

# Proteomic Analysis of Connexin 43 Reveals Novel Interactors Related to Osteoarthritis\*<sup>§</sup>

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We have previously reported that articular chondrocytes in tissue contain long cytoplasmic arms that physically connect two distant cells. Cell-to-cell communication occurs through connexin channels termed Gap Junction (GJ) channels, which achieve direct cellular communication by allowing the intercellular exchange of ions, small RNAs, nutrients, and second messengers. The Cx43 protein is overexpressed in several human diseases and inflammation processes and in articular cartilage from patients with osteoarthritis (OA). An increase in the level of Cx43 is known to alter gene expression, cell signaling, growth, and cell proliferation. The interaction of proteins with the C-terminal tail of connexin 43 (Cx43) directly modulates GJ-dependent and -independent functions. Here, we describe the isolation of Cx43 complexes using mild extraction conditions and immunoaffinity purification. Cx43 complexes were extracted from human primary articular chondrocytes isolated from healthy donors and patients with OA. The proteomic content of the native complexes was determined using LC-MS/MS, and protein associations with Cx43 were validated using Western blot and immunolocalization experiments. We identified >100 Cx43-associated proteins including previously uncharacterized proteins related to nucleolar functions, RNA transport, and translation. We also identified several proteins

involved in human diseases, cartilage structure, and OA as novel functional Cx43 interactors, which emphasized the importance of Cx43 in the normal physiology and structural and functional integrity of chondrocytes and articular cartilage. Gene Ontology (GO) terms of the proteins identified in the OA samples showed an enrichment of Cx43-interactors related to cell adhesion, calmodulin binding, the nucleolus, and the cytoskeleton in OA samples compared with healthy samples. However, the mitochondrial proteins SOD2 and ATP5J2 were identified only in samples from healthy donors. The identification of Cx43 interactors will provide clues to the functions of Cx43 in human cells and its roles in the development of several diseases, including OA. *Molecular & Cellular Proteomics* 14: 10.1074/mcp.M115.050211, 1831–1845, 2015.

Direct intercellular communication is accomplished in nearly all tissues and organs by aqueous, low-resistance pores located in the lipid bilayer of the contacted cells. These pores, named gap junctions (GJ)<sup>1</sup>, are composed of connexins (Cxs) and play critical developmental and functional roles (1, 2). Numerous processes, such as the diffusion of metabolites, nutrients, small RNAs and second messengers, and the rapid transmission of action potentials in heart or neuronal tissue via so-called electrical synapses, are driven by GJ communication (3–7). The junctional channel is composed of two end-to-end hemichannels, each of which is a hexamer of six subunits of Cxs that dock with each other to form a contiguous gap junctional channel. Cxs proteins have a common topology that includes four  $\alpha$ -helical transmembrane domains, two extracellular loops, a cytoplasmic loop, and N- and C termini located on the cytoplasmic membrane face. Cx43 (the 43-kDa isoform) is the most widely expressed GJ protein in different cell types (8). Yet, as many

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Author contributions: RG-F designed and performed the experiments and performed data analyses. PC-F performed the experiments. PF-P and JM performed the mass-spec runs and processed the data. DM performed immunofluorescent analysis. BA and EF provided critical input. FJB participated in design of experiments. MDM conceived the study, designed and performed the experiments (IHC analysis), designed and performed the data and bioinformatic analyses (GO, comparisons with other studies) and wrote the paper. All authors discussed the results and commented on the manuscript.

<sup>1</sup> The abbreviations used are: GJ, gap junction; ACN, acetonitrile; Co-IP, co-immunoprecipitation; CTD, carboxy-terminal domain; Cx43, connexin 43; DTT, dithiothreitol; ECM, extracellular Matrix; GJIC, gap junctional intercellular communication; GO, Gene Ontology; IAA, iodoacetamide; IHC, immunohistochemistry; IP, immunoprecipitation; OA, osteoarthritis; TFA, trifluoroacetic acid.

as 20 murine and 21 human Cx isoforms have been identified (9).

Cx43 has a relatively large carboxy-terminal domain (CTD) that takes part in multiple proteomic interactions. Increasing evidence indicates that gap-junctional Cx43 is part of a multiprotein complex and that Cx43-interacting proteins are thought to form a dynamic scaffolding protein complex, termed the Nexus, that may function as a platform to localize signaling, structural, and cytoskeletal proteins (10, 11). Indeed many aspects of Cx43 function, for example cellular transport, plaque assembly and stability, and channel conductivity are most likely governed by interactions with regulatory and structural proteins that bind to the cytoplasmic domains of Cx43. These proteins include the tight junction protein zonula occludens-1 (ZO-1) (12–14), 14–3-3 (15), Drebrin (16),  $\beta$ -tubulin (17), c-Src, v-Src (10), and other potential Cx43-interacting proteins that target Cx43 to points of cell-cell contact and regulate gap junctional intercellular communication (GJIC) (10).

Initially, the cellular functions of Cxs were attributed exclusively to direct cell-to-cell diffusion; however, some Cx functions seem to be independent of channel function (18). In fact, several reports have suggested that the Cx43 complex might fulfil functions that are not necessarily related to the control of GJIC. For instance, Cx43 mutants lacking the C-terminal tail that were expressed in transformed cells restored the GJIC but inhibited proliferation (19). Moreover, a Cx43 mutant that does not form GJ has been shown to suppress cell growth (20), and the expression of the C-terminal tail alone was sufficient to reduce proliferation (19, 21). On the other hand, the overexpressed C-terminal tail of Cx43 localized to the nucleus and inhibited cell growth (21, 22). These and other studies raise the possibility that the C-terminal tail of Cx43 can control cell-cycle, gene expression, or different signaling pathways via binding proteins independently of its channel function (18).

The channel-independent effects of Cxs might be explained by the (dys)function of Cx-tail interactions proteins. Our group is interested in investigating the physiopathological mechanism that is associated with osteoarthritis (OA). Recent results suggest a direct role of Cx43 in the development of OA that is not necessary related to its channel function (7, 23, 24). For instance, only a few connexin-interacting proteins have been described to date, and previous studies have used artificial systems, such as yeast two-hybrid screens or Cx43-domain fusion proteins. The aim of this study was to identify Cx43-interacting proteins in primary articular chondrocytes from human adults. In this report, lysates of primary articular chondrocytes were subjected to immunoprecipitation (IP) with an antibody directed against the C-terminal tail of Cx43. Cx43 interactors were identified using liquid chromatography and mass spectrometry (MALDI-TOF/TOF). We have identified >100 Cx43 associated proteins. Among these, we have found out novel functional Cx43 interactors involved in human dis-

ease and OA development, which emphasizes the potential importance of Cx43-interactions in normal development and physiology.

#### EXPERIMENTAL PROCEDURES

**Cartilage Collection, Processing, and Primary Culture of Chondrocytes**—Human knee and femoral head articular cartilage samples were generously obtained from adult donors undergoing joint surgery. All donors signed an informed consent form, and the institutional Ethics Committee approved the study (Registration Code Galicia CAEIG: 2012/094 - PI13/00591). *In situ* cartilage samples were frozen immediately in Cryomold® Standard using Tissue-Tek® O.C.T.™ compound and isopentane in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Cartilage from healthy persons was obtained *in situ* from individuals who suffered a knee or hip fracture with no history of joint disease. Healthy samples were assigned on based on their medical record data and histological study. Histological samples (healthy and OA with radiologic diagnosis) were graded using the modified Mankin score (23) (mean age: 71.38 years, range: 63 to 89 years; Mankin score: 0–13 points). The samples (OA patients and age-matched healthy subjects) were divided into four groups: normal/healthy (score = 0–1); early OA (score = 2–3), named grade I; mild OA (score = 4–5), named grade II; moderate OA (score = 6–7), named grade III; and severe OA, named grade IV (score = 8–13). To perform this study, we selected samples from the normal/healthy, early OA and moderate grade III groups. For the isolation and culture of primary chondrocytes, a fresh cartilage was rinsed with saline, and cells were isolated as previously reported (23). The cells (2.5 million) were then seeded into 162-cm<sup>2</sup> flasks and incubated at  $37^{\circ}\text{C}$  in 5% CO<sub>2</sub> and air in DMEM supplemented with 100  $\mu\text{g}/\text{ml}$  Primocin (*In vivo* Gen Primocin™) and 15% FCS until  $\sim 80$ – $90\%$  confluence was reached. The chondrocytes were used in experiments during the third or fourth weeks of primary culture.

**Co-Immunoprecipitation**—Co-immunoprecipitation (Co-IP) was performed using primary chondrocytes from human adults as described previously with minor modifications (7). Cells were resuspended in 1000  $\mu\text{l}$  of IP-lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8, 0.5% v/v Nonidet P-40, 1.0% v/v Triton-X, 150 mM NaCl) or in IP-lysis buffer containing Sarkosyl and SDS (50 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8, 0.5% v/v Nonidet P-40, 0.5% v/v Sarkosyl, 0.1% SDS v/v, 150 mM NaCl). Both IP buffers contained protease inhibitors (Sigma-Aldrich) and 1 mM PMSF. Lysates were precleared by incubating with 50  $\mu\text{l}$  of Protein G Sepharose (50% slurry). Four micrograms of antiCTD-Cx43 antibody (SC-20, sc-6560 from Santa Cruz Biotechnology, Heidelberg, Germany) followed by Protein G Sepharose beads (50  $\mu\text{l}$  of 50% slurry) were added to the precleared lysates and incubated overnight at  $4^{\circ}\text{C}$ . A control IP was performed without antibody. The beads were washed four times with the corresponding IP buffer, and the bead pellets were suspended in loading buffer (10% SDS, 0.2 M Tris pH 6.8, 50% glycerol, 0.1% (w/v) bromophenol blue (5x) containing 1.42 M 2-mercaptoethanol) and incubated for 30 min at  $37^{\circ}\text{C}$  before being boiled for 10 min. Supernatants were stored at  $-80^{\circ}\text{C}$ . Silver stain analysis of immunoprecipitations and Western blotting were used to determine the specificity of the interactions. Silver staining was performed after the electrophoretic separation on polyacrylamide gels. The gels were fixed for 30 min in fixed solution (40% ethanol, 10% acid acetic) and rinsed twice for 10 min in water. After incubating with 0.02% thiosulfate sodium for 1 min, gels were immediately rinsing in water for another min, incubated with silver solution for 30 min (0.2% silver nitrate, 0.075% formalin) and washed for 10–15 s with water. The develop solution contained 3% carbonate potassium, 12.5 mg/ml thiosulfate sodium and 0.025% formalin. The development was stopped by dipping the gel in the stop solution (3% Tris-Buffer, 10%

acid acetic) for 15 min. The stained gels were scanned after thoroughly rinsing in water.

For Western blot assays, anti- $\alpha$ -tubulin antibody (T9026) was supplied from Sigma-Aldrich, Schnellendorf, Germany antinucleolin antibody (PAB12541) was from Abnova, Heidelberg, Germany GmbH, the antiranGTP (sc-1156), antiCx43 (sc-6560), antiCalcyclin (sc-271396), and anti-Integrin  $\alpha$ V (sc-376156) antibodies were from Santa Cruz Biotechnology, antiAlpha B Crystallin (ab13496) from Abcam. For SDS-PAGE gel electrophoresis, 5  $\mu$ l of the denatured, immunoprecipitated proteins were loaded and separated on 10% polyacrylamide gels, which were then electro blotted onto polyvinylidene fluoride (PVDF) membrane (Millipore Co., Bedford, MA). The membranes were blocked with 5% milk in PBS and 0.05% Tween-20 (Sigma-Aldrich) and, after probing with antibody, developed using ECL Western blotting Detection reagent from GE Healthcare.

**Identification of Proteins Using SDS-PAGE and nanoLC-MALDI-TOF/TOF**—Immunoproteins were loaded and separated by SDS-PAGE in 10% acrylamide in-house gels in a Protean® mini-gel system (Bio-Rad, Hercules, CA). Gels were stained and visualized with Coomassie Brilliant blue G250 and the resulting lane was size-fractionated into three sections that were subsequently processed independently. Each section was destained with methanol, diced into small pieces and in-gel digested following a standard procedure (25). Briefly, the samples were desiccated with acetonitrile (26) reduced with 10 mM dithiothreitol (DTT) for 30 min at 56 °C, alkylated with iodoacetamide (50 mM IAA), dehydrated in 100% ACN and trypsin-digested (6.6 ng/ $\mu$ l, Roche Mannheim, Germany) for 16 h at 37 °C. Peptides were then extracted from the gel pieces with 50% ACN/0.1% trifluoroacetic acid (TFA), dried in a speed-vacuum, reconstituted in 0.1% TFA and desalted using homemade stage tips (Empore disk-C18, 47 mm, 3 M O Agilent Technologies, Santa Clara, CA). The peptide fractions were separated using reversed phase chromatography in a Tempo nanoLC system (ABSciex, Madrid, Spain). After precolumn desalting on a trapping column (0.5  $\times$  2 mm, Michrom Bioresources, Auburn, CA) at a flow rate of 15  $\mu$ l/min for 15 min, tryptic digests were separated on a C18 nanocolumn (Integragit C18, Proteopep II, 75  $\mu$ m id, 15 cm, 5  $\mu$ m, 300 Å; New Objective, Woburn, MA) at a flow rate 350 nl/min, with a 30 min linear gradient of 5 to 50% of buffer B (80% ACN containing 0.1% TFA). The column was washed and regenerated for 5 min using 80% of buffer B. After all nonpolar and nonpeptide materials were removed, the column was re-equilibrated under the initial starting conditions for 15 min. The separated peptides were spotted onto a MALDI plate each 15 s and mixed with  $\alpha$ -cyano-4-hydroxycinnamic matrix (3 mg/ml in 0.1% TFA, 70% ACN) at a flow rate of 1.2  $\mu$ l/min using a Sun Collect MALDI Spotter/Micro-Fraction Collector (SunChrom Wissenschaftliche Geräte GmbH, Germany). The MS runs for each chromatogram were acquired and analyzed in a MALDI-TOF/TOF instrument (4800 ABSciex, Framingham, MA). MS spectra from  $m/z$  800–4000 were acquired for each fraction and processed with internal calibration. Angiotensin peptide diluted in the matrix (3 fmol/spot) was used for the internal standard calibration at an  $m/z$  of 1046.5. After screening of all LC-MALDI sample positions in MS positive reflector mode, the fragmentation of automatically selected precursors was performed at collision energy of 1 kV with CID gas (air). Up to 12 of the most intense ion signals per spot position with signal/noise (S/N) above 80 were selected as precursors for MS/MS acquisition, excluding common trypsin autolysis peaks and matrix ion signals. A second MS/MS was acquired excluding the precursors selected in the previous MS/MS run. Precursors with a S/N above 25–40 were selected to identify proteins that were not identified in the previous run. For protein identification, MS/MS spectra acquired by 4000 Series Explorer software v.3.5.1 were searched with ProteinPilot v.3.0 (ABSciex) against the Uniprot-SwissProt protein database (release version 2012\_05; 536,029 entries), using the

Paragon Algorithm. The Paragon Algorithm in ProteinPilot software served as the default search program with trypsin as the digestion agent with one missed cleavage allowed, iodoacetamide as a fixed modification of cysteine and biological modifications. Only proteins with a threshold >95% confidence (>1.3 Unused Score) were considered for protein identification. False positive identification rate is around 5%.

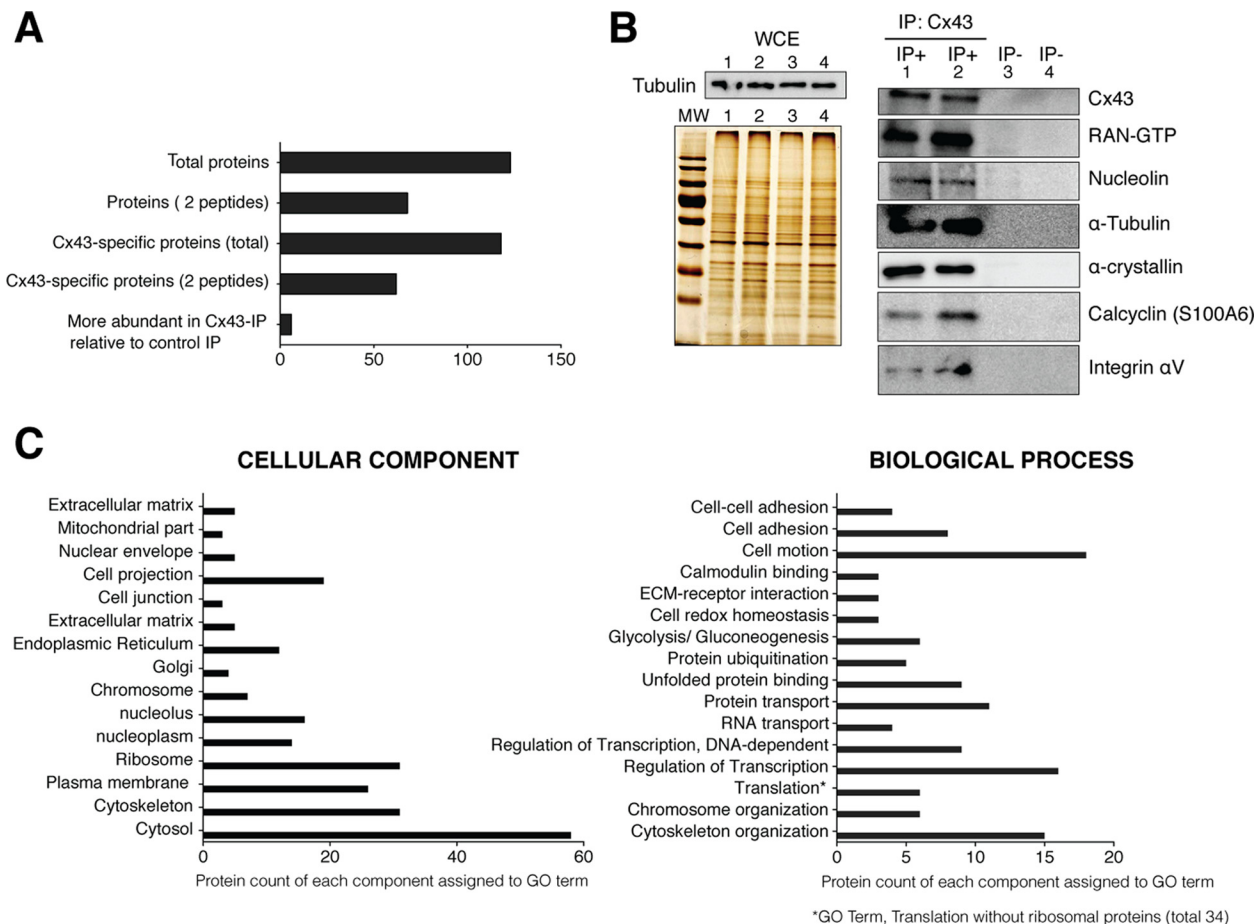
**Gene Ontology (GO) Analysis**—To analyze the MS data and for the representation (protein enrichment) of GO terms, MS data were analyzed using the Protein Information and Knowledge Extractor (PIKE, <http://proteo.cnb.csic.es/pike/>), which links to the DAVID web system to provide a quality measure of the protein relationships regarding GO (27, 28).

**Immunohistochemistry Assays**—For immunohistochemistry (IHC), cultured cells on chamber slides were fixed with 4% formaldehyde for 10 min at room temperature as previously described with minor modifications (23). Cells on chamber slides were counterstained with hematoxylin/eosin or counterstained with 4',6-diamidino-2-phenylindole (DAPI). The antiCx43 antibody (610062) was supplied from BD Transduction Laboratories, antiHSP90 $\beta$  antibody (sc-1057) was from Santa Cruz Biotechnology, and the antivimentin antibody (ab8069) was from Abcam. Negative controls (omitting the primary antibody) were performed to test the specificity of each antibody. For immunofluorescence, cultured cells on chamber slides were fixed with cold methanol for 5 min at –20 °C and permeabilized with 1% Triton-X-100 (v/v) for 5 min at room temperature using the fluorescent-tagged secondary antibodies Polyclonal Rabbit anti-Mouse Immunoglobulins/FITC (F0313, Dako Cytomation), Polyclonal Goat anti-Mouse Immunoglobulins/RPE (R-0480, Dako), and Polyclonal rabbit anti-Goat Immunoglobulins/PE (SC-3755, Santa Cruz Biotechnology). The cells were counterstained with DAPI and mounted in glycerol mounting medium (C0563, DAKO). For IHC, samples were analyzed on an Olympus BX61 microscope using a DP71 digital camera (Olympus, Hamburg, Germany), and calibration and quantification of the images was performed using the AnalySIS<sup>D</sup> 5.0 software (Olympus Biosystems, Hamburg, Germany). Immunofluorescence assays were acquired in a Leica TCS-Sp5 (AOBS) Laser scanning confocal (Leica Microsystems, Wetzlar, Germany) using a 63X HCX PLAPO 1.4 N.A. oil immersion objective and the acquisition software LAS AF 2.6 (Leica Microsystems). Intensity line profile for each channel was performed by using Image J 1.47s (NIH).

## RESULTS

**Mass Spectrometric Identification of Cx43-Associated Proteins**—When Cx43-interacting proteins from chondrocytes of healthy donors were analyzed, 123 proteins were identified, of which 68 proteins were represented by at least two unique peptides (Fig. 1A). One hundred and eighteen proteins were specific to the Cx43 immunoprecipitation (IP) and not identified in the control IP, which was performed without antibody (See supplemental Tables S1 and S2). Among these, 62 proteins were represented by at least two unique peptides. We considered a second category called “more abundant proteins” ( $n = 6$ ) that included proteins such as annexin A2, prelamin-A/C and vimentin. The “more abundant proteins” corresponded to the proteins identified in both, the control IP and Cx43 IP but with more peptides at 95% confidence and with higher coverage and score in the Cx43 IP than in the control IP (supplemental Tables S1 and S2). In all cases, only proteins with >95% confidence and score >1.3 were considered significant.





**FIG. 1. Analysis of Cx43-associated proteins identified using mass-spectrometry.** Primary chondrocytes in culture were lysed in the presence of the nonionic surfactants Igepal and Triton X-100 using a near-physiological-salt concentration of 150 mM NaCl to pull-down the Cx43 (nonjunctional and junctional Cx43). Immunoprecipitated proteins using an antibody that specifically recognized the C-terminal tail of Cx43 were identified using MS. Four different samples of chondrocytes from four healthy donors were analyzed. Overall, proteins were identified with 95% peptide confidence (minimum value 1.3 of unused cutoff) *A*, Summary of the number of identified proteins. Proteins identified as specific to the Cx43-IP but absent from the control IP (no antibody). The “more abundant proteins” in Cx43-IP relative to control IP correspond to the number of proteins identified as most likely more abundant in the Cx43-IP than in the control-IP (peptide difference  $\geq 2$ ). See [supplemental Tables S1 and S2](#) for a complete list of proteins identified in the Cx43-IP and control IP. *B*, Whole-cell extracts (WCE) and proteins immunoprecipitated with (IP+) or without (IP-) an antiCTD-Cx43 antibody were analyzed by silver staining and Western blotting. The protein content of the WCE (input, without antibody) (images shown on the left) for each experiment (IP) (images shown on the right) was previously analyzed by silver staining and Western blotting (Tubulin) (image shown on the top-left). Lanes represent 0.5% of the total WCE used for each IP. The numbers (1–4) represent the WCE utilized for the corresponding experiment (IP). For the images shown on the right, lanes represent the 25% of each IP *C*, Gene Ontology clustering of the protein set. GO analysis (27, 28) for identified proteins reveals a strong enrichment for GO terms associated with cytoskeleton, nuclear localization, translation processes, cell adhesion, and motion and carbohydrate metabolism. See [supplemental Tables S1 and S2](#) for a complete list of the identified proteins.

The Co-IP methodology is the referenced method to investigate interacting proteins or protein complexes. However, this technique has limitations and does not necessarily indicate whether interactions are direct. Besides, many protein–protein interactions are temporary and proteins can be found in different cellular compartment. Other limitations include the nonspecific binding to protein A/G and other contaminants. Despite this, the purification strategies used in this report resulted in a low number of proteins identified in the control IP ([supplemental Table S2](#)). Nevertheless, a selection of possible Cx43-interacting proteins identified in the MS

analysis was further validated by a combination of IP and Western blotting (Fig. 1*B*). Two IP samples using the Cx43 antibody (IP+) and two IP samples performed without antibody (IP-) confirmed the presence of previously identified proteins in the Cx43 IP. Cx43, nucleolin and the GTP-binding nuclear protein Ran among other interactors were detected in Cx43 IP samples, but not in control IPs performed without antibody. The same number of cells was used for each IP, and the whole-cell extracts that were used for IPs were previously resolved by SDS-PAGE and visualized using silver staining (Fig. 1*B*).

TABLE I

Example of Cx43-interacting proteins likely related to Cx trafficking, GJ assembly, GJIC, and turnover (chondrocytes from healthy donors). The cytoskeletal proteins that interact or colocalize with Cxs have a critical functional importance in Cx trafficking and assembly of Cx into GJs. Microfilaments and microtubules are involved in turnover mechanisms that compromise the control of GJIC. The values of peptide probability or score, sequence coverage and number of peptides found are shown in [supplementary Fig. S1](#). Only proteins that show >95% confidence and score >1.3 were considered for this study

Protein Group\Name	Gene name
$\alpha$ - <sup>a</sup> and $\beta$ -tubulin <sup>ab</sup>	TUBA1B\TUBB1\TUBB2A\ TUBB2C
$\alpha$ -actin <sup>a</sup> and actin related proteins	ACTB\ACTR3B\ACTN4
Translocon-associated protein subunit delta	SSR4
Myosin motor proteins <sup>a</sup> (Myosin IC <sup>b</sup> )	MYL12B\MYH9\MYO1B
Vimentin <sup>a</sup>	VIM
Vinculin <sup>ab</sup>	VCL
Talin-1 <sup>b</sup>	TLN1
Filamin A, alpha	FLNA
Dihydropyrimidinase-related protein 2	DPYSL2
SEC22 vesicle trafficking protein homolog B	SEC22B
Gelsolin	GSN
Profilin-1	PFN1
Erzin <sup>ab</sup>	EZR
Radixin <sup>b</sup>	RDX
Moesin <sup>b</sup>	MSN
Annexin A1 <sup>b</sup> \A2 <sup>b</sup> \A5	ANXA1\ANXA2\ANXA5
Caldesmon	CALD1
Endoplasmic	HSP90B1
Tropomyosin $\alpha$ 4	TPM4
Proteasome subunit beta type-2	PSMB2
Ubiquitin-40S ribosomal protein S27a	RPS27A
Cytoskeleton-associated protein 4	CKAP4
Thy-1 membrane glycoprotein <sup>b</sup>	THY1
Protein S100-A6\A11\A10	S100A6\S100A11\S100A10

<sup>a</sup> Previously reported interactions of Cx43.

<sup>b</sup> Constituent part of the plasma membrane.

To explore the functions of the proteins associated with Cx43, we analyzed the GO terms of the specific and more-abundant identified proteins (Fig. 1C). We found significant enrichment for GO processes directly linked with cytoskeleton, ribosome, plasma membrane, nucleus, and cell projection. Cytoskeletal proteins are an important class of contaminant proteins that was identified in a study to bind directly to commonly used immunoprecipitated matrices (30). However, the identified cytoskeletal proteins were enriched in our specific IP, but not in our control IP ([supplemental Table S1](#)). Among the 31 identified cytoskeletal-related proteins, 15 were implicated in cytoskeleton organization (Fig. 1C, Table I and [supplemental Table S1](#)), and some of the identified cytoskeletal proteins are known to interact with Cx43 ( $\alpha$ -/ $\beta$ -tubulin,  $\alpha$ -actin, Ezrin, Myosin, Vimentin, or Vinculin).

The GO term translation (for Biological Process) is caused by the presence of ribosomal proteins and other translation-

related proteins in the Cx43 IP (six proteins) compared with the control IP ([supplemental Table S1](#)). Note that in this case, the 40S and 60S ribosomal proteins have not been counted in the GO term translation (Fig. 1C). MS results revealed several interactors directly linked to nuclear activity, including several histones and nucleolar-related proteins (Table II and [supplemental Table S1](#)). Interestingly, a number of proteins related to metabolic pathways (glycolysis/gluconeogenesis), protein folding and transport, cell adhesion, cell motion, calmodulin binding, and cell-redox homeostasis was also identified to interact with Cx43 (Fig. 1C and Table II).

**Cx43 Nuclear and Perinuclear-Related Interactors**—The MS results suggest that Cx43 interacts with two components of the nuclear translocation system: the GTP-binding nuclear protein Ran (RAN) and importin  $\beta$  (KPNB1) (Table II and Fig. 2A). Additionally, using cNLS Mapper to predict nuclear localization signals (NLSs) specific to the importin pathway, we identified two NLSs sites located on the C-Terminal tail of the Cx43 sequence (Fig. 2B). In fact, the images obtained from immunohistochemistry (IHC) experiments using an antibody that specifically recognizes the C-terminal tail of Cx43 and DAPI nuclear staining showed multiple positive spots for Cx43 in the nucleus (Fig. 2C). Cx43 was localized across the nuclear margin and in nuclear foci (one to six per cell).

The identified nuclear-related proteins with a score < 1.3 are listed in [supplemental Table S1](#) and summarized in Fig. 2A and Table II. Among the transcription-related interactors, such as the TBP-interacting protein CAND1, several nucleolus-related proteins were identified. The MS results suggest that Cx43 interacts with the major nucleolar protein nucleolin and with the transcription release factor PTRF, which is required for rRNA transcription and termination.

**Cx43 Interactors Colocalize with Cx43**—To determine whether the Cx43-associated proteins that were identified using MS also associate with Cx43 *in situ*, we achieved immunofluorescence techniques using antivimentin, antiHSP90B, and monoclonal antiCx43 antibodies to determine if these two candidate proteins colocalize with Cx43 (Fig. 3). Articular chondrocytes in monolayer culture contain high levels of vimentin that is mainly localized in the cytoplasm. Multiple positive spots for Cx43 (green) were found to colocalize with positive staining for vimentin (red) in the cytoplasm, at cell margin (membrane), and in the cellular projections (Fig. 3A). The chaperone HSP90 $\beta$  was also identified by mass spectrometry as a Cx43 interactor, and immunofluorescence assays suggested that this protein colocalized with Cx43 in the cytoplasm and in the plasma membrane (margin of the cells) (Fig. 3B).

**Cx43-Associated Proteins Related to OA**—Analysis of the Cx43-associated proteins that were isolated from chondrocyte samples from early phase OA patients (grade 1, following Mankin score, see material and methods) identified 157 proteins, of which 153 were specific to the Cx43 IP and not identified in the control IP performed without antibody (Fig. 4A, [supplemental Tables S2 and S3](#)). Eleven proteins, which in-

TABLE II

Cx43-interacting proteins (represented as gene name) from chondrocytes of healthy donors using the Cx43 IP-based proteomic approach. The values of peptide probability score, sequence coverage and number of peptides found are shown in [supplementary Fig. S1](#). Only proteins that show >95% confidence and score >1.3 were considered for this study

Group\Complex	Gene name
Nucleosome. Histones	HIST1H1T, HIST1H2AC, H2AFJ, HIST2H2BF, H3F3A, HIST1H4A
Transcription related factors	PTRF, NCL, CAND1, SND1, STAT1, RAN
RNA-binding proteins	MVP*, HNRNPA1*, HNRNPA2*, HNRNPA3, HNRNPK, NLC, RAN*, PTRF
RNA transport*	
RNA splicing	HNRNPA1, HNRNPA2, HNRNPA3, HNRNPK, SNRPD3
Ribosome	RPS16, RPS18, RPS19, RPS24, RPS26, RPS4X, RPS5, RPS7, RPS8, RPS9, RPLP2, RPL11, RPL12, RPL15, RPL17, RPL21, RPL24, RPL26, RPL27A, RPL3, RPL35, RPL35A, RPL36, RPL4, RPL6, RPL7, RPL7A
Translation	EEF2, EEF1AL3, EEF1G, EIF5AL1
Endomembrane system (transport within the cell)	KPNB1#, RAN#, LMNA, MVP#
Nuclear Pore Complex#	
Mitochondrial	SOD2, ATP5J2
Antioxidant activity	PRDX1, SOD2
Generation of precursor metabolites and energy	PKM2, ENO1, GAPDH, LDHA, ALDOA, PYGB, PGK1, SOD2
Protein folding and other-related proteins	CRYAB, HSPA8, HSP90AB1, HSP90B1, PPIB, PPIA, HSPA5, P4HB, PDIA3, HSPB1, SERPINH1

cluded annexin A2, glutathione S-transferase and vimentin, were more highly represented in the Cx43 IP than in the control IP ([supplemental Table S3](#)); 73 proteins were identified as Cx43-interactors in healthy and OA chondrocyte groups ([supplemental Table S3](#), see peptide difference); and 80 interactors, including the 14-3-3 protein, integrin  $\alpha$ -5 and integrin  $\beta$ -1 were exclusively identified in chondrocytes from OA patients ([supplemental Table S3](#), see specific to OA versus healthy).

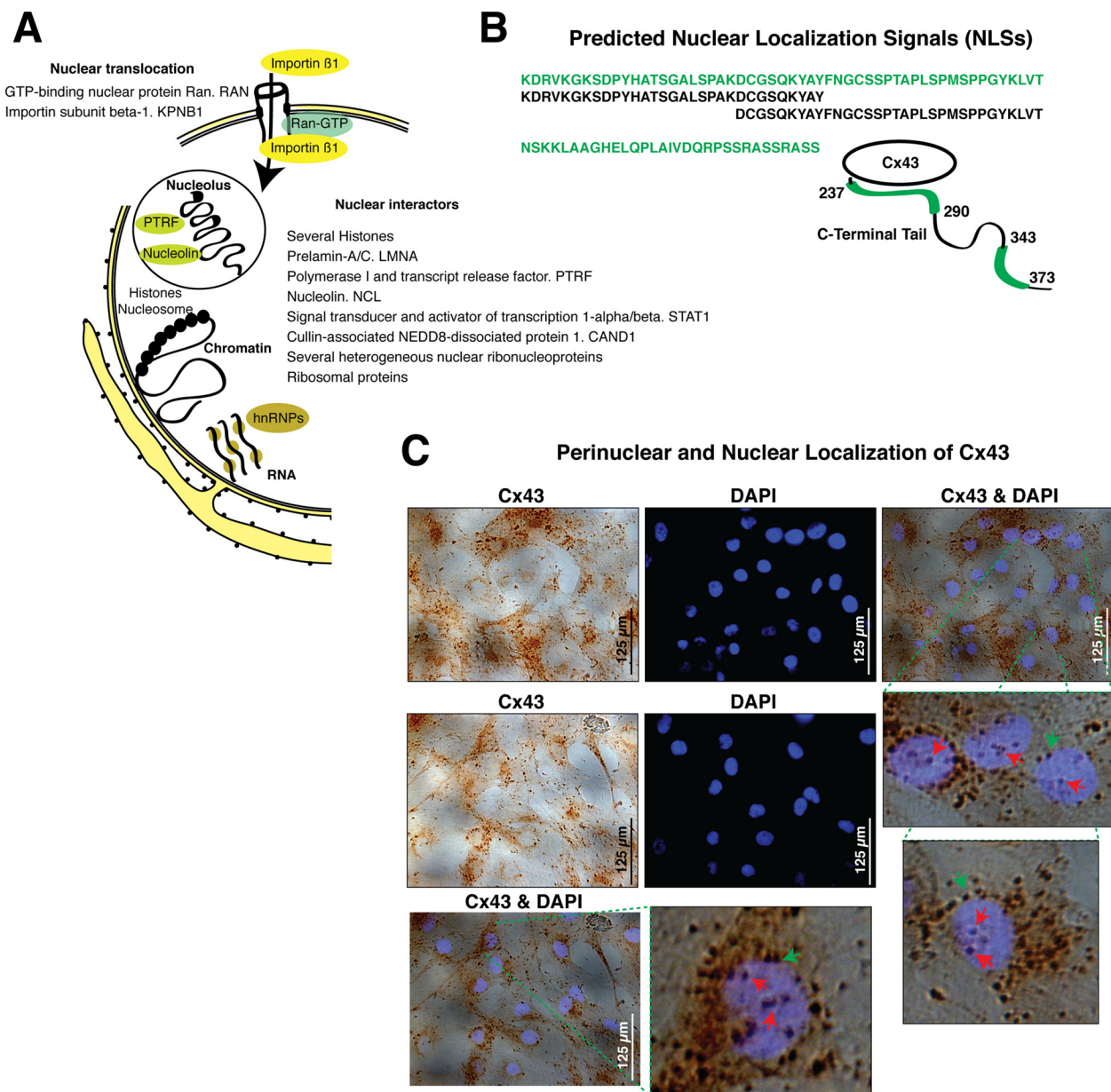
We next analyzed the GO terms of interactors identified in OA samples and compared them to the results of the proteins identified in healthy samples (Fig. 4B, gray bars). The results showed an enrichment of Cx43 interactors related to cell adhesion, extracellular matrix interaction, calmodulin binding, nucleolus, translation and cytoskeleton-related proteins in OA compared with healthy samples. Critical components for cartilage structure and function, such as collagen XII or collagen VI, which exclusively localize to and delimit the pericellular matrix, were only identified in OA samples (Table III and [supplemental Table S3](#)). However, the mitochondrial proteins SOD2 or ATP5J2 were only identified in samples from healthy donors ([supplemental Table S1](#)).

The analysis of four different samples from patients with moderate-stage disease (grade 3), revealed a set of 130 Cx43-interactors that were specific to the Cx43 IP ([supplemental Table S4](#)). Eighty-eight proteins were identified as Cx43-interactors in both groups in the early and moderate stages of the disease; 64 proteins were identified as interactors in the healthy and moderate stage groups; and 32 interactors, including HMG-1 and Zyxin, were exclusively identified in chondrocytes from patients with moderate-phase disease ([supplemental Table 4](#)). The Cx43 interactors that were exclusively found in healthy and OA samples and the common interactors arranged by peptide difference are shown in [supplemental Tables S3 and S4](#) and summarized in

Table IV. The proteins were organized into different groups: exclusive to moderate OA, exclusive to early OA, exclusive to healthy, exclusive to OAs (both groups), specific to moderate OA versus early OA and peptide differences between common interactors found in each group.

The IP and MS methodologies have limitations, and several Cx43-interactors, such as the scaffolding protein zonula occidens-1 (ZO-1) (13, 14) and several phosphatases that are known to interact with Cx43, were not detected in this study. Some of these interactions might depend on the cell cycle stage in which the cells were taken for the IP experiments and the methodology that was used (31, 32). The low expression of tyrosine phosphorylated proteins and other signaling proteins makes these proteins difficult to detect using this methodology. On the other hand, a key factor in maintaining complex formation throughout the steps required for co-IP technique is the lysis (IP) and wash buffers. We used a buffer with low ionic strength and nonionic detergents (Nonidet P-40 and Triton X-100) to maintain the protein-protein interactions during the IP incubations. Using these conditions, we identified more than 100 Cx43-bound proteins ([supplemental Tables S2–S4](#)), several of which are a constituent part of the plasma membrane. However, the release of proteins from membranes presents significant challenges. To increase the chance that a particular membrane protein that interacts with Cx43 being detected by MS, we mechanically disrupted the cells using a 25-G needle in the presence of SDS and Sarkosyl. The presence of these ionic detergents favors the solubilization of membrane proteins but affects protein-protein interactions during the IP incubations. Using these conditions, we were only able to identify 32 Cx43-interactors ([supplemental Table S5](#)), and 29 were already identified as Cx43 interactors in samples from OA patients. Only Cadherin-23, the four-span transmembrane protein 2 (MS4A7) and Myosin-14 were exclusively identified as Cx43 interactors





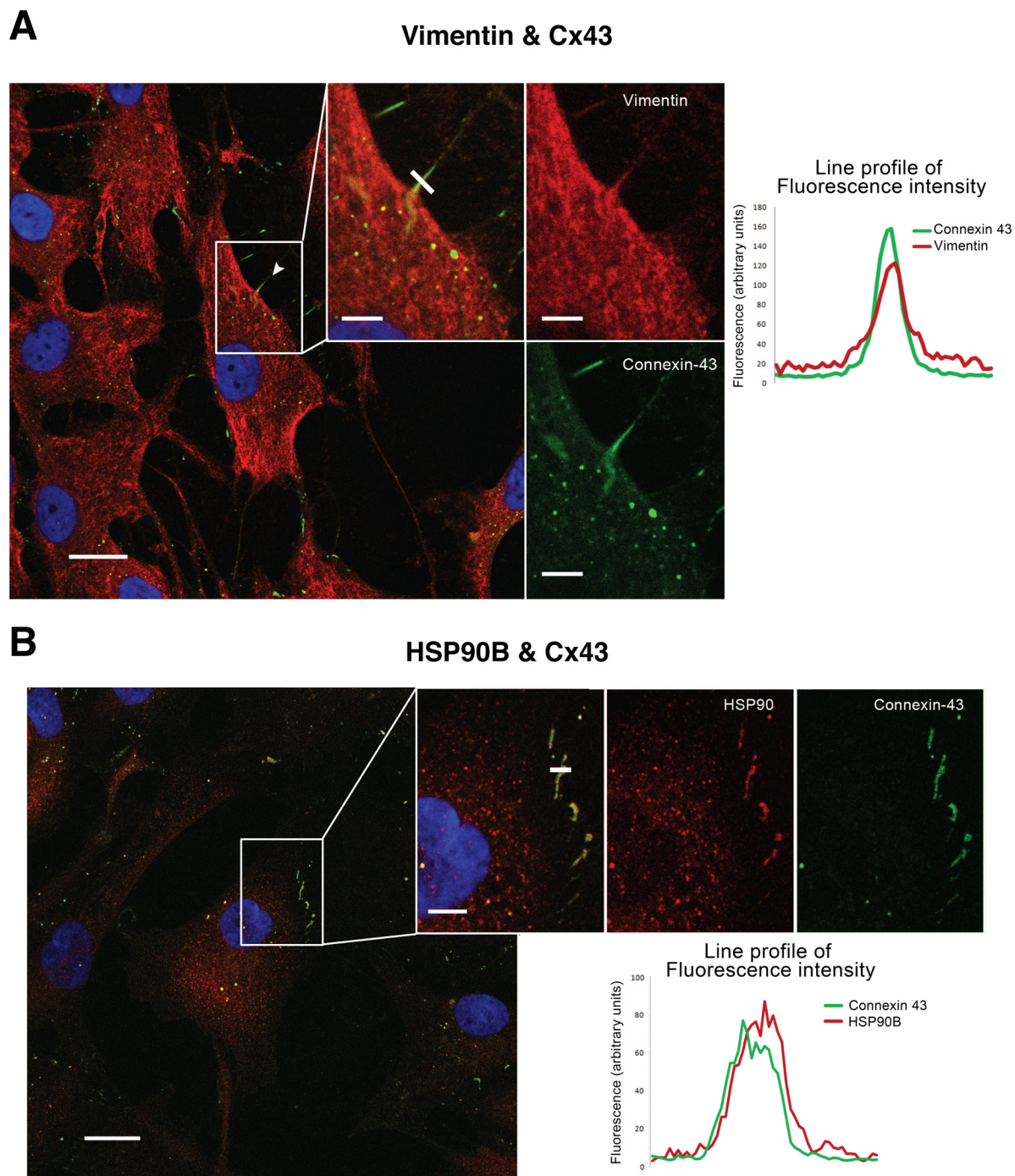
**FIG. 2. Cx43-nuclear and perinuclear interactors.** **A**, Cx43-associated proteins related to nuclear import and nuclear and perinuclear localization. Schematic view of the nucleus and endoplasmic reticulum; black spots represent ribosomes. The nuclear envelopment is composed of two lipid bilayer membranes, nuclear pore complexes, and the nuclear lamina. The outer nuclear membrane is continuous with the endoplasmic reticulum. Some of the Cx43 nuclear-related interactors specific to Cx43-IP are represented and listed in the schematic representation (unique peptides, 95% confidence). The complete list of identified proteins, number of identified peptides, sequence coverage, and score is found in [supplemental Table S1](#). **B**, Schematic representation of the C-terminal tail of the Cx43 protein showing the position of the two candidate NLSs that are specific to the importin  $\alpha/\beta$  pathway and identified using cNLS Mapper (93). The amino acid sequence and positions of each NLS are indicated in green. **C**, DAPI staining and IHC using an antiCx43 antibody on primary human articular chondrocytes in monolayer culture. Merged images and magnifications are shown. Red arrows indicate nuclear staining for Cx43; and green arrows indicate perinuclear Cx43-positive spots.

under these experimental conditions ([supplemental Table S5](#)). In addition, the analysis of membrane proteins by MS remains challenging because of the hydrophobicity of transmembrane domains, which are resistant to proteolysis. The resulting hydrophobic peptides are difficult to extract from in-gel digestions and are difficult to solubilize in buffers compatible with MS. These characteristics, together with the poor ionization of hy-

drophobic peptides, results in the loss of peptides during the process and makes the detection of low-abundance peptides in a complex spectra by MS difficult (33, 34).

#### DISCUSSION

The mapping of protein interactions with Cx43 have aided in understanding the formation and regulation of gating Cx43-

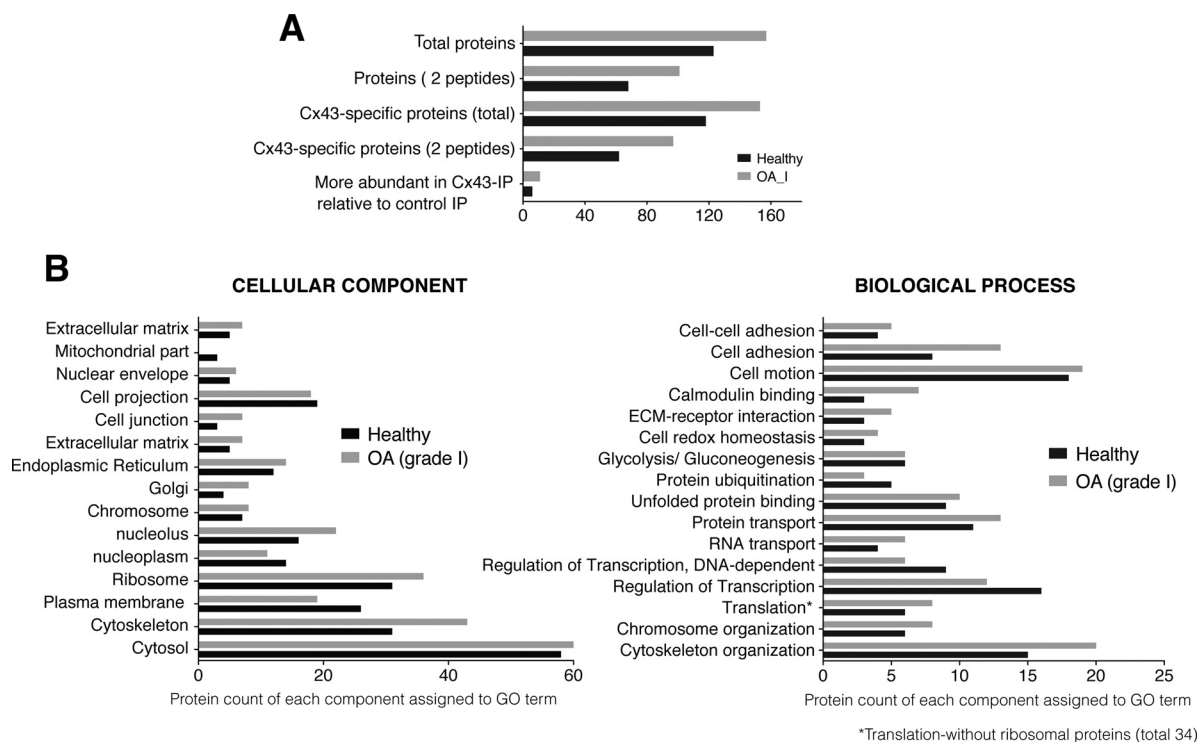


**FIG. 3. Colocalization of Cx43 with vimentin and HSP90 $\beta$  in articular chondrocytes.** Immunofluorescence confocal microscopy was used to study colocalization of two antigens in one cell. *A*, Images represent the double staining and DAPI in chondrocytes in monolayer culture that were obtained from healthy human donors. Cx43 is detected in green, and vimentin is in red. *B*, Colocalization between HSP90 $\beta$  and Cx43 was also studied in human chondrocytes in monolayer culture. Cx43 is shown in green, and HSP90 $\beta$  is in red color. Right panels; colocalization graphs show the fluorescence intensity profile from a line crossing through the cell protrusions, the coincidence of fluorescence intensity peaks, for both red and green, marks the colocalization of both proteins.

based junctions in molecular detail and contributed to understanding the pathogenesis of diseases such as cardiac disease (35). Biochemical and molecular biology methods, such as GST tag pull-down assays, yeast two-hybrid approaches,

*in vitro* studies, colocalization and co-IP, have been utilized to identify Cx43-interactors (16, 17, 32, 36–45). However, to our knowledge this study is the first, to combine the isolation of human native Cx43 by IP and LC-MS/MS identification. The





**FIG. 4. Analysis of Cx43-associated proteins identified in the OA samples.** Four different samples of chondrocytes from four OA patients were analyzed. **A**, A summary of the number of the identified proteins. Gray represents the Cx43 interactors identified in samples from OA grade I. Proteins identified as specific to the Cx43-IP but absent from the control IP (no antibody). The number of proteins identified as most likely more abundant in the Cx43-IP than in the control-IP (peptide difference  $\geq 2$ ). See [supplemental Table S3](#) for a complete list of the proteins identified in the Cx43-IP and control IP. **B**, GO analysis for the identified proteins. Overall, the proteins were identified with 95% peptide confidence (minimum value 1.3 of unused cutoff). See [supplemental Tables S1–S3](#) for a complete list of the identified proteins. Gray represents the Cx43 interactors that were identified in samples from OA grade I patients.

meta-analysis to determine the degree of overlap between the identified interactors in this study and the identified proteins in previous studies revealed a set of proteins ( $n = 108$  for healthy and  $n = 129$  for chondrocytes from OA patients) that were exclusive to the present study (see Fig. 5) and 15 (for healthy) and 28 (for chondrocytes from OA patients) common interactors. The differences in protein detection between the present and previous studies are most likely because of differences in the cell type, methodology, detection sensitivity, and purification strategies used.

Following our methodology, we have identified a large number of known and novel Cx43 interactors, some of which are responsible for the development of human diseases. These include proteins, such as Serpin H1, HnRNP-A2/B1, 78 kDa glucose-regulated protein, collagens, and PPLase B, that are related to connective tissue disorders and others, such as alpha(B)-crystallin, which is responsible for the development of myofibrillar myopathy and cataracts. HspB1 is related to Charcot-Marie-Tooth disease, neuropathy and neurodegeneration; vinculin is related to cardiomyopathy; and Profilin-1 is related to the development of amyotrophic lateral sclerosis and neurodegeneration. Moreover, several interactors that were identified in samples from healthy chondrocytes, such as SOD2, vimentin, vinculin, gelsolin, or alpha-enolase, have

been previously reported to be altered in chondrocytes from OA patients (46–53) (Table III) and early to severe stages of the disease contain very high levels of Cx43 (23).

IP experiments were performed *in vitro* using cultured chondrocytes. Chondrocytes were actively proliferating in an asynchronous culture where cells are in different stages of the cell cycle, whereas they were mainly in a resting stage in normal tissue. This difference, together with physiological conditions and the type of ECM molecules that interact with chondrocytes in tissue, may affect the number of the Cx43-associated proteins identified in this study and in particular identification of Cx43 interactors related with cytoskeletal proteins and proteins involved in cell-matrix adhesion and cell-to-cell communication. Cartilage is very challenging tissue because it is solid and mainly composed of collagen-rich ECM with low cellularity. Extraction of proteins will require the use of chaotropic agents incompatible with protein interactions and IP experiments. These limitations make it impossible to perform IP experiments using tissue as the starting material. Still, the Cx43-associated proteins identified in this and other studies finally depend on the Cx43 amino acid sequence and domains. We have identified a number of multiple Cx43 interacting proteins that might help in understanding the different cellular functions of Cx43. Further studies will be

## Identification of Binding Partners of Connexin 43

TABLE III

Example of Cx43-interacting proteins identified in this study that were previously reported to be altered in chondrocytes from OA patients (46–53). # Interactors exclusively identified in samples from healthy donors. \* Interactors that were only identified in samples from OA patients. The remaining interactors were identified in both samples (healthy and early/moderate OA). Protein Pilot Score, Amino acids sequence coverage for the identified proteins (Cov.) and number of peptides (No. pept.) masses matching/not matching the top hit from MS-Fit PMF. Only proteins that show >95% confidence and score >1.3 were considered for this study

Group/Complex	Protein name	Gene name	Healthy OA (g. III)	Score	Cov.	No. pept.	
Extracellular matrix structural constituent	Collagen alpha-1(I) chain	COL1A1	Healthy OA (g. III)	6 8.24	23.9 50	3 4	
	Collagen alpha-2(I) chain	COL1A2	Healthy OA (g. III)	4 2	31.3 40.7	2 1	
	Collagen alpha-1(VI) chain*	COL6A1*	OA (g. III)	21.28\10.29	57.1\51.3	12\6	
	Collagen alpha-2(VI) chain*	COL6A2*	OA (g. III)	14.25\6	56.1\36.3	11\4	
	Collagen alpha-3(IV) chain*	COL6A3*	Healthy	2.84	7.4	2	
	Collagen alpha-1(XII) chain*	COL12A1*	OA (g. III)	95.44\61.62	53.9\47.5	54\34	
Carbohydrate metabolic processes	Alpha-enolase	ENO1	Healthy OA (g. III)	8 26.89\26.98	21 65\58.1	4 13\14	
	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Healthy	10.41	30.5	5	
	Fructose-biphosphate aldolase A	ALDOA	Healthy	4.04\9.96	42.4\31.6	2\5	
			OA (g. III)	2.01	8.8	1	
	Cytoskeleton related-proteins	Vimentin	VIM	Healthy	2.1\7.56	43.7\38.5	1\4
				OA (g. III)	81.76	87.1	48
Gelsolin		GSN	Healthy	124.3\78.96	100\94.2	158\53	
			OA (g. III)	2	6.8	1	
Vinculin		VCL	Healthy	2\ 9.81	17.4\39.1	1\5	
			OA (g. III)	5.29	20.3	3	
Actin, cytoplasmic 1		ACTB	Healthy	4.32\13.21	40.5\45.3	2\7	
			OA (g. III)	25.29	53.6	16	
	Healthy		29.04	62.9	21		
	OA (g. III)		2	3.9	1		
Chaperone and Protein folding-related proteins	Alpha-crystallin B chain	CRYAB	Healthy	10.02\12.35	59.1\53	6\7	
			OA (g. III)	4	26.3	2	
	Heat shock cognate 71 KDa protein	HSPA8	Healthy	13.73\4.18	65.7\47.4	7\2	
			OA (g. III)	10.72	22.5	5	
	Heat shock protein HSP 90-beta	HSP90AB1	Healthy	3.04\13.06	35.8\42.9	2\8	
			OA (g. III)	7.26	17.4	4	
	Endoplasmic	HSP90B1	Healthy	2.81\16.29	28\34.7	2\8	
			OA (g. III)	5.64	5.6	3	
	78 kDa glucose-regulated protein	HSPA5	Healthy	3.82\22.57	31.1\38.5	2\11	
			OA (g. III)	22.93	30.3	11	
Others	Annexin A1	ANXA1	Healthy	6.14\32.82	53.4\60.6	3\17	
			OA (g. III)	10	30.6	5	
	Annexin A2	ANXA2	Healthy	10.87\ 6.15	47.4\27.2	6\3	
			OA (g. III)	52.03	65.8	30	
	Annexin A5	ANXA5	Healthy	54.59\48.92	84.4\86.1	40\30	
			OA (g. III)	2	19.7	1	
	Superoxide dismutase [Mn], mitochondrial	SOD2#	Healthy	2.05\2	32.8\20.6	1\1	
			OA (g. III)	2	15.3	1	
	Prelamin-A\C	LMNA	Healthy	26.38	44.7	15	
			OA (g. III)	39.97\16.48	82.7\55.1	21\8	
	Eukaryotic translation initiation factor 5A-2	EIF5A2	Healthy	2	40.5	1	
			OA (g. I)	14.35	21.1	7	
	Elongation factor 2	EEF2	Healthy	9.2\19.24	35.9\34.4	5\9	
			OA (g. I)	2.37	18.9	1	
Dihydropyrimidinase-related protein 2	DPYSL2	Healthy	7.31	35.1	4		
		OA (g. III)	2	3.9	1		
Major vault protein	MVP	Healthy	2.3	31	1		
		OA (g. III)	4	11.6	2		
GTP-binding nuclear protein RAN	RAN	Healthy	2.01	34.7	1		
		OA (g. I)	2.03	20.9	1		
Integrin alpha-V	ITGAV*	Healthy	2\2	18.9\8.4	1\1		
		OA (g. III)	2\2	18.9\8.4	1\1		

TABLE IV

Cx43-interacting proteins exclusively identified in samples from healthy, early OA and moderate OA patients. The table represents some of the Cx43-interacting proteins that were exclusively identified for each group. For the complete list of interactors, see [supplementary Table S4](#). The values of peptide probability score, sequence coverage and number of peptides found are shown in supplementary figures. Only proteins that show >95% confidence and score >1.3 were considered for this study

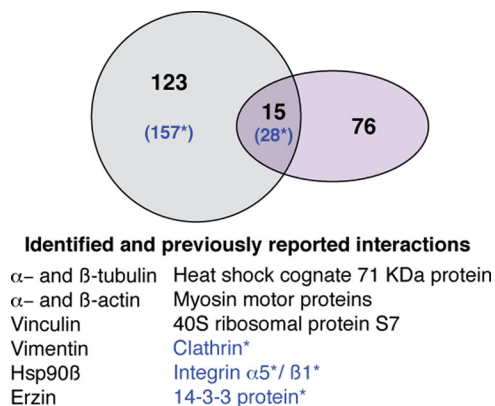
	Protein name	Gene name	Score	Cov.	No. pept.
Healthy	ATP synthase subunit f, mitochondrial	ATP5J2	2	13.8	1
	Erzin	EZR	2	3.9	1
	Protein S100-A10	S100A10	2	14.4	1
	Protein S100-A11	S100A11	2	10.5	1
	Protein S100-A6	S100A6	2	16.7	1
	Ras-related protein Rap-1A	RAP1A	2	7.6	1
	Superoxide dismutase [Mn], mitochondrial	SOD2	2	15.3	1
	Thy-1 membrane glycoprotein	THY1	4	24.2	2
	Transforming protein RhoA	RHOA	2	17.6	1
	Ubiquitin-40S ribosomal protein S27a	RPS27A	2.3	24.4	1
	Vesicle-trafficking protein SEC22b	SEC22B	2	18.1	1
Early OA (grade I)	14-3-3 protein zeta/delta	YWHAZ	4	28.2	2
	1,4-alpha-glucan-branching enzyme	GBE1	2	13	1
	Myosin-10	MYH10	11.36	46.5	7
	Protein piccolo	PCLO	1.81	19.2	1
	Coatomer subunit alpha	COPA	2	28.8	1
	Dextrin	DSTN	2.14	44.2	1
	Integrin alpha-V	ITGAV	2.03	20.9	1
	Matrin-3	MATR3	2.05	27.4	1
	Septin-2	SEPT2	2	21.3	1
	Transforming growth factor-beta-induced protein ig-h3	TGFBI	2.32	19.8	1
	Vigilin	HDLBP	3.84	34.6	2
Moderate OA (grade III)	26S proteasome non-ATPase regulatory subunit 2	PSMD2	2	7.3	1
	A-Kinase anchor protein 2	AKAP2	2	24.7	1
	AP-2 complex subunit alpha-1	AP2A1	2.82	16	2
	ATP-citrate synthase	ACLY	3.96	18.1	2
	Coatomer subunit beta and gamma	COPB1	2.03	16.8	1
		COPG	6.47	24.5	4
	Heterochromatin protein 1-binding protein 3	HP1BP3	2	17.9	2
	High mobility group protein B1	HMGB1	1.73	23.7	2
	LIM domain and actin-binding protein 1	LIMA1	3.07	28.7	2
	LIM domain only protein 7	LMO7	2.53	25.9	2
	Stress-70 protein, mitochondrial	HSPA9	5.26	31.1	3
	Transgelin	TAGLN	1.39	42.3	1
	Zyxin	ZYX	2.01	14.3	1

necessary to fully understand the molecular roles of these interactions under physiological conditions and in healthy and disease states.

Especially noteworthy is the identification of several Cx43-interactors related to the generation of precursor metabolites and energy (Table II). Some of the Cx43-interactors identified in this study are enzymes responsible for glycolysis and gluconeogenesis (ALDOA, GAPDH, PGK1, ENO1, and PKM2). Notably, the glycolytic enzymes that were identified as Cx43-binding proteins, except Lactate Dehydrogenase A, which is implicated in the final step of anaerobic glycolysis, are involved in the energy-generation phase of glycolysis, which results in the net synthesis of ATP and NADH molecules. The interactions between Cx43 and these enzymes most likely play an important role in the cellular use of glucose. In fact, the eye lens and articular cartilage of adults are avascular organs, and because no blood supply reaches these tissues, it has

been reported that cells in these tissues are maintained by the movement of fluids, ions, nutrients (e.g. glucose), and metabolites through a network of GJs (7, 54, 55). In addition, results from different reports strongly suggest that inhibition of GJIC modifies glucose uptake and that glucose levels modify Cx43 gene expression (56). Reduced levels of Cx43 alters the expression of the glucose transporters GLUT-1 and GLUT-3 and type I and II hexokinases, which are enzymes responsible for the phosphorylation of intracellular glucose to obtain a charged molecule that cannot be transported through the plasma membrane (57, 58). On the other hand, high-glucose conditions have been shown to reduce Cx43 expression and GJIC in normal conditions (59–61). However, increased levels of Cx43 have been detected in pathological conditions, e.g. during wound healing in diabetic rats and humans (62–64). Consequently, cell metabolism and Cx43 appear to be interconnected. It would be interesting to study the role of Cx43 in





**FIG. 5. Meta-analysis of Cx43 mass-spectrometric data.** Proportional Venn diagram comparing the Cx43-associated proteins that were identified in the present study (dark gray circle) to those from previous reports (purple circle). Overlap (green) includes proteins present in both groups. Blue letters and asterisk (\*) represent Cx43 interactors identified in samples from OA grade I. Some of these previous studies used co-IP, colocalization or affinity techniques and nuclear magnetic resonance using different types of cells and tissues from rat, mouse, human, porcine, goldfish, rabbit, or chick. See the reference list of some of the selected reports and reviews for meta-analysis (16, 17, 32, 36–45). None of the previous studies were performed using cartilage or articular chondrocytes.

glucose metabolism in particular chondrocytes because diabetes has been suggested as a possible risk factor for developing OA and OA chondrocytes showed significantly elevated Cx43 protein levels (23).

Recent studies have shown that Cx43 localizes to mitochondria (65–68), forms functional hemichannels, and plays an important role during ischemic preconditioning (65, 66, 68, 69). During prolonged ischemia, ATP levels decrease, leading to lowering of the pH and accumulation of reactive oxygen species (ROS), which ultimately result in cell death. Several studies have shown that mitochondrial Cx43 creates a protective, preconditioning effect during the early stages of cardiac ischemia by most likely favoring ATP and/or glutathione release to generate preconditioning and cell-protective functions. In this report, the MS data revealed that Cx43 might interact with the mitochondrial protein SOD2 and the mitochondrial membrane ATP synthase (ATP5J2). In addition, the Hsp90 chaperone, which has been reported to be involved in the translocation of Cx43 to the mitochondria (67, 70), has been identified in this study and by other authors (67) as a Cx43 binding protein. Importantly, SOD2 and ATP5J2 were only identified as Cx43-interactors in samples from healthy donors, and not in OA chondrocytes (supplemental Table S4). The functional relation between Cx43 and the identified mitochondrial interactors will most likely help in understanding the importance of normal mitochondrial function in degenerative disorders or preconditioning.

Gene array analyses have indicated that Cx43 might represent a central node in the regulation of genome-wide expression (71–73). Full-length Cx43 and its C-terminal tail have

been localized to the nucleus, and translocation of the Cx43-CTD into the nucleus modified cell growth, proliferation and gene expression (22). It has been suggested that Cx43 might regulate gene expression through the recruitment of transcription factors to the proximal promoter element termed the connexin response element (CxRE) (74, 75). Yet, how Cx43 can translocate into the nucleus and modify gene expression has not been described. IP and MS analysis has identified two possible Cx43-interactors involved in the RAN-GTP nuclear transport cycle that could be involved in the translocation of Cx43 to the nucleus, the GTP-binding nuclear protein Ran (RAN) and importin  $\beta$  (KPNB1) (Fig. 3A). Additionally, the C-terminal tail of Cx43 contains two potential NLSs that are recognized by the importin  $\alpha/\beta$  complex, which imports proteins to the nucleus through the RAN-GTP cycle (Fig. 3B). However, cNLS Mapper does not predict an importin  $\alpha$ -independent NLS. Thus, we cannot rule out that additional sites in the Cx43 sequence might be implicated in the translocation of the protein to the nucleus. The identified Cx43-nuclear interactors include several histones, transcription factors and nucleolar proteins, such as nucleolin or the polymerase I and transcript release factor (PTRF), which suggests that Cx43 or its C-terminal tail might affect chromatin organization and nucleolar activity.

The presence of possible interactors with known functions directly linked to downstream transcription processes, such as RNA splicing, processing, export and translation (Table II), together with the perinuclear localization of Cx43 (Fig. 2C) suggest a potential role of Cx43 in these processes. We have identified several Cx43-interactors involved in post-transcriptional regulation and mRNA processing. Some of these interactors are integrated into the nuclear or endoplasmic reticulum membranes. Together with the identification of several Cx43-binding proteins involved in RNA transport, such as hnRNPs, and translation, which include several ribosomal proteins (Table II), these results suggest a possible role of Cx43 in translation and protein biosynthesis that might be related to RNA species transport across the nuclear and endoplasmic reticulum system. On the other hand, the interaction of Cx43 with the Major Vault Protein (MVP) might be related with the transport of different types of RNA, including mRNA (76, 77), along cytoskeletal elements into dendritic microtubules or the axonal compartment (78–81).

Components of the cell junction and cell projections and proteins related to cell adhesion, motility, and extracellular matrix interactions have been identified using MS as Cx43-associated proteins, and these include several collagens, vinculin, talin, and annexins. The cell adhesion and ECM-receptor interaction proteins collagen VI and XII and integrin  $\alpha$ -V and  $\beta$ -I were only detected in samples from OA patients. Moreover, it has been reported that residues 136–158 of the cytoplasmic loop region of Cx43 contains a calmodulin-binding motif (42), and the MS data in this report revealed that calmodulin-interacting proteins, such as calde-

smo1, several types of myosin and a diversity of cytoskeletal proteins, also associate with Cx43 in OA samples. These results suggest that Cx43, particularly in OA chondrocytes, might play a (dys)functional role in cell adhesion and migration. In addition, caldesmon plays important roles in the migration of nonmuscle cells via regulating the actin-myosin system (82), and caldesmon binding to myosin has been reported to result in the enhancement of axon extension (83). Interestingly, we have found that chondrocytes from OA patients contain longer cellular extensions (more than 200  $\mu\text{m}$  in length) when compared with chondrocytes in healthy cartilage (7). Further studies will be necessary to reveal the role of Cx43 in these processes and to discern its function in OA chondrocytes.

Cx43 is a complex protein with multiple functions and cellular localizations. The results reported here suggest that the complex proteomic interactions with Cx43 may play important functions in cell metabolism, gene transcription and translation, cytoskeleton organization, motility, and cell–cell and cell–matrix interactions. It is well documented that Cx43 is up- or down-regulated in a number of diseases such as cancers, inflammation, and disorders such as Alzheimer's disease, Huntington's disease, or OA (18, 23, 84–88). The literature also indicates that mutations in the Cx43 sequence, including single mutations in its C-terminal tail, are responsible for the development of diseases such as oculodentodigital dysplasia (ODDD) (89–92). Many of these observations lack the mechanistic understanding that is required for the development of an effective therapy. The Cx43-interactors identified in this report will serve to gain a better understanding of the cellular functions of Cx43 in health and disease. Although the Cx43 sequence is well conserved from humans to fish, several domains are only highly conserved among primates; therefore, the results presented here can be extended as a guide for further studies of Cx43 interactions in different human cell types.

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 This article contains supplemental Tables S1 to S5.

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Ethics approval: The study was conducted with the approval of the local ethics committee in Galicia, Spain.

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