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Original Article

The transcription co-factor JAB1/COPS5, serves as a potential oncogenic hub of human chondrosarcoma cells *in vitro*

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Abstract: Chondrosarcoma (CS) is the second most common skeletal malignancy in humans. High-grade CS is aggressive and extremely resistant to chemo- and radio-therapies. The lack of effective treatment options warrants the development of novel therapies. The evolutionarily conserved transcriptional co-factor JAB1 (also known as COPS5/CSN5) has emerged as a novel regulator of tumorigenesis. JAB1 overexpression occurs in many common cancers and is associated with poor prognosis. However, the role of JAB1 in CS pathogenesis was completely unknown. To study JAB1's function in CS, we performed shRNA knockdown (KD) of *JAB1* in two high-grade human CS cell lines, SW1353 and Hs819.T, and observed significantly decreased proliferation and colony formations, and increased apoptosis in both CS cell lines upon JAB1-KD. Interestingly, we found that endogenous JAB1 interacted with endogenous SOX9, a potent oncogene and a master regulator of skeletogenesis, in chondrosarcoma cells, but not in primary chondrocytes. JAB1 also binds to the same SOX9-mediated chondrocyte-specific enhancer elements in CS cells. Furthermore, we found that a recently developed, novel, potent, and JAB1-specific small molecule inhibitor, CSN5i-3, can significantly increase apoptosis, drastically alter the activities of several signaling pathways, and modulates the expression of specific Cullin-ring-ligases (CRLs) in CS cells. Finally, our RNA-sequencing analysis in *JAB1-KD* CS cells identified a total of 2945 differentially expressed genes. Gene set enrichment analysis revealed that *JAB1* regulates several essential pathways such as DNA damage response and cell cycle regulation. In conclusion, our study showed that JAB1 might regulate a distinct pro-tumorigenic regulatory network to promote chondrosarcoma pathogenesis.

Keywords: JAB1/COPS5, SOX9, chondrosarcoma, COP9 signalosome, transcriptome

Introduction

Chondrosarcoma (CS) is the second most common skeletal cancer and represents up to 27% of all bone malignancies [1]. It is usually diagnosed between the ages of 30 to 60 years, and the 5-year survival rates for grade-I and grade-2 CS is 83% and 50%, respectively [2]. CS can originate from any cartilaginous tissue; however, the most commonly affected sites are near the pelvis, femur, and humerus. The most common clinical symptoms are local pain, edema, teeth displacement, and hearing loss. The lungs are the primary site of CS metastasis, and there are very limited therapeutic options available for high-grade CS, barring aggressive surgical resection [3]. Indeed, high-grade chondrosarcoma is aggressive and is extremely resistant to chemo- and radio-therapies [4]. Comprehensive genomic analysis of 49 cases of CS patients identified hypermutability of the genes *COL2A1* and *TP53,* loss-of-function in RB and IHH signaling pathways, and neoplastic gain-of-function mutations of *IDH2* [5]. A most recent study has proposed the use of mRNA-based approaches to identify patients with high-risk CS [6]. However, our incomplete understanding of the molecular basis of CS pathogenesis hinders the development of novel treatments.

Table 1. Upregulated and downregulated pathways upon silencing *JAB1* in SW1353 human chondrosarcoma cells identified by gene set enrichment analysis

Name of the pathways	# of genes	FDR q-value
Upregulated pathways in *JAB1-KD* cells		
Chromosome Segregation	169	0
Condensed Chromosome	111	0
Cell Cycle G2/M Phase Transition	98	0.003
Cellular Response to DNA Damage Stimulus	431	0.003
Cullin Ring Ubiquitin Ligase Complex	78	0.20
Downregulated pathways in *JAB1-KD* cells		
Extracellular Matrix	207	0
Calcium Ion Binding	315	0
Biological Adhesion	461	0
Skeletal System Development	239	1.30E-04
Endochondral Bone Morphogenesis	32	1.27E-04

The evolutionarily conserved transcriptional cofactor, JAB1, also known as COPS5/CSN5, has emerged as an attractive target for cancer treatment. *JAB1* was originally cloned as a Jun activation domain-binding protein 1, and acts as a c-Jun or JunD co-activator to promote AP-1 complex activity [7]. JAB1 plays important roles in cell cycle progression, apoptosis, DNA damage repair, various signaling pathways, and a vast array of developmental processes [8-10]. The constitutive deletion of *Jab1* in mice results in early embryonic lethality by E8.5, with impaired proliferation and increased apoptosis [11, 12]. JAB1 is also the fifth subunit of the COP9 signalosome (CSN), an evolutionarily conserved proteolysis regulator [9, 13]. The COP9 signalosome is composed of eight subunits (CSN1-8). The CSN is essential for regulating the largest family of E3 ubiquitin ligases, the Cullin-ring ligases (CRLs), which are central mediators of tumorigenesis. Within the CSN, JAB1 is the only subunit containing a zinc-binding JAMM motif, which constitutes the catalytic center for cleaving NEDD8 (an ubiquitin-like small molecule) from the Cullin subunit (deneddylation) [14]. JAB1 plays an essential role in regulating CRL homeostasis [13]. The deregulation of any CSN subunit, particularly JAB1, has detrimental effects on various cellular functions, such as DNA fidelity maintenance, angiogenesis, and microenvironment homeostasis, all of which are critical for tumor development [8, 15]. Indeed, clinical studies reveal that the expression and localization of JAB1 correlate with disease progression in diverse tumor types [8]. JAB1 amplification and overexpression also confer tamoxifen-resistance in ER-alpha-positive breast cancer [16]. However, the role of JAB1 in chondrosarcoma pathogenesis has not been reported.

In the present study, we found that the knockdown (KD) of *JAB1* reduced tumorigenic properties and elevated apoptosis of human CS cells *in vitro*. We demonstrated that CSN5i-3, a novel and highly specific small molecule inhibitor of JAB1, can induce apoptosis of human CS cells and has specific effects on the ubiquitin-proteasome system. Co-immunoprecipitation studies revealed that JAB1 interacts with SOX9, a master regulator of chondrogenesis and a potent oncogene, in human and rat CS cell lines, but not in mouse primary chondrocytes. A reporter assay revealed that several key signaling pathways were also significantly altered in CSN5i-3 treated human CS cells. Finally, we employed RNA-sequencing and identified a large and distinct JAB1-mediated transcriptome in human CS cells.

Materials and methods

Cells and drugs

The two human chondrosarcoma cells lines SW1353 (ATCC# HTB-94, female) and Hs819.T (ATCC# CRL-7891, male) were procured from ATCC. A widely used human immortalized chondrocyte cell line C20A4 (Cat# SCC041, male) was procured from Millipore-Sigma. Mouse primary rib chondrocytes were isolated following a sequential digestion by pronase (2 mg/ml in PBS) for 30 min at 37°C, collagenase D (3 mg/ml in DMEM) for 1.5 h at 37°C, and overnight at 37°C in collagenase D (1.5 mg/ml in DMEM). Cells were maintained in DMEM-F12 (Gibco) supplemented with 10% FBS (Invitrogen) and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco), at 5% CO_2 and 37°C. For lentiviral infection, SW1353 and Hs819.T cells were infected with Mission shRNAs (Sigma-Aldrich, St. Louis, MO, USA) specifically targeting *JAB1* or a Non-target

Table 2. List of the ChIP-qPCR primers used in this study [20]

Genomic element	Primer sequence (5'-3')	Detected region
rSox9-SOM Enh1	GCAGTTAGCTAGGAGCTTCAG	~650 bp upstream from SOM a region
rSox9-SOM Enh1	GGTAGTCTGACTATTGGATCTG	
rSox9-SOM Enh4	GTTACCACCATCTCATAAA	~650 downstream from SOM b region
rSox9-SOM Enh4	TGTCTCACCACCTTCTTGAAA	
rAcan Enh2	GATGACCAATCCCTCAAGAA	~250 bp upstream from Sox9 binding motif
rAcan Enh2	GCGCTGTTTATGTGGGTGTTTC	
rCol2a1 Enh1	GCTGTGCATTGTGGGAGAG	Flanked the Sox9 binding site
rCol2a1 Enh1	CTGTGAATCGGGCTCTGTATG	
rCol2a1 Enh2	TCAGGTCTTTGGGTCCTGT	~350 bp upstream from Sox9 binding motif
rCol2a1 Enh2	TCGGTCTCTTACGATTCTGCATCT	
rGapdh	TACGTGCACCCGTAAAGC	Gapdh promoter region
rGapdh	CTTGGTGCGTGCACATTTC	

shRNA control (Supplementary Table 1) at MOI=5, per the manufacturer instructions. 48 hours after infection, cells were changed to puromycin (2 μg/mL; Sigma)-selection media and grew for another 72 hours.

CSN5i-3 was a generous gift from Dr. Eva Altmann at Novartis.

Chondrosarcoma functional assays

All functional assays were performed in SW1353- and Hs819.T *JAB1*-knockdown and control cells as described [17, 18]. For the MTT assay, SW1353 and Hs819.T *JAB1*-knockdown and control cells were plated at a density of 10^4 cells per well of 96-well plates. Cells were allowed to grow for 24 or 96 hours. 10 μl of a 5 mg/mL MTT reagent (Invitrogen) was added to the cells and incubated at 37°C in the dark for 4 hours. Cells were then washed with PBS, and after the addition of DMSO, readings were taken at 570 nm using the Tecan GENios microplate reader (Männedorf, Switzerland). For the colony formation assay, *JAB1*-knockdown (*JAB1-KD*) and control cells were plated at a density of 1000 cells/cm^2 in 60 mm dishes and cultured for 3 days. Cells were fixed for 10 min in cold methanol on ice, and placed in 0.5% crystal violet (Sigma-Aldrich) in 25% methanol for 10 min at room temperature. Cells were then washed with distilled water and allowed to air dry overnight. Images were taken and processed for colony quantification using ImageJ. For the wound healing assay, *JAB1*-knockdown and control cells were plated at 10^6 cells/well in 6-well plates, and cultured overnight. Scrapes were made using a 200 μl micropipette tip. Cells were then allowed to grow for 72 hours and the distance was measured using Image J.

Chromatin immunoprecipitation (ChIP)-qPCR

Chromatin was prepared and immunoprecipitated as described [19] with an anti-JAB1 (Santa Cruz Biotechnology) antibody, or an IgG control (Santa Cruz Biotechnology). Primers used to amplify the enhancer elements for Sox9's binding to the *Aggrecan* and *Col2a1* genes were described previously [20], and are listed in **Table 2**.

RNA-sequencing analysis

RNA-sequencing was performed at the Genomics Core at Case Western Reserve University, and the bioinformatics analysis was performed at the Case Computational Biology Core Facility. The dataset has been deposited into the NCBI Gene Expression Omnibus under the accession number GSE137683.

Statistical analysis

All experiments were independently performed using at least triplicates. The statistical significance was determined using the Mann-Whitney rank sum test. *P* values <0.05 were considered statistically significant.

A complete and detailed methodology can be found in Supplementary Materials and Methods.

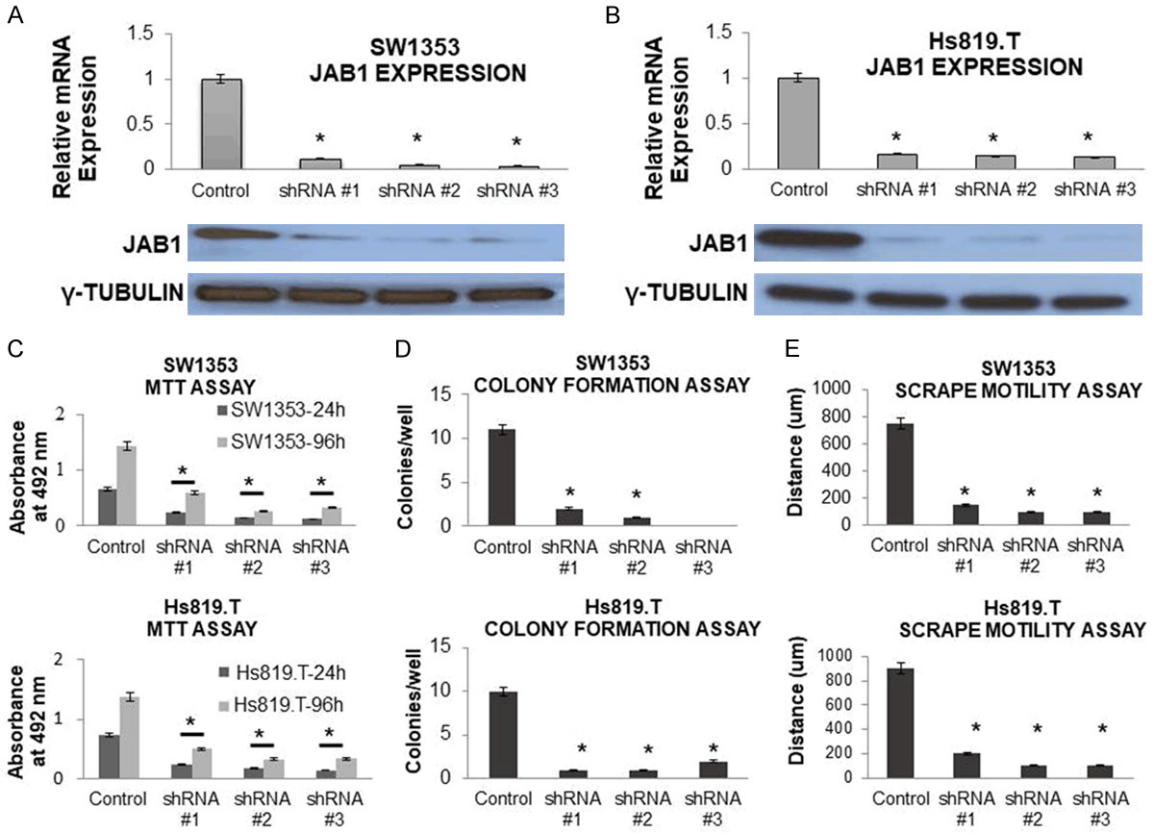


Figure 1. *JAB1*-knockdown reduced tumorigenic properties in human chondrosarcoma cell lines (CS) SW1353 and Hs819.T. A, B. Real-time qPCR and Western blot analysis of *JAB1* knockdown. C. Cell proliferation status as measured with the MTT assay. D. Colony formation assay. E. The scrape motility assay. N=5-6. Error bars represent means ± SD. *P<0.05 when compared with controls. All controls in these experiments are a scrambled non-target shRNA.

Results

JAB1-knockdown significantly reduces the oncogenic properties of human chondrosarcoma cells

To test the hypothesis that the loss of *JAB1* reduces the tumorigenic properties of human chondrosarcoma (CS) cells, we performed lentiviral shRNA knockdowns in two commonly used, high-grade human CS cell lines, SW1353 and Hs819.T. Three independent shRNAs were used to avoid off-target effects in both human CS cells along with a scrambled non-target control (NTC) shRNA (Supplementary Figure 1A). Both real-time qPCR and western blot analysis confirmed the robust JAB1 knockdown (*JAB1-KD)* in SW1353 and Hs819.T cells (**Figure 1A**, **1B**). To understand the functional relevance of *JAB1-KD* in CS cells, we performed standard tumorigenic functional assays. First, the MTT assay showed that cell proliferation or viability was significantly reduced at both 24 hours and 96 hours in *JAB1-KD* SW1353 and Hs819.T cells compared with the controls (**Figure 1C**). Next, relative to the controls, *JAB1-KD* significantly reduced colony formations in both human CS cell lines (**Figure 1D**). Finally, an *in vitro* wound-healing assay revealed that there was a significant reduction in cell migration in both SW1353 and Hs819.T cells upon *JAB1* depletion (**Figure 1E**). Thus, the loss of JAB1 in human CS cells results in significantly reduced malignant properties.

JAB1 is a potential therapeutic target for CS treatment

The NEDDylation pathway is known to trigger the activation of the largest family of E3 ubiquitin ligases, the Cullin-RING ligases (CRLs) [21]. In brief, as illustrated in **Figure 2E**, in a cascade analogous to ubiquitin transfer, NEDD8 activation enzyme (NAE) conjugates NEDD8, a

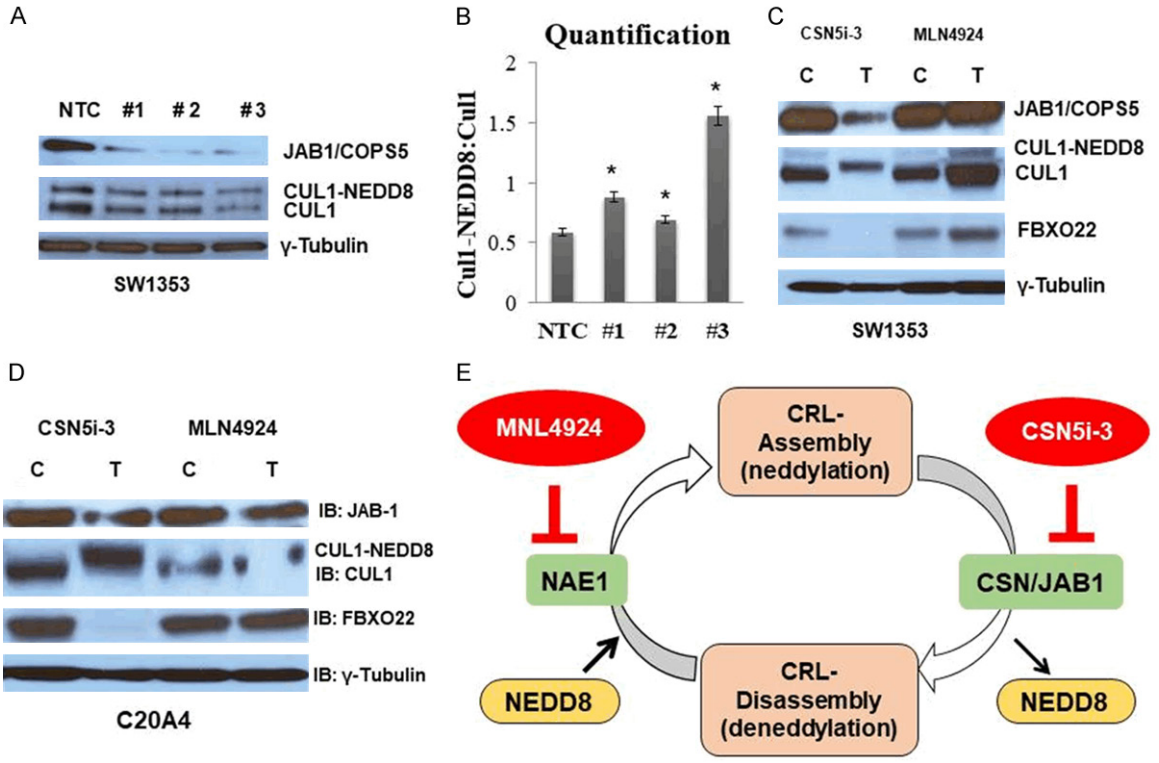


Figure 2. Targeting the neddylation pathway in human chondrosarcoma (SW1353) and human immortalized chondrocyte (C20A4) cell lines. (A) Western blot analysis of CULLIN1 in *JAB1*-KD SW1353 CS cells. (B) Densitometry quantification of CULLIN1-Nedd8/CULLIN1 ratio in *JAB1*-KD vs. Non-target control SW1353 cells with western blot images in (A). (C) Western blot analysis of CSN5i-3- and MLN4924-treated SW1353 cells. (D) Western blot analysis of SOX9, CULLIN1, and FBXO22 in C20A4 cells treated with CSN5i-3 or MLN4924. (E) The schematic representation of the NEDDylation cycle of the CULLIN-RING Ligase (CRL), as activated by NAE1 and inactivated by JAB1. Error bars represent means ± SD. *P<0.05 when compared with controls.

small ubiquitin-like protein, to the Cullin subunit of CRLs, and thus traps the Cullin in an active, NEDDylated state [22]. On the other hand, JAB1, the catalytic subunit of the COP9 signalosome complex, catalyzes the removal of NEDD8 from the Cullin subunit to keep it in an inactive, deNEDDylated state and maintaining their cellular homeostasis [14, 21] (**Figure 2E**). In recent years, the NEDDylation pathway has emerged as an attractive therapeutic target for cancer treatment [23-25]. Indeed, MLN4924, a specific inhibitor of NAE1, is currently in clinical trials for the treatment of various cancers [25]. Moreover, a highly specific small molecule inhibitor of JAB1, CSN5i-3, has recently been developed [26]. MLN4924 inhibits NAE1, thus rendering CRLs in their deNEDDylated state, whereas CSN5i-3 inhibits JAB1 and traps CRLs in their NEDDylated state (**Figure 2E**).

It was previously reported that CSN5i-3-mediated JAB1 inhibition reduced cell viability in a large panel of cell lines, as well as repressed the growth of lymphoma xenografts in mice [26], but CSN5i-3's effects in CS have not been studied. Thus, we employed CSN5i-3 and MLN4924 to determine whether disrupting the NEDDylation pathway can affect the growth of human CS cells, SW1353 cells (Supplementary Figure 1B, 1C). CRLs are key regulators of cellular homeostasis and their activities can be modulated by neddylation. Indeed, our western blot analysis in SW1353 cells demonstrated that, as expected, the ratio of NEDDylated Cullin1 to Cullin1 was increased upon *JAB1*-KD (**Figure 2A**, **2B**). Interestingly, western analysis also revealed that, as expected, CSN5i-3 treatment caused the accumulation of NEDDylated Cullin1 in both SW1353 and C20A4 cells (an immortalized human chondrocytes cell line) (**Figure 2C**, **2D**), whereas MLN4924 treatment increased Cullin1 levels in SW1353 cells (**Figure 2C**) but not in C20A4 cells (**Figure 2D**). Interestingly, consistent with another group's findings [26], the expression of FBXO22 [27,

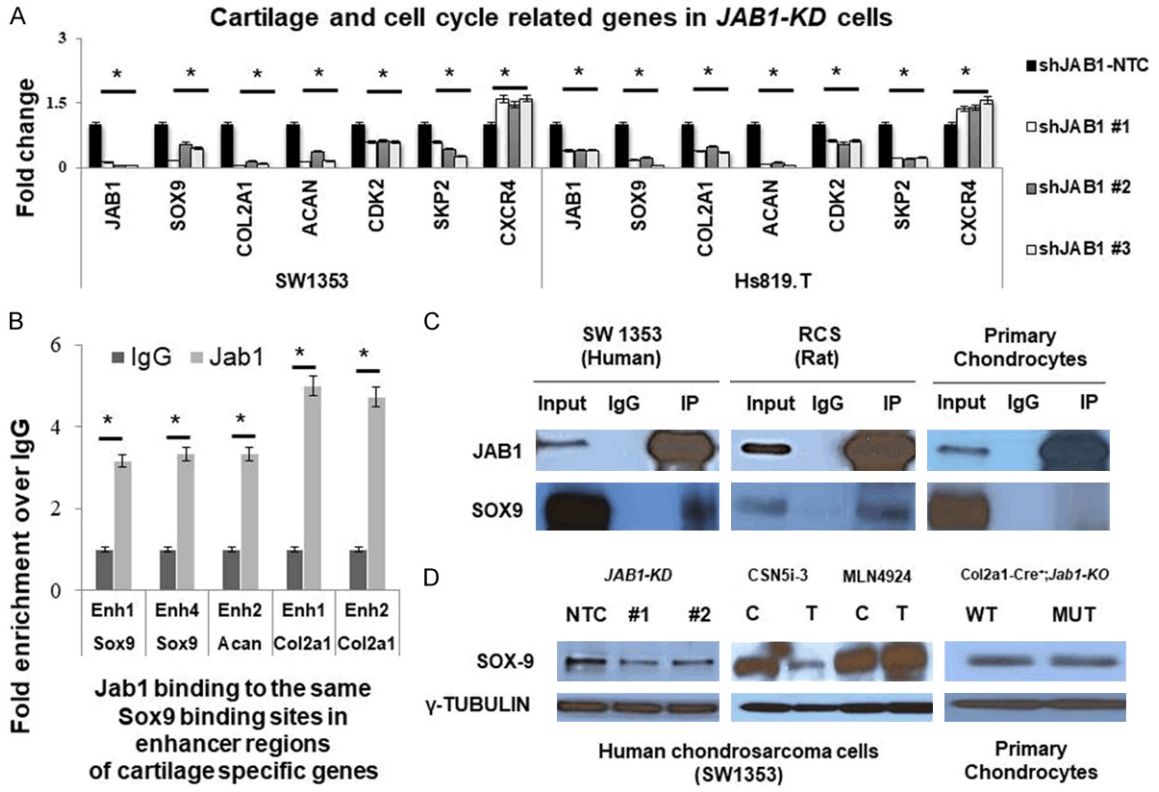


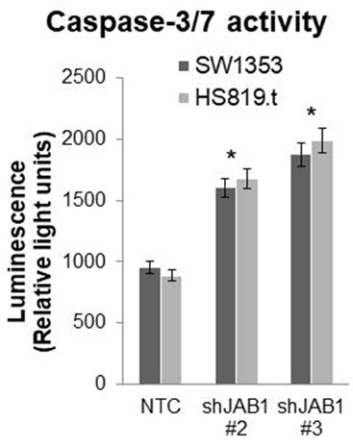
Figure 3. The JAB1-SOX9 axis likely promotes chondrosarcoma pathogenesis. A. Real-time qPCR analysis in *JAB1*-KD SW1353 cells. B. ChIP-qPCR showed that JAB1 was significantly enriched at the same SOX9-binding sites within the SOX9 enhancer regions in rat chondrosarcoma cells (RCS). C. JAB1 immunoprecipitations in SW1353 human chondrosarcoma, rat chondrosarcoma cells (RCS), and mouse primary chondrocytes to detect JAB1-SOX9 interaction. D. Western blot analysis of SOX9 expression in both human chondrosarcoma cells and mouse *Jab1*-knockout primary chondrocytes. Error bars represent means ± SD. *P<0.05 when compared with controls.

28], a poorly characterized F-box protein, was completely abolished when SW1353 and C20A4 cells were treated with CSN5i-3 (**Figure 2C**, **2D**), but slightly elevated in MLN4924-treated SW1353 cells (**Figure 2C**), and no noticeable differences in MLN4924-treated C20A4 cells (**Figure 2D**). These results suggest that FBXO22 might be a specific target of JAB1 in CS cells. As both MLN4924 and CSN5i-3 target the neddylation pathway, it would be interesting to investigate whether JAB1's unique enzymatic activity of deneddylation is necessary for its oncogenic function in future studies.

JAB1 directly interacts with SOX9 specifically in CS cells

Sox9 is a master regulator of cartilage development and controls all aspects of skeletogenesis [19, 29, 30]. In recent years, it has become increasingly clear that many developmental master regulators become reactivated in adult life to drive tumorigenesis. Indeed, SOX9 has been implicated as a potent oncogene in breast and prostate cancers [31, 32]. Interestingly, a high-level endogenous SOX9 expression was confirmed in human CS, and targeting SOX9 via MiR-145 has been proposed as a therapeutic strategy in CS [33]. Thus, to determine the effect of *JAB1*-KD on SOX9 expression and SOX9 downstream targets, we performed RT-qPCR and discovered a significant downregulation of *SOX9* and its downstream targets *COL2A1* and *AGGRECAN* in *JAB1*-KD CS cells (**Figure 3A**). Next, we performed ChIP-qPCR analysis in a well-characterized rat chondrosarcoma cell line (RCS) [34] and demonstrated that there was significant enrichment of JAB1 at the same SOX9 binding sites in the cartilage-specific enhancer regions of *SOX9, AGGRECAN and COL2A1* genes in the RCS cells (**Figure 3B**). More importantly, our IP experiments demonstrated that endogenous JAB1 interacts with endogenous SOX9 in both human and rat chondrosarcoma cells, but not

A



B

SW1353	Control	CSN5i-3	MLN4924	NTC	shJAB1 #3
Sub-G0	0.99 %	12.5 %	26.56 %	1.89 %	19.5 %
G1	69.8 %	57.1 %	35.5 %	69.7 %	65.1 %
S	9.74 %	2.99 %	7.64 %	8.15 %	2.67 %
G2/M	19.47 %	27.41 %	30.3 %	19.1 %	12.13 %
HS819.t	Control	CSN5i-3	MLN4924	NTC	shJab1 #3
Sub-G0	1.09 %	11.2 %	22.2 %	1.21 %	16.1 %
G1	72.6 %	49.3 %	36.4 %	70.8 %	66.5 %
S	5.11 %	8.4 %	7.9 %	8.2 %	4.51 %
G2/M	20.93 %	32.6 %	34.1 %	20.1 %	12.5 %

C

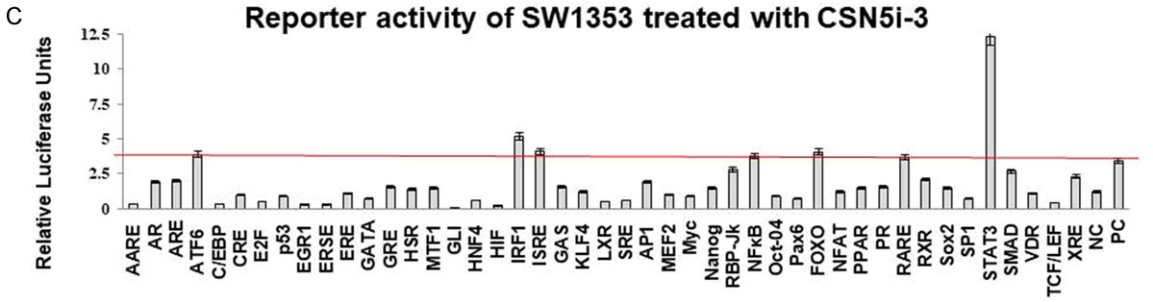


Figure 4. *JAB1*-silencing led to increased apoptosis and chemo-sensitivity in human CS cells. A. The caspase 3/7 glo assay in *JAB1*-KD human chondrosarcoma cells. B. Cell cycle analysis by propidium iodide flow cytometry in *JAB1*-KD, CSN5i-3-treated, and MLN4924-treated human chondrosarcoma cells. Error bars represent means ± SD. *P<0.05 when compared with controls. C. Cignal reporter assay in SW1353 cells showed that JAB1 deficiency altered the activities of several key signaling pathways. Red line indicates the reporter activities that were correlated with positive control (PC). Abbreviations of the reporters are listed in the Supplementary Table 3.

in mouse primary chondrocytes (**Figure 3C**). Thus, we speculate that JAB1 might promote CS tumorigenesis by differentially regulating SOX9 activity in cancer versus normal cells. Interestingly our western blot results revealed that SOX9 was significantly downregulated in both *JAB1-KD* and CSN5i-3-treated human CS cells (**Figure 3D**). However, the SOX9 protein levels were not grossly affected in *Jab1*-knockout mouse primary chondrocytes (**Figure 3D**). This strongly suggests that in CS cells, but not in the normal chondrocytes, JAB1 can directly regulate SOX9, another potent oncogene, at both transcriptional and translational levels, to drive the tumorigenesis.

JAB1-KD reduces chemo-resistance and increases apoptosis in human CS cells

Chondrosarcomas are highly resistant to chemotherapy [35]. Thus, to test if *JAB1*-KD in human CS cells affects their chemo-sensitivity, we treated *JAB1*-KD human CS cells with a standard chemotherapeutic drug, etoposide [36]. Interestingly, upon etoposide treatment, apoptosis, as measured by Caspase 3/7 activity, was significantly increased in *JAB1*-KD CS cells compared with the control cells (**Figure 4A**), suggesting that *JAB1*-KD CS cells have increased sensitivity to chemotherapy. Moreover, our cell cycle analysis revealed the significantly increased accumulation of cells in the sub-G0 phase in CSN5i-3-treated, MLN4924-treated, and *JAB1-KD* CS cells when compared with their respective controls (**Figure 4B**). This suggests that there might be increased cell death upon JAB1-inhibition in CS cells. There was also a drastic decline in the number of cells in the G1-phase of the cell cycle in CSN5i-3-treated, MLN4924-treated, and *JAB1-KD* CS cells (**Figure 4B**). Moreover, the cells in the S- and G2/M-phases were both significantly

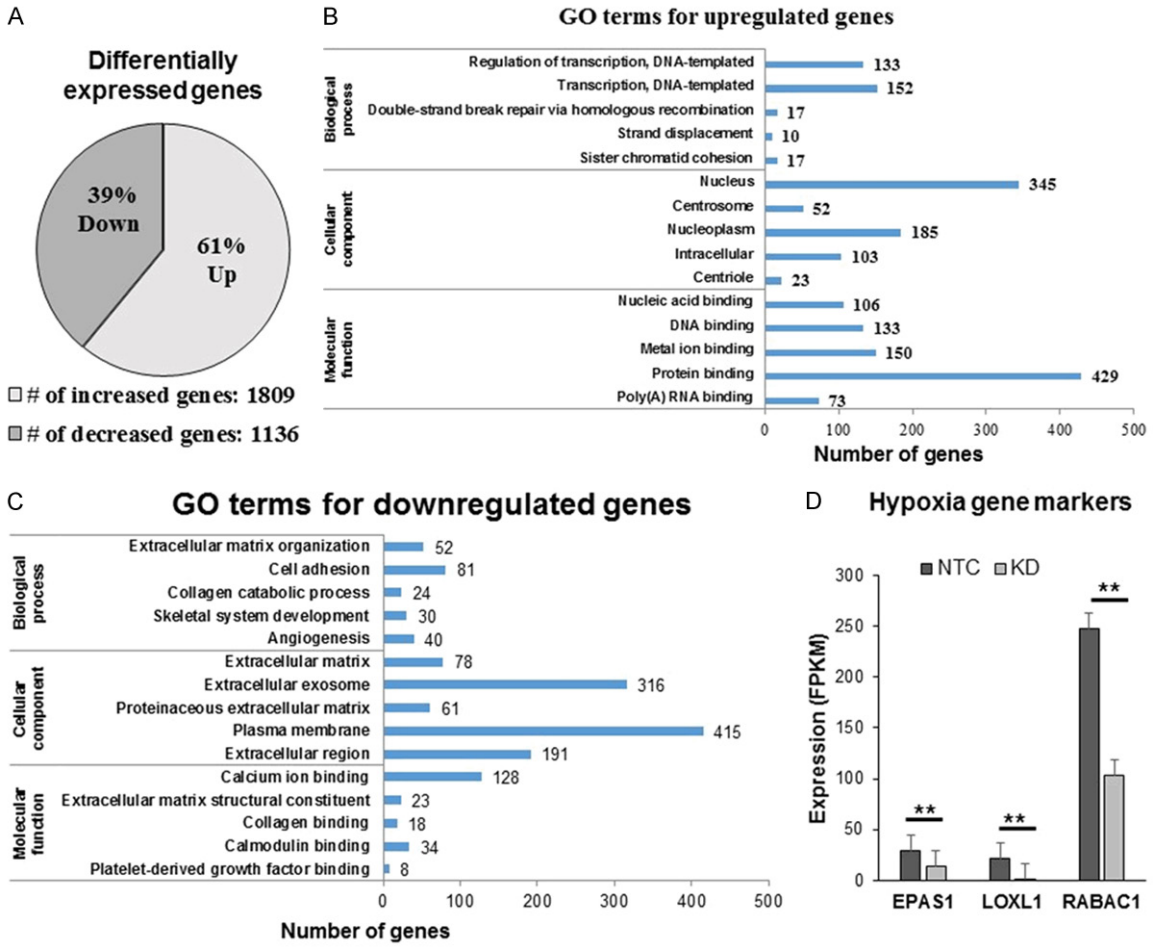


Figure 5. RNA-sequencing analysis of *JAB1*-KD SW1353 human chondrosarcoma cells reveals a large and specific JAB1-mediated transcriptome. A. Pie chart showing the number of and percentage of significantly up- and downregulated genes upon *JAB1*-KD. B. Significantly enriched Gene Ontology (GO) terms for upregulated genes upon *JAB1*-KD in SW1353 cells by DAVID analysis. C. Significantly enriched GO terms for downregulated genes upon *JAB1*-KD in SW1353 cells by DAVID analysis. D. Hypoxia pathway markers including *EPAS1*, *LOXL1*, and *RABAC1* were significantly downregulated in SW1353 cells upon *JAB1*-KD. Error bars represent means ± SD. *P<0.05 when compared with controls.

altered in CSN5i-3-treated, MLN4924-treated, and *JAB1-KD* CS cells compared with their controls (**Figure 4B**). Next, to identify the significantly altered signalling pathways in CSN5i-3-treated CS cells, we performed a large-scale, unbiased functional reporter assay screening. Interestingly, we found that the Signal Transducer and Activator of Transcription-3 (STAT3) and Interferon Regulatory Factor 1 (IRF1) reporter activities were both significantly increased (**Figure 4C** and Supplementary Table 3). These results confirmed that JAB1 might regulate numerous signalling pathways in CS pathogenesis. However, the significance of these specific findings remains to be determined.

RNA-sequencing analysis demonstrates that JAB1 controls a large and unique transcriptome in human chondrosarcoma cells

As a transcriptional co-factor, JAB1 modulates the transcriptional activity of many transcription factors [7, 8]. However, the JAB1 downstream targets in CS at the whole transcriptome level were unknown. To this end, we performed RNA-sequencing in *JAB1-KD* and control SW1353 cells. We identified a large JAB1-mediated transcriptome in CS, with 1809 and 1136 genes that were up- and downregulated, respectively, in *JAB1*-KD SW1353 cells (**Figure 5A**). The top 50 induced and repressed genes were listed in Supplementary Figure 2. We next

performed the standard Gene Set Enrichment Analysis (GSEA) to identify the key biological pathways that are altered in CS cells upon *JAB1*-KD (**Table 1**). GSEA using the Molecular Signature Database demonstrated that the top signatures enriched in the upregulated genes were those related to chromosome segregation, condensed chromosome, cell cycle G2/M phase transition, cellular response to DNA damage stimulus, and Cullin ring ubiquitin ligase complex (**Table 1** and Supplementary Figure 3A). On the other hand, the significantly enriched pathways in the downregulated genes are those related to extracellular matrix, calcium ion binding, biological adhesion, skeletal system development, and endochondral bone morphogenesis (**Table 1** and Supplementary Figure 3B), many of which are also among previously identified SOX9 downstream targets [19, 30]. These results suggest that JAB1 downstream target genes are mainly involved in cell cycle regulation and skeletal development in CS cells. To further interpret the *JAB1*-KD CS RNA-sequencing results, we used the standard bioinformatics tool, Database for Annotation, Visualization, and Integrated Discovery (DAVID) [37], to identify the most significantly altered Gene Ontology (GO) terms regarding molecular functions, cellular components, and biological processes (**Figure 5B** and **5C**). Interestingly, for the significantly upregulated genes, a large number of genes were involved in transcription and protein binding, and mainly localized in the nucleus (**Figure 5B**). On the other hand, for the significantly downregulated genes, a large number of genes were involved in cell adhesion and extracellular matrix, and mainly localized in the extracellular matrix and plasma membrane (**Figure 5C**). Interestingly, a recent network analysis has identified hypoxia-inducible factor 2 alpha (HIF-2 alpha, encoded by *EPAS1*) as an upstream regulator that governs the chondrosarcoma malignance network [38]. To explore the possible link between JAB1 and the hypoxia pathway, we analyzed the expression of major hypoxia-related gene markers such as *EPAS1*, *LOXL1* and *RABAC1* upon *JAB1*-KD in SW1353 cells. Interestingly, the expression levels of these hypoxia genes were all significantly downregulated in SW1353 cells upon *JAB1*-KD compared with the non-target control (NTC) cells (**Figure 5D**).

Discussion

Our lab previously showed that the Jab1 was required for the successive stages of skeletal development *in vivo* [39-42]. We also demonstrated that Jab1 promotes osteosarcoma progression in mice via its inhibitory effect on p53 [43]. In the present study, we aimed to investigate the function of JAB1 in human CS pathogenesis. Our results are consistent with many previous *in vitro* studies, which showed that the knockdown of *JAB1* results in decreased oncogenic properties in human gastric [44], pancreatic [45], nasopharyngeal [46], and colorectal [47] cancer cell lines. Additionally, some recent xenograft studies demonstrated that *JAB1*-silencing can suppress the *in vivo* tumor growth in mice [26, 48, 49].

In this study, we first confirmed that *JAB1-KD* reduced the oncogenic properties of CS cells, with increased apoptosis, an enhanced sensitivity to the chemotherapeutic agent etoposide, and altered cell cycle progression. Interestingly, our IP data demonstrated that JAB1 forms a complex with SOX9 in both human and rat CS cells, but not in mouse Jab1-knockout primary chondrocytes (**Figure 3C**). SOX9 has been well studied as a master transcriptional factor regulating cartilage development [29], and is also a known oncogene [50]. Interestingly, our ChIP results confirmed JAB1 binding to the same SOX9 binding sites in the cartilage-specific enhancer regions of *SOX9, AGGRECAN and Col2a1* genes (**Figure 3B**). Together, these results strongly suggest that JAB1 drives CS tumorigenesis at least in part by promoting SOX9 activity.

In this study, we also tested the effect of CSN5i-3 and MLN4924, two small molecule inhibitors that specifically target CRL homeostasis via the neddylation pathway (**Figure 2E**), in human CS cells. Finally, we demonstrate that JAB1 regulates a large oncogenic transcriptome in CS, and might represent an oncogenic hub to promote CS pathogenesis. Interestingly, we noticed that JAB1 downregulation via its knockdown or treatment with the JAB1-specific inhibitor CSN5i-3 in human CS cells both caused a significant reduction in cell proliferation and viability (**Figure 1** and Supplementary Figure 1). These results are consistent with a recent study which showed that CSN5i-3 induces apoptosis and suppresses the growth of breast cancer cells *in vitro*,

and suppresses tumor growth *in vivo* [48]. Thus, it is possible that CS and other cancers are addicted to the oncogenic function of JAB1. Mechanistically, JAB1 might regulate CS by controlling proliferation (**Figure 1**), cell cycle (**Figures 3A** and **4B**), apoptosis (**Figure 4A** and **4B**), and the stability of specific F-box proteins such as FBXO22 (**Figure 2C**, **2D**).

Interestingly, our *JAB1* knockdown in human CS cells led to an enhanced response to the standard chemotherapeutic drug etoposide (**Figure 4A**). These results support recent findings that silencing *JAB1* expression sensitizes cancer cells to cisplatin treatment in biliary tract cancer, nasopharyngeal carcinoma, and breast cancer [51, 52]. Therefore JAB1 is not only likely to be vital for cancer cell survival, its silencing might also sensitize cancer cells to chemo treatment [51, 53]. Thus, ours and other groups' studies support the notion that *JAB1*-silencing or inhibition is an effective approach to treat human chondrosarcoma and many other cancers. Moreover, our RNA-sequencing results demonstrate that *JAB1* regulates a large oncogenic transcriptome in CS. The GSEA and DAVID analysis of our RNA-sequencing results revealed that JAB1 regulates key oncogenic pathways in human CS cells, including chromosomal alterations, cell cycle, DNA damage response, and CRL-ubiquitin ligase pathways (**Table 1**; **Figure 5**). Our recently published microarray analysis showed that JAB1 also regulates multiple key pathways, including cell cycle control, DNA replication, p53, BMP/TGF-β, TNF, MAPK, and steroid hormone biosynthesis pathways in prostate cancer cells [54]. Overall our present study, along, with others', suggests that JAB1 might control a unique set of downstream targets in each type of human cancer types to promote tumorigenesis.

In summary, this study demonstrates that JAB1 specifically interacts with another potent oncogene, SOX9, to drive the oncogenesis of human CS formation. CSN5i-3, a novel, specific and potent small molecule inhibitor of JAB1, might be a promising drug for the treatment of human CS.

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Disclosure of conflict of interest

None.

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Supplementary Materials and Methods

Materials and methods

RNA isolation, cDNA synthesis and real-time RT-PCR

Total RNA was extracted from SW1353 and Hs819.T using the TRIzol reagent (Invitrogen) and the PureLink RNA Mini kit (Invitrogen) according to the manufacturer's protocol. 1 μg of total RNA was reverse transcribed to cDNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The ABI 7500 real-time PCR System (Applied Biosystems) was used to perform real-time qPCR analysis with the Power SYBR Green Master Mix (Applied Biosystems) and gene-specific primers. Gene expression was quantification using the comparative threshold cycle ($\Delta\Delta C_t$) method as described [1]. *GAPDH* was used as an internal control.

RNA-sequencing analysis

RNA sequencing libraries were prepared using the TruSeq Stranded Total RNA with Ribo-Zero Gold kit (Illumina, San Diego, CA, USA). This kit allows the generation of strand-specific data while simultaneously removing both cytoplasmic and mitochondrial rRNA. Libraries were sequenced on the HiSeq 2500 instruments and 50 million 50-base pair single end reads were produced per sample. Data was extracted, de-convoluted based on dual index reads and analyzed using CLC Genomics Workbench software (Qiagen, Venlo, Netherlands). Sequencing reads generated from the Illumina platform were assessed for quality using FastQC (Babraham Institute, Babraham, UK). The reads were then trimmed for adapter sequences using TrimGalore (Babraham Institute). Reads that passed quality control were then aligned to the human reference genome (hg38) using STAR aligner. The alignment for the sequences was guided using the Gencode gene annotation for human version 25. The STAR aligned reads were then analyzed for differential gene expression using cufflinks, a RNASeq analysis package which reports the fragments per kilobase of exon per million fragments mapped (FPKM) for each gene. Differential genes were identified using a significance cutoff of FDR<0.05. These genes were then subjected to gene set enrichment analysis using the MSigDB from the Broad Institute to determine any relevant processes that may be differentially over-represented for the conditions tested. For GSEA, FDR Q-value <0.25 is significant.

Western analysis

Total protein was extracted from SW1353, Hs819.T, C20A4, and mouse primary chondrocytes. Western blot analysis was performed as described [2]. Antibodies and their dilutions used in this study can be found in Supplementary Table 2.

Co-immunoprecipitation

SW1353 and Rat chondrosarcoma (RCS) cell pellets were lysed, followed by immunoprecipitation as described [3]. Antibodies and their dilutions used in this study can be found in Supplementary Table 2.

Caspase 3/7 glow assay

Human CS cells were seeded in 24-well plates at a density of 25,000 cells per well. After 24 hrs, cells were treated with 500 mM etoposide (Sigma, St Louis, MO). After another 24 hrs, apoptosis was measured using the Caspase-Glo 3/7 assay as described [2].

Cell cycle analysis with propidium iodide (PI)

JAB1-KD SW1353 cells, JAB1-KD Hs819.T cells, and SW1353, Hs819.T cells treated with either CSN5i-3 or MLN4924, were subjected to cell cycle analysis as described [4], using FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) at the Case Flow Cytometry Core Facility.

Cignal reporter assay

The Cignal Reporter Assay (Qiagen# CCA-901L-12) was performed according to the manufacturer's protocol. Briefly, CSN5i-3-treated and control SW1353 cells were reverse transfected in 96-well plates. The reporter activity was measured 48 hours after transfection, using the Dual Luciferase Assay (Promega) according to the manufacturer's protocol. A detailed list of the pathways and the targeted transcription factors in the Cignal Reporter Assay is provided in Supplementary Table 3.

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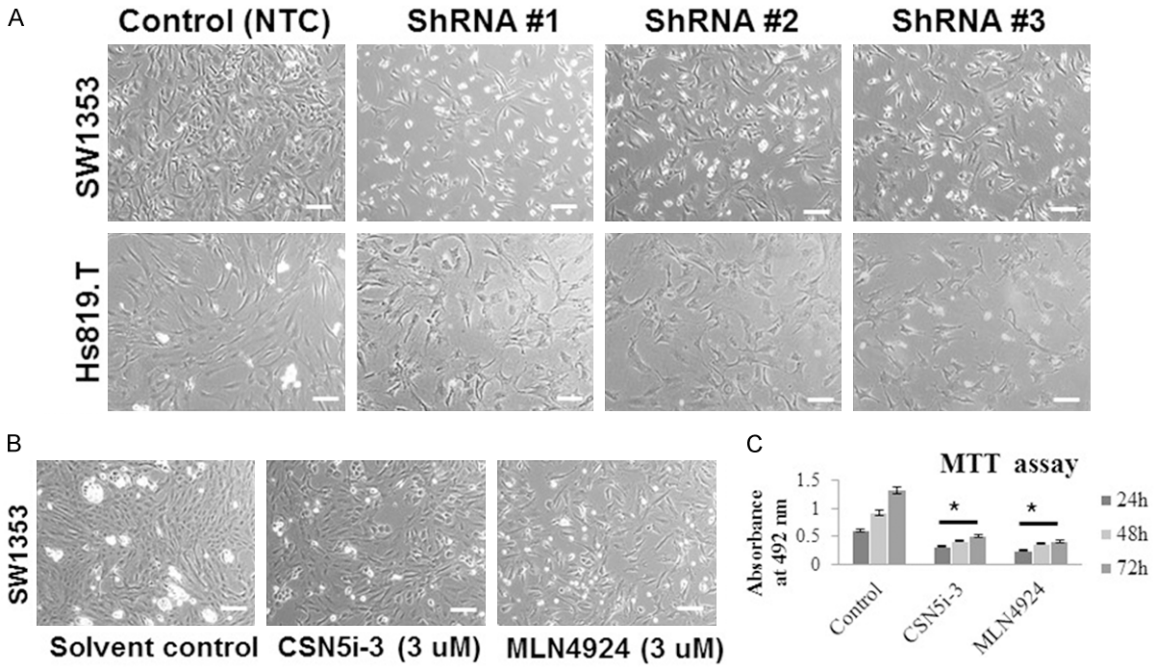
Supplementary Table 1. JAB1 targeting shRNAs along and the non-target control shRNA

shRNA	Sigma catalog#
Non-target control shRNA	SHC002V
shRNAs #1	TRCN0000019201
shRNAs #2	TRCN0000343836
shRNAs #3	TRCN0000343837



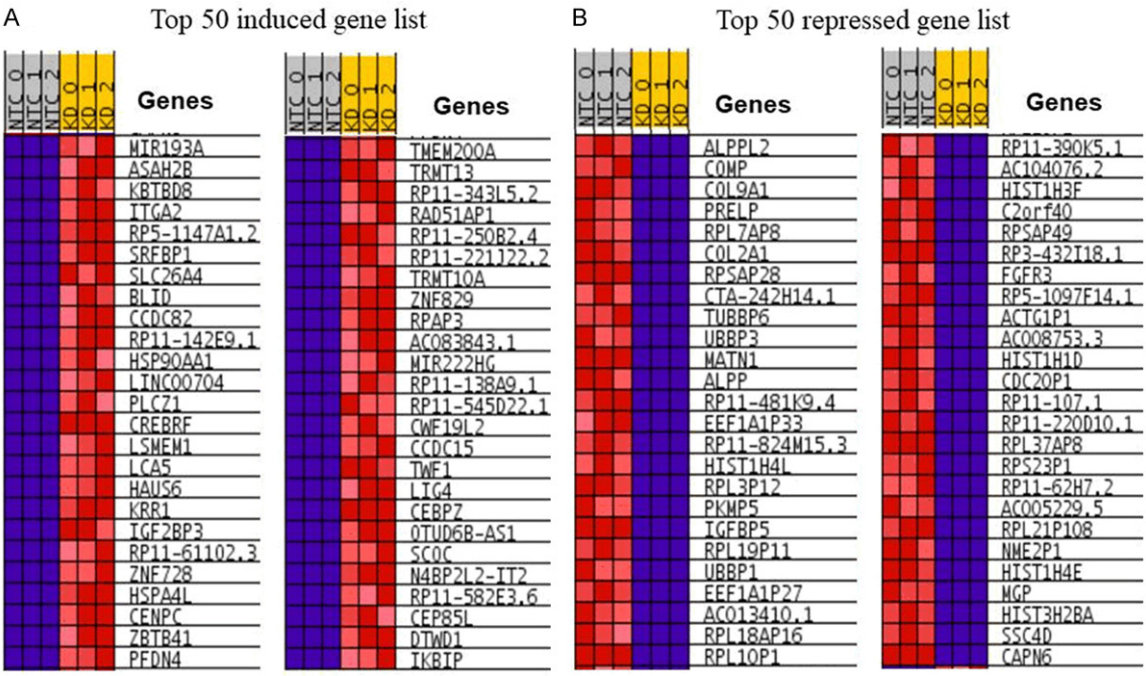
Supplementary Figure 1. The effect of shRNA-JAB1, CSN5i-3 and MLN4924 on human chondrosarcoma cells. A. The morphology of two human chondrosarcoma cells SW1353 and Hs819.T, upon *JAB1*-KD, compared with non-target controls. B. The morphology of SW1353 upon treatment with CSN5i-3 and MLN4924. C. The cell proliferation was measured with the MTT assay in, SW1353, treated with CSN5i-3 or MLN4924. NTC: non-target control. Error bars represent means ± SD. $^{*}P<0.05$ when compared with controls. All controls in these experiments are a scrambled non-target shRNA.

Supplementary Table 2. Details of the antibodies used in this study

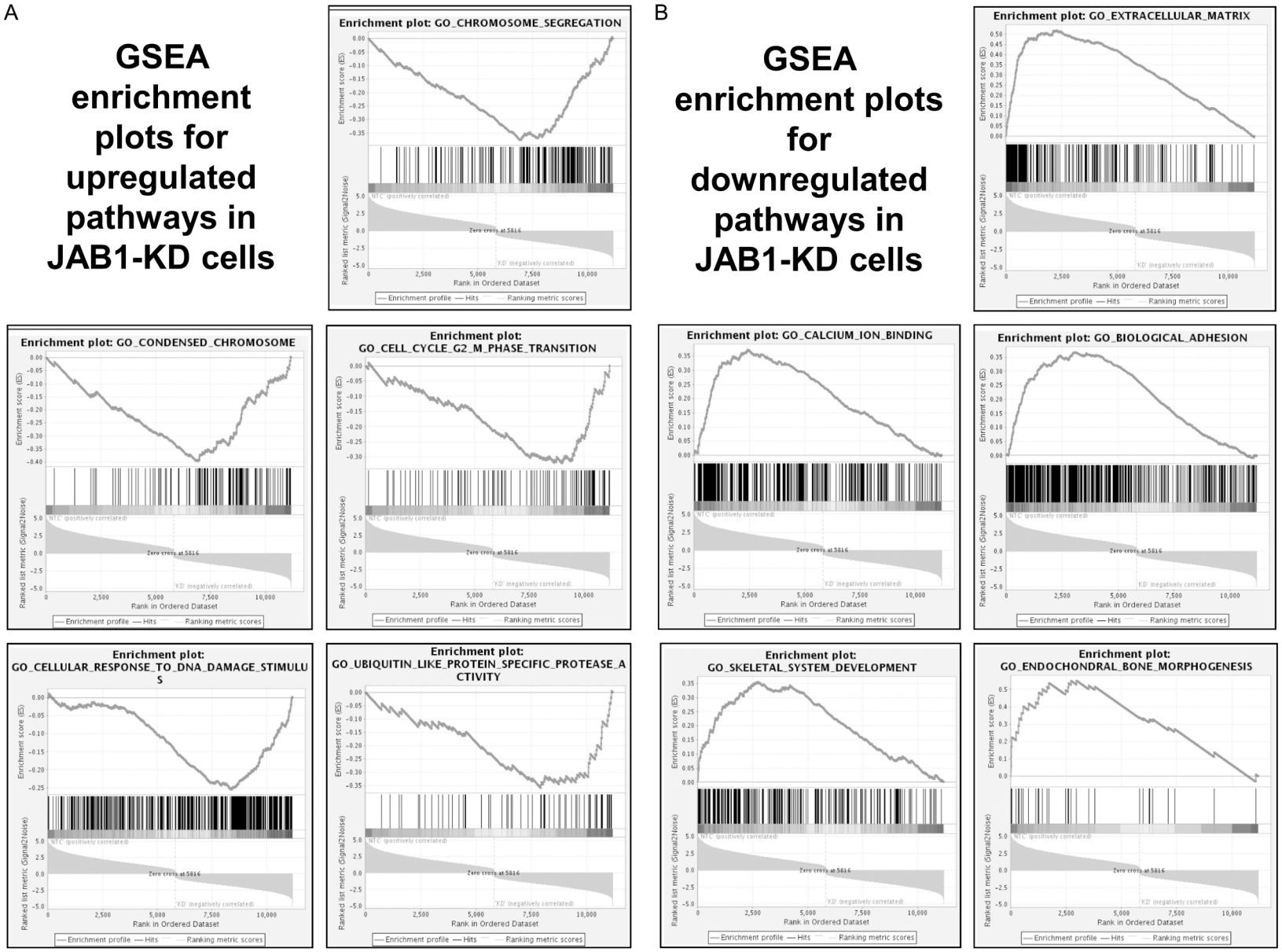
Antibody	Company	Dilution	Catalog#
γ-Tubulin	Sigma	1:1000	T6557
JAB1	Santa Cruz	1:500	Sc-9074x
CUL1	Eiptomics	1:200	2432-1
FBXO22	GeneTex	1:200	GTX117774
SOX9	Millipore Sigma	1:200	AB5535

Supplementary Table 3. List of pathways and the targeted transcription factors used in the cignal reporter assay

Position	Abbreviation	Pathway	Transcription Factor
1	AARE	Amino Acid Deprivation	ATF2/3/4
2	Androgen	Androgen	AR
3	ARE	Antioxidant Response	Nrf2/Nrf1
4	ATF6	ATF6	ATF6
5	C/EBP	C/EBP	C/EBP
6	CRE	cAMP/PKA	CREB
7	Cell cycle	Cell cycle	E2F
8	DNA damage	DNA damage	p53
9	EGR1	EGR1	EGR1
10	ERSE	ER Stress	CBF/NF-Y/YY1
11	Estrogen	Estrogen	ER
12	GATA	GATA	GATA
13	Glucocorticoid	Glucocorticoid	GR
14	HSR	Heat Shock Response	HSF-1
15	MTF1	Heavy Metal Stress	MTF-1
16	GLI	Hedgehog	GLI
17	HNF4	HNF4	HNF4
18	HIF	Hypoxia	HIF-1α
19	Interferon regulator	Interferon regulator	IRF1
20	ISRE	Type I Interferon	STAT1/STAT2
21	GAS	Interferon Gamma	STAT1
22	KLF4	KLF4	KLF4
23	LXR	Liver X	LXR
24	SRE	MAPK/ERK	SRF/Elk-1
25	MAPK/Jnk	MAPK/Jnk	AP-1
26	MEF2	MEF2	MEF2
27	Myc	Myc	c-Myc
28	Nanog	Nanog	Nanog
29	Notach	Notch	RBP-Jk
30	NFκB	NFκB	NFκB
31	Oct4	Oct4	Oct4
32	Pax6	Pax6	Pax6
33	FOXO	PI3K/Akt	FOXO
34	NFAT	PKC/Ca^{++}	NFAT
35	PPAR	PPAR	PPAR
36	Progesterone	Progesterone	PR
37	Retinoic acid	Retinoic acid	RAR
38	Retinoid X	Retinoid X	RXR
39	Sox2	Sox2	Sox2
40	SP1	SP1	SP1
41	STAT3	STAT3	STAT3
42	SMAD	TGFβ	SMAD2/3/4
43	Vitamin D	Vitamin D	VDR
44	Wnt	Wnt	TCF/LEF
45	XRE	Xenobiotic	AhR
46	NC	Negative Control	
47	PC	Positive Control	



Supplementary Figure 2. Top 50 genes that were significantly altered in SW1353 cells upon *JAB1*-KD.



Supplementary Figure 3. GSEA analysis of *JAB1*-KD human chondrosarcoma. A. GSEA enrichment plots for upregulated pathways in *JAB1*-KD cells for: chromosome segregation, condensed chromosome, cell cycle G2/M phase transition, cellular response to DNA damage stimulus, and cullin ring ubiquitin ligase complex respectively. B. GSEA enrichment plots for downregulated pathways in *JAB1*-KD cells for: extracellular matrix, calcium ion binding, biological adhesion, skeletal system development, and endochondral bone morphogenesis respectively.