of apoptosis. p53 is inactivated and further degraded after interaction with JAB1. Our data suggest that higher amounts of p53 associated with JAB1 may result in enhanced inactivation of p53. The mechanism suggested by these data would be a potential explanation for the higher proliferative potential of RA FLSs as compared to OA FLSs. The results of JAB1/CSN-mediated regulation of p53 stability in RA FLSs may also be of clinical importance for future anti-TNF therapy. Further study of the effects of JAB1-p53 interaction on the proliferation of RA FLSs was beyond the scope of this study, but is an important issue for defining the mechanisms underlying hyperproliferation of RA FLSs in the future.

In summary, our data suggest that JAB1 is critically involved in the regulation of TNF- α -mediated anti-apoptosis in rheumatoid synovial fibroblasts. Based on evidence that knockdown of endogenous JAB1 leads to enhancement of TNF- α -mediated apoptosis, we suggest that overexpression of JAB1 may contribute to defects of RA FLS apoptosis in general and to the development of synovial hyperplasia and tissue invasion in RA. Furthermore, as we found that JAB1 regulation of TNF- α -induced apoptosis responses is not restricted to RA FLSs, these findings may be of relevance to other TNF- α -mediated inflammatory diseases.

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