frontiers

# Sox9 determines translational capacity during early chondrogenic differentiation of ATDC5 cells by regulating expression of ribosome biogenesis factors and ribosomal proteins

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**Abstract**

Introduction: In addition to the well-known cartilage extracellular matrix-related expression of Sox9, we demonstrated that chondrogenic differentiation of progenitor cells is driven by a sharply defined bi-phasic expression of Sox9: an immediate early and a late (extracellular matrix associated) phase expression. In this study we aimed to determine what biological processes are driven by Sox9 during this early phase of chondrogenic differentiation.

Materials: Sox9 expression in ATDC5 cells was knocked-down by siRNA transfection at the day before chondrogenic differentiation or at day 6 of differentiation. Samples were harvested at 2 hours, and 7 days of differentiation. The transcriptomes (RNA-seq approach) and proteomes (Label-free proteomics approach) were compared using pathway and network analyses. Total protein translational capacity was evaluated with the SuNSET assay, active ribosomes with polysome profiling and ribosome modus with bicistronic reporter assays.

Results: Early Sox9 knockdown severely inhibited chondrogenic differentiation weeks later. Sox9 expression during the immediate early phase of ATDC5 chondrogenic differentiation regulated the expression of ribosome biogenesis factors and ribosomal protein subunits. This was accompanied by decreased translational capacity following Sox9 knockdown, and this correlated to lower amounts of active mono- and polysomes. Moreover, cap- versus IRES-mediated translation was altered by Sox9 knockdown. Sox9 overexpression was able to induce reciprocal effects to the Sox9 knockdown.

Conclusion: Here we identified an essential new function for Sox9 during early chondrogenic differentiation. A role for Sox9 in regulation of ribosome amount, activity and/or composition may be crucial in preparation for the demanding proliferative phase and subsequent cartilage extracellular matrix-production of chondroprogenitors in the growth plate *in vivo*.

## Introduction

Chondrogenesis, or chondrogenic differentiation, is the differentiation path of progenitor cells via early mesenchymal condensation into chondrocytes that synthesize a cartilaginous extracellular matrix (ECM) (1-3). Aside from formation of articular cartilage and its maintenance, skeletal development also depends on chondrogenic differentiation. Development of the long bones of the mammalian skeleton depends on the activity of growth plates; cartilaginous entities at the ends of developing bones in which chondrocytes differentiate from progenitor cells (1-3). In contrast to articular chondrocytes, differentiating growth plate chondrocytes are predestined to undergo hypertrophic differentiation and apoptosis. The remaining cartilaginous matrix is subsequently remodelled by osteoclastic/osteocytic activity, resulting in *de novo* synthesized bone tissue (1-3). *In vivo*, chondrogenic differentiation is almost exclusively initiated from local mesenchymal progenitor cells that reside in the cartilaginous tissue (growth plate resting zone (4), articular cartilage superficial layer(5)) or in surrounding fibrous tissues (e.g. periosteum (6, 7)). However, *in vitro* chondrogenic differentiation has been reported from various primary (mesenchymal) progenitor cell sources including synovial membrane/fluid, bone marrow, adipose tissue, fibroblasts, and induced pluripotent stem cells (8-10). In addition to high amounts of oligosaccharides (mostly hyaluronic acid, heparan sulphate, chondroitin sulfate), important cartilage ECM proteins are type II collagen (Col2a1) and aggrecan (Acan)(2, 11, 12).

The master regulator of chondrogenic differentiation is the transcription factor SRY (sex determining region Y)-box 9 (Sox9). Mutations in *SOX9* were originally identified as the cause for campomelic dysplasia (13, 14), a severe skeletal dysplasia associated with XY sex reversal and disproportionally short stature, as well as general lack of cartilaginous tissue formation. *SOX9* was found to be essential for murine early chondrogenic lineage determination (15). Upon nuclear translocation (16, 17), Sox9 binds as a homodimer to its consensus DNA recognition sequence (A/T)(A/T)CAA(A/T)G(18), which includes the highly conserved AACAAT motif recognized by the HMG-box domain shared amongst

Sox and Sry protein family members. In chondrogenic differentiation Sox9 drives the transcription of, and cooperates with L-Sox5 and Sox6 for efficient transcription of the *COL2A1* and *ACAN* genes (15, 19-22). Other cartilage ECM genes have also been demonstrated as under transcriptional control of SOX9; including *COL9A1* (23), *COL27A1* (24), and *MATN1 (25, 26)*. Besides L-Sox5 and Sox6, another important factor for Sox9-mediated transcription is Smad3. Smad3 modulates the interaction between Sox9 and CBP (CREB-binding protein)/p300 (27), thereby possibly explaining the pro-chondrogenic effect of bone morphogenetic proteins (BMPs) and transforming growth factor beta (TGFβs) on chondrogenic differentiation (28, 29).

During chondrogenic differentiation of progenitor cells *in vitro*, induction of Sox9 expression is biphasic (30). In the first hours after initiation of chondrogenic differentiation Sox9 expression is transiently induced (immediate early Sox9 induction), together with the other members of the "Sox-trio". Sox9 expression increases a second time, in parallel with the synthesis of cartilage ECM molecules (late Sox9 induction). Previously, we demonstrated that this immediate early Sox9 expression is in part regulated by the immediate early response gene 1 (Egr1) (31) as well as by NFκB/p65 (30). Similar expression patterns were also found in growth plate sections (30). The function of the early Sox9 induction itself remains elusive. In the present work we therefore determined the transcriptomic and proteomic consequences of the abrogation of early Sox9 expression during ATDC5 chondrogenic differentiation, and uncovered the biological processes that are driven by Sox9 during the early phase of chondrogenic differentiation.

## Materials and methods

### ATDC5 cell culture

ATDC5 cells (RIKEN BRC, Japan, STR profiled)(32) were cultured in a humidified atmosphere at 37°C and 5% $CO_2$ in culture media consisting of Dulbecco's Modified Eagle Medium (DMEM)/F12 (Life Technologies, Waltham, Massachusetts, USA), 5% fetal calf serum (Life Technologies), 1% antibiotic/antimycotic (Life Technologies) and 1% non-essential amino acids (NEAA)(Life Technologies). Chondrogenic differentiation was induced by plating the cells in triplicates at 6400 cells/$cm^2$, or 20,000 cells/$cm^2$ in transfection experiments, and the addition to culture media of differentiation supplements 10 μg/ml insulin (Sigma-Aldrich, St. Louis, Missouri, United States), 10 μg/ml transferrin (Roche, Basel, Switzerland), and 30 nM sodium selenite (Sigma–Aldrich). Media was refreshed every two days.

### Sox9 loss and gain of function

A small interfering RNA (siRNA) duplex for Sox9 ("Sox9 RNAi") (sense: 5'-GACUCACAUCUCUCCUAAUTT-3', anti-sense: 5'-AUUAGGAGAGAUGUGAGUCTT-3') and a scrambled siRNA duplex ("Control RNAi", Eurogentec, Seraing, Belgium) were transfected (100 nM) one day prior to initiation of chondrogenic differentiation or at day 6 of differentiation using HiPerFECT according to manufacturers' protocol (Qiagen, Hilden, Germany). A custom made DNA strand containing a start codon and 3xFLAG sequence (derived from p3xFLAG CMV7.1) and the mSox9 coding sequence (NM_011448.4:376-1899) without start codon was flanked by 5'EcoRI and 3'XbaI restriction sites (GeneCust, Boynes, France). This fragment was cloned directionally into the

pLVX-EIF1α-IRES puro MCS (Takara, Saint-Germain-en-Laye, France) to generate a pLVX-EIF1α-mSox9-IRES puro transfer plasmid. Lentiviral particles were generated according to manufacturer's instructions with the 4$^{th}$ generation VSV-G envelope Lenti-X system (Takara, Saint-Germain-en-Laye, France). Lentiviral titers were determined by p24 ELISA (enzyme-linked immunosorbent assay; INNOTEST HIV antigen mAb, Fujirebio, Zwijnaarde, Belgium). Viral transductions were performed by incubation of 1 ng lentivirus/cell in the presence of 8 µg/mL polybrene (Sigma-Aldrich) for 8 hours, followed by an overnight incubation with 1.6 µg/mL polybrene.

RNA isolation

RNA was isolated using TRIzol (Life Technologies), collecting the aqueous phase after centrifugation. RNA was precipitated with isopropanol (VWR International, Radnor, Pennsylvania, USA) (-80°C) and pellet by centrifugation. RNA pellets were washed in 80% ethanol (VWR International) and dried. RNA was dissolved in DNase/RNase-free pure water. RNA quantity and purity were determined spectrophotometrically (Biodrop, Isogen Life Sciences, Utrecht, The Netherlands).

Quantitative real time PCR

Total RNA was reverse transcribed into cDNA using standard procedures and random hexamer priming as previously described (33). Real-time quantitative PCR (RT-qPCR) was performed using Mesagreen qPCR master mix plus for SYBR Green (Eurogentec, Liège, Belgium). A CFX96 Real-Time PCR Detection System (Biorad, Hercules, California, United States) was used for amplification: initial denaturation 95°C for 10 minutes, followed by 40 cycles of amplification (denaturing 15 seconds at 95°C and annealing 1 minute at 60°C). Validated primer sequences are shown in Supplemental Table

1. Data were analyzed using the standard curve method, mRNA expression was normalized to a reference gene and gene expression was calculated as fold change as compared to control conditions or t=0.

RNA-sequencing and analysis

Isolated RNA was checked for quality and integrity on the Agilent 2100 Bioanalyzer (Santa Clara, California, USA) via 2100 an Expert Eukaryote Total RNA Nano chip according manufacturer's protocol. The mRNA sequencing library was generated using TruSeq mRNA sample preparation kit (Illumina, Eindhoven, the Netherlands). In short, mRNA was enriched using magnetic beads coated with poly-dT, followed by fragmentation. The fragmented mRNA enriched samples were subjected to cDNA synthesis by reverse transcriptase, followed by dA-tailing and ligation of specific double-stranded bar-coded adapters. Next, the library was amplified and following cleanup the sizes of the libraries were determined on an Agilent 2100 Bioanalyzer via a DNA 1000 chip according manufacturer's protocol. Pooled libraries consisting of equal molar samples were sequenced on a high-output 75bp single read on the NextSeq500 (Illumina). For each sample, the number of reads covering one or more exons of a given transcript were extracted. Triplicates of samples that were treated with either Scrambled or Sox9 siRNAs, at two different time points, were grouped separately. A transcript was defined as expressed when all replicates of a group had at least 5 reads extracted within the transcript's region. The grouped data were then compared to one another. The fold-change difference and the p-value were calculated using R-package edgeR (34, 35), after which the p-value was corrected for multiple testing (false discovery rate (FDR)-corrected). Transcripts having an FDR-corrected p-value <0.05, and a fold change of at least 1.5 were considered differentially expressed transcripts. RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-10333. EnrichR (36) software was used to display the pathways of interest obtained from the enrichment of down or up-regulated proteins.

The top three pathways of interest were considered from both Wikipathways and KEGG software (version 2019, Mouse) based on the combined score of the p-value and the adjusted p-value scores.

Label-free proteomics

At indicated time points, plates were rinsed 3 times with 1% phosphate buffered saline (PBS). A mixture containing complete Mini Protease Inhibitor Cocktail (Roche, Basel, Switzerland) in 25 mM ammonium bicarbonate (ABC) buffer (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 6M urea (GE Healthcare, Eindhoven, The Netherlands) was added to the plates. Cells were collected by scraping with a rubber policeman and the samples were transferred to Eppendorf tubes. Triplicates were pooled and sonicated for 10 minutes and centrifuged at 12.000g for 10 min in 4°C. The supernatant containing proteins was transferred into new tubes and a Bradford assay (Biorad, Lunteren, the Netherlands) was performed to assess protein concentration. The concentrations were adjusted to 0.2µg/µL in order to normalize for the following steps. Samples were reduced with 20mM of Dithiothreitol (DTT) (Sigma-Aldrich) for 45 minutes and alkylated with 40 mM of iodoacetamide (IAM; Sigma-Aldrich) for 45 minutes in the dark. The alkylation step was stopped by adding 20 mM of DTT. Samples were then digested using LysC and trypsin (Promega, Leiden, The Netherlands) added at a ratio of 1:25 (enzyme:protein) and incubated for 2 hours at 37°C in a water bath. Finally, 200 µL of 25 mM ABC buffer was added to the samples before overnight incubation at 37°C. The digestion was stopped by adding formic acid (FA; Sigma-Aldrich) and acetonitrile (Biosolve) at a final concentration of 1% and 2%, respectively. 200 ng of each sample were injected in duplicate for liquid-chromatography mass spectrometry (LC-MSMS) analysis. The separation of the peptides was performed on a Thermo Fisher Scientific Dionex Ultimate 3000 Rapid Separation ultrahigh-performance liquid-chromatography (HPLC) system (Thermo Scientific, MA, USA) equipped with an Acclaim PepMap C18 analytical column (2µm, 75µm*150 mm, 100Å). The samples were first trapped on an online C18 column for desalting. The peptides were then separated on the analytical column with a 90-minutes linear gradient

from 5% to 35% acetonitrile/0.1% FA and a flow rate set at 300 nL/min. The HPLC system was coupled to a high mass resolution Orbitrap MS instrument (Q-Exactive HF, Thermo Scientific, Waltham, MA). The mass spectrometer was operated in data-dependent acquisition (DDA) mode with the following settings: Full MS scan of the mass range *m/z* 350-1,650 at a resolution of 120,000 at *m/z* 400, followed by tandem mass spectrometry (MS/MS) scans for the fragmentation of the 15 most intense ions at a resolution of 30,000. The ions already selected for fragmentation were dynamically excluded for 20s. External calibration of the instrument was performed using a standard calibration solution for positive ion mode (Thermo Scientific). For protein identification, raw files were processed within the Proteome Discoverer software version 2.2 (Thermo Scientific) using the search engine Sequest with the Swiss-Prot database *Mus musculus* version 2017-10-25 (TaxID 10090). The following parameters were used for the database search: carbamidomethylation of Cysteine for fixed modifications; oxidation of Methionine and acetylation of protein N-terminal for variable modifications; trypsin for enzyme with a maximum of two missed cleavages; y and b for the ion types with a mass tolerance of 10 ppm and 0.02 Da for the precursors and the fragments, respectively; minimum and maximum peptide length of 6 and 144, respectively. Normalization of the data was performed on the total peptide amount. Percolator was used for the decoy database search and the FDR was fixed at 1% maximum. Finally, a list of 23 commonly detected contaminants were removed manually (data not shown). For protein quantitation, the Minora Feature Detector node in the processing step and the Feature Mapper node combined with the Precursor Ions Quantifier node in the consensus step were used with default settings. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (37) partner repository with the dataset identifier PXD024715. ANOVA test and principal component analysis (PCA) were performed within the Proteome Discoverer software. PCA was performed to visualize protein abundance changes between groups in an unsupervised manner. ANOVA test was used to analyze the statistical significance of variation observed in protein abundances between the conditions. The proteins were

considered modulated with a p-value ≤0.05 and a fold change (FC) ≥2. The modulated proteins were then imported within the EnrichR software (36) to display the top 3 pathways of down or up-regulated proteins ranked by the combined score. WikiPathways and KEGG were used as databases (version 2019, Mouse).

Immunoblotting

Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0,5% Sodium deoxycholate, 0,1% SDS, 50 mM Tris pH 8.0, 5.0 mM Ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.5 mM dithiothreitol (DTT) supplemented with cOmplete Mini Protease Inhibitor Cocktail (Roche) and PhosSTOP phosphatase inhibitor (Sigma-Aldrich)). Extracts were sonicated and protein concentrations were determined with a bicinchoninic acid assay (BCA) assay (Sigma-Aldrich). Proteins were separated by SDS-PAGE (sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes by electroblotting. Primary antibodies for immunodetection were anti-Sox9 (Abcam, Cambridge, UK; ab3697) and anti-Tubulin (Sigma-Aldrich; T6074). Bound primary antibodies were detected using immunoglobulins conjugated with HRP (horseradish peroxidase; DakoCytomation, Glostrup, Denmark) and visualized by enhanced chemoluminescence (ECL). ECL signals were quantified using Image J 1.46f software (Figure 1B). Relative differences in Sox9 levels, corrected for background and Tubulin levels, were determined as compared to t=0 conditions.

sGAG assay

The sulphated glycosaminoglycan (GAG) content was measured using a modified dimethyl methylene blue (DMB) assay (38). The absorbance of samples was read at 540 and 595 nm using a spectrophotometer (Multiskan FC, Life Technologies) and GAG concentrations were calculated using

a chondroitin sulfate standard curve (Sigma-Aldrich) and corrected for total protein content using a BCA assay.

SUnSET assay

Protein translational capacity of ATDC5 cultures (in sextuplicates) was assessed with the SUnSET assay (39, 40). 5.4 µM puromycin (Sigma-Aldrich) was incubated for 15 minutes in the cell culture medium, immediately followed by washing in PBS and fixation for 20 minutes with 10% formalin (VWR, Radnor, Pennsylvania, United States). Permeabilization was performed for 10 minutes with PBS supplemented with 0.1% Triton X-100. Wells were rinsed with PBS with 0.1% Tween (PBS-T) and blocked for 1.5 hour with 1% (m/v) skimmed milk powder (ELK, Campina, Zaltbommel, the Netherlands) in PBS-T, followed by overnight incubation at 4°C with the primary anti-puromycin antibody 12D10 (Sigma-Aldrich). After washing with PBS-T, wells were incubated for 1 hour at room temperature with the secondary goat anti-mouse Alexa488 antibody (Life Technologies). The fluorescent signal intensity was determined using a TriStar$^2$ LB942 (Berthold, Bad Wildbad, Germany) equipped with excitation filter F485 and emission filter F535. Fluorescent data was normalized to DNA-content from the same well (41). To this end, wells were washed with HEPES-Buffered Saline (HBS), followed by 1 hour incubation with 5 µg/mL DAPI (Life Technologies) plus 5 µg/mL HOECHST 33342 (Life Technologies) in HBS. After subsequent washing steps with HBS, fluorescent signal intensity was determined using a TriStar$^2$ LB942 (Berthold), using the excitation filter F355 and emission filter F460.

Polysome fractionation

Polysome fractionation was performed as described previously (42). Three 15 cm plates with ATDC5 cells were used to generate a single sample. At the day of sample collection, cells were differentiated for 2 hours, then pre-treated for 5 minutes with 100 µg/ml Cycloheximide (Sigma), washed twice in 0.9% NaCl with Cycloheximide and collected by scraping with a rubber policeman in cold 0.9% NaCl. Pelleted cells were lysed for 10 minutes in 1.8 ml polysome extraction buffer (20 mM Tris-HCl (pH7.5), 100 mM KCl, 5 mM MgCl2, 0.5% Nonidet P-40, 100 µg/ml Cycloheximide, complete protease inhibitor cocktail (Roche) and RNasin (Promega, 40U/ml)) on ice. Nuclei and cellular debris were removed by centrifugation at 12.000x g for 10 minutes at $4^0$C and 9/10th of the total volume was transferred to fresh tubes and measured spectrophotometrically. Total yield was the same for siCtrl and siSox9 treated cells. Sucrose gradients (linear 10-50%) were made using the Gradient Master (BioComp) in ultracentrifuge tubes (Seton, SW41 tubes). Cytoplasmic extracts (250 µg/sample) were loaded to each gradient in a fixed volume (400 µl). Gradients were run on an ultra-centrifuge (Beckman L60, Brea, California, USA) at 39.000 rpm for 1.5 hours at $4^0$C with max acceleration and deceleration 9. Samples were fractionated into 24 x 0.5 ml fractions using a Piston Gradient fractionator (BioComp, Fredericton, Canada) and fraction collector (Gilson FC203B, Middleton, Wisconsin, USA) with continuous A260 monitoring (Triax FC-1).

Bicistronic reporter assay

Reporter constructs for the CrPv IGR IRES, the CrPv CCGG IGR IRES mutant, the HCV and the P53 IRES were a kind gift of Dr. S. Thompson (UAB, USA). One day post plating, maxi-prep DNA (0.5 µg/well) and 100 nM siRNA were transfected into 24-wells wells (n=3/group) using Mirus Transit-X2 according to manufacturer's instructions. The next day differentiation was induced for 24 hours,

samples were collected by washing cells with 0.9% NaCl and incubation in 100 µl passive lysis buffer for 15 minutes (Promega). Subsequently, samples were transferred to Eppendorf tubes and centrifuged for 10 minutes at 12.000x g in a tabletop centrifuge. Next, 50 µl lysate was used for dual luciferase measurements (Promega) using a Berthold injection system (Reeuwijk, the Netherlands;10 seconds counting time per cistron). Data is represented as fold change of the ratio Fluc/Rluc in control cells for each IRES.

Statistics in other than proteomics or transcriptomic analysis

Statistical significance was determined by two-tailed student t-tests using Graphpad PRISM 5.0 (La Jolla, CA, USA). Error bars in graphs represent mean ± standard error of the mean. Significance for all tests was set at $p \leq 0.05$.

## Results

### Early Sox9 peak in ATDC5 chondrogenic differentiation

Induction of Sox9 expression is biphasic during chondrogenic differentiation of progenitor cells *in vitro* (30, 31). In the first (2-4) hours after initiation of chondrogenic differentiation of ATDC5 cells Sox9 expression was transiently induced on mRNA (Figure 1A) and protein level (Figure 1B). Sox9 expression increased a second time around day 7 in differentiation (Figure 1A), in parallel with the expression of the important Sox9 transcriptional target Col2a1 (21) (Figure 1C) and gain of sulphated glycosaminoglycan (GAG) content (2, 19) (Figure 1D). The immediate early transient Sox9 expression peak at 2 hours in differentiation did not correlate with the induction of expression of well-known Sox9 transcriptional targets such as Col2a1. Hence, we questioned what the function of the early Sox9 expression peak (2 hours) was and how it differs from the later Sox9 activity (day 7). We approached this by performing a loss-of-function experiment and comparing the Sox9-dependent transcriptome and proteome in an unbiased manner.

### Transcriptome and proteome analysis at 2 hours and 7 days in ATDC5 chondrogenic differentiation under Sox9 knockdown

To target early Sox9 expression, a siRNA for Sox9 or scrambled control siRNA were transfected prior to initiation of differentiation (t = -1 day) (Figure 2A). At t=0 chondrogenic differentiation was induced and cells were differentiated for 14 days. We established effective knockdown of Sox9 at t=0 and t=2 hours in differentiation, while at day 5, 7 and 14 in differentiation Sox9 mRNA levels returned to scrambled siRNA control conditions (Figure 2B; black bars and Figure 2C). In parallel, ATDC5 cells were differentiated and the siRNA for Sox9 or scrambled siRNA was transfected at day 6 in differentiation, to specifically target expression of “late” Sox9 induction. Effective knockdown of Sox9

312 at day 7 and day 14 was observed at mRNA as well as (Figure 2B; grey bars) at the protein level (Figure
313 2C). Knockdown of the early Sox9 expression peak resulted in decreased Col2a1 expression, and
314 increased Col10a1 expression at early time-points. At day 7 and 14 in ATDC5 differentiation, Sox9
315 siRNA treatment was not effective anymore (Figure 2B; white versus black bars), however, a major
316 induction of Col2a1 mRNA expression at day 7 and 14 in differentiation was prevented in this
317 condition (Figure 2D). A similar but opposite effect was seen for Col10a1 expression in the early Sox9
318 peak knockdown condition (Figure 2E). Knockdown of the "late" Sox9 peak (grey bars) also resulted
319 in a decreased Col2a1 and increased Col10a1 expression at day 7 and 14 in differentiation, with a
320 smaller magnitude. These data indicate that the early Sox9 expression peak is paramount for successful
321 chondrogenic differentiation of ATDC5 cells (see Supplemental Figure 1 for confirmation with
322 independent Sox9 siRNA). Differential expression of mRNAs and proteins was determined between
323 the scrambled versus Sox9 siRNA at 2 hours as well as at 7 days in ATDC5 differentiation, to target
324 the early and late Sox9 expression peaks (Figure 2D).

325 The extracted RNA was used for RNA sequencing and Principal Component Analysis (PCA) of the
326 transcriptome confirmed that samples from the 4 groups separated (Supplementary Figure 2).
327 Noteworthy, the separation between the scrambled siRNA and Sox9 siRNA conditions was evident at
328 2 hours, while separation between the scrambled siRNA and Sox9 siRNA conditions at 7 days was less
329 clear. At 2 hours in ATDC5 chondrogenic differentiation, knockdown of Sox9 led to the differential
330 expression of 2422 genes, with 1235 upregulated genes and 1187 downregulated genes (Figure 3A/B).
331 At 7 days in differentiation 493 genes were differentially expressed (203 up and 290 down) due to
332 knockdown of Sox9. From these differentially expressed genes, 203 genes were upregulated (only 15
333 overlapped with the 2 hour time point) and 290 genes were downregulated (49 genes overlapped with
334 the 2 hours condition) (Figure 3A/B). All genes that were differentially expressed ($FC \geq 2$; $p < 0.05$) at 2

hours and at 7 days in ATDC5 chondrogenic differentiation following Sox9 knockdown, are shown in Supplementary Tables 2 and 3, respectively.

Proteins from control conditions and Sox9 knockdown conditions at 2 hours and 7 days in ATDC5 differentiation were quantified using a label-free proteomics approach. PCA plotting confirmed that control samples at 2 hours clearly separated from the Sox9 knockdown samples at 2 hours in ATDC5 differentiation. Separation between control and Sox9 knockdown conditions was also confirmed at 7 days in chondrogenic differentiation. However, and in concert with the PCA plot of the RNA sequencing data (Supplementary Figure 2), the separation between control and Sox9 knockdown conditions appeared to be most obvious at 2 hours in differentiation (Supplementary Figure 3A). At 2 hours in differentiation, knockdown of Sox9 caused the differential expression of 90 proteins (29 up and 61 down)(Figure 3C/D and Supplemental Figure 3B). At 7 days in differentiation, the knockdown of Sox9 induced differential expression of 19 proteins (9 up and 10 down). There was no overlap between the Sox9-dependent differentially expressed proteins at 2 hours or at 7 days in differentiation (Figure 3C/D and Supplemental Figure 3B). The proteins that were differentially expressed ($FC \geq 2$; $p < 0.05$) at 2 hours and at 7 days in ATDC5 chondrogenic differentiation following Sox9 knockdown, are shown in Supplementary Tables 4 and 5, respectively.

These data indicate that at 2 hours in chondrogenic differentiation the knockdown of Sox9 induced different changes in the ATDC5 transcriptome and proteome when compared to knockdown of Sox9 at 7 days in differentiation. In addition, the consequences of Sox9 siRNA treatment appears to be stronger at 2 hours than at 7 days of differentiation, as indicated by larger separation in the PCA plots and larger number of differentially expressed genes and proteins. The role of the immediate early Sox9 expression was further investigated by comparing the Sox9-dependent differentially expressed mRNAs and proteins at 2 hours in differentiation. Four overlapping mRNAs and proteins are upregulated in the early Sox9 knockdown condition (Rps30/Fau, Avan, Eefsec, Rpl38; Figure 3E and 3F, Supplementary

Table 6) and 3 overlapping mRNAs and proteins downregulated in the early Sox9 knockdown condition (Ube2d3, Dclk1, Svil; Supplementary Table 6). Except for Rpl38, these overlapping targets are unique for the 2 hour in differentiation time point. The relative low number of overlapping genes and proteins (Figure 3E and F) might be explained by the different control of expression regulation at different levels for RNA and protein (43) and the notion that not all mRNAs are instantaneously translated into protein. Overlapping targets might be involved in a biological process which reacts fastest in a way that is visible in both RNA and protein expression.

Immediate early Sox9 expression is involved in ribosomal protein expression

To determine which prominent pathways link to the Sox9-dependent differential transcriptome and proteome at 2 hours in differentiation, we performed pathway analyses. Two independent Pathway analyses revealed that “Cytoplasmic Ribosomal Proteins” and “Ribosome” pathways were in the top three of identified enriched pathways (Figure 4). This strong overrepresentation of the “Cytoplasmic Ribosomal Proteins” and “Ribosome” pathways was not obvious in the Sox9-dependent differential transcriptome and proteome at day 7 in differentiation (Supplementary Table 7). Further analysis revealed the differential expression of 29 ribosomal protein encoding genes from the large (60S) ribosomal subunit (Rpls) and 10 ribosomal proteins from the small (40S) ribosomal subunit (Rpss) in the Sox9 knockdown condition at 2 hours in ATDC5 chondrogenic differentiation (Figure 5A and Supplementary Table 2). In addition, the 2 hours proteomics datasets demonstrated the differential expression of 5 ribosomal Rpl and Rps proteins (Figure 5A and Supplementary Table 4). Notably, the 4 overlapping mRNAs and proteins (Rps30/Fau, Avan, Eefsec, Rpl38) that were upregulated in the Sox9 knockdown condition at 2 hours in ATDC5 chondrogenic differentiation (Figure 3E and Figure 5B, Supplementary Figure 4A) are all linked to protein translation and represent either ribosomal

protein subunits or factors with a known function in ribosome biogenesis. Additional factors involved in ribosome biogenesis, but only differentially expressed in either the 2 hours transcriptomics or proteomics datasets were SBDS, Nop10 and Brix1 (Figure 5B. Supplementary Tables 2 and 3). Where expression of Sox9 was significantly increased at two hours in ATDC5 differentiation, expression of Rpl38, Rps30/Fau, Nop10 and SBDS was significantly decreased compared to t0 (Supplementary Figure 4C). Since ribosomes do not only consist of proteins, but depend on structural and catalytically active ribosomal RNAs (rRNAs), we investigated whether rRNA levels were affected by the Sox9 knockdown at 2 hours in differentiation in ATDC5. Expression of 18S rRNA, 28S rRNA and 5.8S rRNA was not significantly different between the conditions (Figure 5C) (see Supplemental Figure 4B for confirmation with independent Sox9 siRNA). Together, these data indicate that the Sox9 expression during early ATDC5 chondrogenic differentiation is involved in expression of ribosomal proteins and proteins involved in ribosome biogenesis.

Early Sox9 expression regulates protein translation capacity and ribosome translation modus

Since we identified the differential expression of ribosomal protein subunits and ribosome biogenesis factors, combined with unaltered rRNA expression levels, we hypothesized that ribosomes of early Sox9 knockdown ATDC5 cells are functionally distinct. To address this, we measured total translational capacity, performed polysome fractionation and evaluated ribosome translation modus in Sox9 knockdown and control ATDC5 cells. Following the knockdown of immediate early Sox9 expression a reduction of the total protein translational capacity was observed at 2 hours in chondrogenic differentiation (Figure 6A). The abrogation of early Sox9 expression also caused a reduction of total protein translational capacity at day 7 in differentiation (while Sox9 levels normalized at 7 days following the early knockdown (Figure 2B)). This impact on translation capacity was lost at day 14 in differentiation (Figure 6A). In contrast, late knockdown of Sox9 expression did not affect ATDC5 translational capacity at 7 days in chondrogenic differentiation. This is consistent with

transcriptome and proteome data. To assess if Sox9 knock-down had a specific effect on polysomal distribution of ribosomes, we performed sucrose density gradient separation of ribosomal subunits. Knockdown of the immediate early Sox9 expression resulted in an overall lower abundance of ribosomal subunits, which is in agreement with reduced translational activity (Figure 6B). In addition to total ribosome translation capacity (Figure 6A), the modus of translation is also subject to regulation. Thus, we evaluated the activity of IRES (internal ribosome entry site)- over cap-mediated protein translation using well-characterized bicistronic reporter constructs (CrPv IGR, HCV and P53 IRES) (44-46). We observed a 1.5-fold induction of the ITAF (IRES trans-acting factor) independent CrPv IGR IRES activity and 5-fold down regulation of both the HCV and P53 IRES activity in Sox9 knockdown ATDC5 cells compared to controls (Figure 6C, and Supplementary Figure 5). We next investigated whether overexpression of Sox9 levels during early ATDC5 differentiation may have a reciprocal effect on the translation capacity as opposed to the Sox9 knockdown conditions. Overexpression of Sox9 was confirmed (Figure 7A) and the mRNA expression of the Sox9 transcriptional target Col2a1 was significantly induced by Sox9 overexpression (Figure 7A). The overexpression of Sox9 resulted in a significant increase in translational capacity at 2 hours in ATDC5 differentiation (Figure 7B). This increase in translational capacity in the Sox9 overexpression condition was not accompanied by an increase in expression of rRNAs (Figure 7C). Contrary to the knockdown of Sox9 (Figure 5B), expression of ribosomal protein subunits Rpl38 and Rps30/Fau was significantly downregulated when Sox9 was overexpressed (Figure 7D). This was also the case for ribosome biogenesis factors Nop10 and SBDS (Figure 7D).

Taken together, knockdown of early Sox9 expression during ATDC5 chondrogenic differentiation reduced protein translational activity over a seven-day time period, which was reflected by a general reduction of $A_{260}$ signal in a polysome fractionation experiment at 2 hours in differentiation. This was associated with a differential effect on ribosome translation modus during the first day of

differentiation. Sox9 overexpression during early ATDC5 differentiation was able to induce reciprocal effects of the Sox9 knockdown on total protein translation.

## Discussion

Early chondrogenic lineage commitment during mesenchymal condensation is driven by Sox9 (15, 47, 48). Following lineage commitment, Sox9 is a key regulator of cartilage ECM synthesis, by driving expression of key ECM molecules such as Acan, Col2a1 and others (15, 19-26). Sox9 also safeguards maintenance of articular cartilage homeostasis by influencing chondrocyte Nkx3-2 levels, a transcriptional repressor of chondrocyte hypertrophy (49, 50). This dual action of Sox9 is recapitulated in *in vitro* models of chondrogenic differentiation, as we have previously reported on bi-phasic expression dynamics of Sox9 in ATDC5 and bone marrow derived stem cells (BMSC) chondrogenic differentiation (30, 31). In this earlier work we demonstrated that early (hours) transient induction of Sox9 expression driven by NFκB/p65 or Egr1 in ATDC5 chondrogenic differentiation is a prerequisite for late-stage (days) expression of cartilage ECM genes. As a key transcription factor for cartilage, the vast majority of investigations on the downstream functions of Sox9 have mainly focussed on its role in the transcriptional regulation of cartilage ECM genes (15, 19-26). In addition, a function in epigenetic reprogramming has been suggested for early Sox9 (31) , as well as a role for Sox9 in activating super-enhancers in chondrocytic cells (51). However, its downstream cell biological consequences during early chondrogenic differentiation are incompletely understood. The present study demonstrates that Sox9 expression during the very early phase of ATDC5 chondrogenic differentiation regulates the expression of ribosomal protein subunits, as well as proteins that are involved in ribosome biogenesis that together modulate ribosome activity and translation modus. These data, for the first time, connect the Sox9 transcription factor to regulation of protein translation during chondrogenic differentiation.

Ribosomopathies are severe genetic diseases caused by mutations in genes involved in ribosome biogenesis and -function and are, amongst others, associated with developmentally-related skeletal

malformations, caused by impairment of chondrogenic development of the growth plates (52, 53). This indicates that chondrogenic differentiation is particularly susceptible to disturbances in ribosome protein translation activity. Indeed, during chondrogenesis, a large amount of cartilage ECM is produced by the developing growth plate and disturbances in ribosome activity are likely to impair ECM synthesis, with consequences for the development of skeletal elements. It should however be noted that the link between Sox9 and chondrocyte translation activity in the current work was particularly present during early rather than late differentiation. This is highlighted by the deregulated expression of ribosomal subunits following Sox9 knockdown in early ATDC5 chondrogenic differentiation (Figure 4 and 5). In addition, the knockdown of Sox9 specifically impacted total protein translation throughout differentiation upon early knockdown, while not having an effect on protein translation when knocked-down during later in chondrogenic differentiation (Figure 6). The link between early Sox9 and chondrocyte protein translation suggests that protein translation is likely to be paced through Sox9 during early chondrogenic differentiation. However, it remains to be determined how early Sox9 is specifically able to influence chondrocyte translational capacity. Our present data suggest that expression of ribosomal protein subunits, ribosome biogenesis factors and ancillary ribosomal factors (such as IRES trans-acting factors (ITAFs)) depends on Sox9 during early chondrogenic differentiation, with downstream consequences for translation in the later differentiation program. Since we found these ribosomal genes and proteins differentially expressed after Sox9 knockdown, we studied the supplementary data of previously published Sox9 ChIP-seq and $Sox9^{-/-}$ mouse studies (48, 51). In supplementary data of a Sox9 ChIP-seq study we found 24 Rps and Rpl genes that were enriched in Sox9 occupancy and 14 with Sox6 occupancy of which 10 overlapped (51). Notably, this included Rpl38, which we found to be differentially expressed at the mRNA and protein level in our present Sox9 knockdown condition.

Aside from ribosome core components, we identified Sox9-dependent differential expression of several factors regulating the mode of protein translation. The rate-limiting step of cap-mediated translation is eukaryotic initiation factor 4 (54). Interestingly, EIF4BP2 was downregulated at the protein level at 2 hours of differentiation in Sox9 knockdown cells. EIF4 binding proteins were shown to regulate cell proliferation, but not cell size (55). Unexpectedly, we found strong differences in IRES activity upon early Sox9 knockdown after 24 hours of differentiation. Of note, the CrPv IGR IRES (a type IV IRES (56)) does not require ITAFs and is able to recruit the ribosome directly for translation (57). The activity of this IRES was increased and might be regulated by specific Rps/Rpls that transiently interact with the core ribosome components. The HCV (a type III IRES) and P53 IRES do require additional ITAF co-factors (58). Based on the increased expression of the ITAF Rpl38 (59), it is tempting to speculate that other ITAFs that facilitate HCV/P53 IRES translation are down regulated and may contribute to alternative use of ribosome translation modus. Rpl10a, Rpl11, Rpl38 and Pdcd4 were shown to regulate IRES-mediated translation (58). Rpl10a is known to activate the *IGF2, APP*, *Chmp2A* and *Bcl-2* IRESs. Gain- and loss-of-function studies in *Drosophila* showed that *Rpl10a* regulates insulin signaling (60). Moreover, *Rpl10a* was found to be preferentially translated by a sub pool of ribosomes in embryonic stem cells that required ribosome-associated Rpl10a (61). Insulin and insulin-like growth factor I (IGF1) signaling are crucial for ATDC5 differentiation (32). Rpl11 induced the *BAG1*, *CSDE1* and *LamB1* IRESs, while *Rpl38* activated a Hox gene IRES (59, 62). Upregulation of *Rpl11* led to stabilization of P53 and reduced proliferation of breast cancer cell-lines (63). In contrast, we found a reduction in *P53* IRES activity. Of note, P53 and ribosome biogenesis were recently coupled through SBDS (Ribosome Maturation Factor)(63). In our dataset, SBDS was down regulated at the protein level at 2 hours in differentiation in Sox9 siRNA treated cells, which matches the observed reduction in ribosomes and/or ribosome activity. Pdcd4 activated or inhibited the P53, INR, IGF1R, BcL-XL and XIAP IRESs (58). The IGF1R IRES might again be relevant for the ATDC5 differentiation model. Finally, Rpl38 was the only ITAF that was found to be upregulated at both the

mRNA and protein level. It was found to control HOX gene translation during murine embryonic development (59) and knockout led to ectopic mineralization in certain soft tissues (64).

We identified multiple connections between immediate early Sox9 expression during ATDC5 chondrogenic differentiation and downstream consequences for expression of ribosomal protein subunits, ribosome biogenesis factors and ITAFs. The connection between Sox9 expression and protein translation appears to be centered in early chondrogenic differentiation, with consequences for protein translation in later stage of chondrogenic differentiation. This suggests a role for early Sox9 in the priming of progenitor cells in the chondrogenic differentiation program for cartilaginous ECM production later in differentiation. This provides a new level of understanding how Sox9 controls the fate of chondrogenic differentiation at the level of protein synthesis. In this respect it is tempting to speculate on the classification of campomelic dysplasia (OMIM #114290) (65), as links between Sox9 and genes involved in ribosomopathies (52, 66) were identified in the present work (SBDS, Rpl11, Rps26). In conclusion, we collected essential new data on the regulation by Sox9 during early chondrogenic differentiation, uncovering an unanticipated role of Sox9 in ribosome biogenesis and protein translational capacity.

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**Contribution to the Field Statement**

In our previous research, we demonstrated that chondrogenic differentiation of progenitor cells is driven by a sharply defined bi-phasic expression of Sox9: an immediate early and a late phase expression. The late phase expression of Sox9 is associated with cartilage extracellular matrix formation, while the function of the early Sox9 induction itself remained elusive. In this study we aimed to determine what biological processes are driven by Sox9 during the early phase of chondrogenic differentiation. We identified an essential new function for Sox9 during early chondrogenic differentiation. A role for Sox9 in regulation of ribosome amount, activity and composition was found and may be crucial in preparation for the demanding proliferative phase and subsequent cartilage extracellular matrix-production of chondroprogenitors in the growth plate in vivo.

**Conflict of interest**

MMJ Caron and TJM Welting are inventor on patents WO2017178251 and WO2017178253 (licensed to Chondropeptix). LW van Rhijn and TJM Welting have shares in Chondropeptix and are CDO and CSO of Chondropeptix. All other authors have no competing interests to declare.

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**Data Availability Statement**

RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-10333. Proteomics data can be found in the ProteomeXchange Consortium via the PRIDE (37) partner repository with the dataset identifier PXD024715. All other data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## Figure legends

Figure 1: Sox9 expression has a bi-phasic peak expression during chondrogenic differentiation of ATDC5 cells

**A**: Sox9 mRNA expression showed bi-phasic peak pattern during ATDC5 differentiation (h=hours, d=days) as measured by RT-qPCR (30, 31). Results were normalized to β-Actin mRNA expression and presented relative to t=0. **B**: Sox9 protein expression peak during early ATDC5 differentiation as measured by immunoblotting. α-Tubulin was used as loading control. Molecular weight markers (in kDa) are shown on the left and relative quantifications are depicted on top of immunoblot. **C**: In similar samples from (A), Col2a1 mRNA expression was measured during ATDC5 differentiation by RT-qPCR and showed an increase in expression from day 6 onwards. **D**: GAG content (by Alcian Blue staining and corrected for total protein expression) was determined during ATDC5 differentiation. Experiments were performed in triplicate, bars represent mean±SEM. ns= not significant, *=$p<0.05$, **=$p<0.01$, ***=$p<0.0001$.

Figure 2: Elucidating the function of early Sox9 expression by transcriptome and proteome analyses

**A**: Schematic representation of experimental set-up for Sox9 knockdown experiment. Specific Sox9 RNAi (100 nM) or scrambled control RNAi (100 nM) were transiently transfected “early” at t=-1d or “late” t=6d. ATDC5 cells were differentiated from day 0 onwards and harvested for transcriptome and proteome analysis at at t=0, 2h, 5d, 7d, 14d. **B**: Sox9 mRNA expression during ATDC5 differentiation in Control and Sox9 RNAi conditions (h=hours, d=days) as measured by RT-qPCR. Results were normalized to β-Actin RNA expression and presented relative to t=0. Bars represent mean±SEM. ns=

not significant, *=p<0.05, **=p<0.01, p=<0.0001. **C**: Sox9 protein expression at t=2h (for "early knockdown condition) and t=7d (for "late" knockdown conditions) time point in Control and Sox9 RNAi conditions as measured by immunoblotting. α-Tubulin was used as loading control. Molecular weight markers (in kd) are shown on the left. **D**: Col2a1 mRNA expression in similar samples from (B). **E**: Col10a1 mRNA expression in similar samples from (B). **F**: Schematic representation of time points and comparisons for transcriptomics and proteomics analysis.

Figure 3: Changes in transcriptome and proteome after Sox9 knockdown

**A**: Venn diagram (67) showing the numbers of genes whose expression was significantly upregulated upon Sox9 knockdown at 2 hours versus 7 day condition. **B**: Venn diagram showing the numbers of genes whose expression was significantly downregulated upon Sox9 knockdown at 2 hours versus 7 day condition. **C**: Venn diagram showing the numbers of proteins whose expression was significantly upregulated upon Sox9 knockdown at 2 hours versus 7 day condition. **D**: Venn diagram showing the numbers of proteins whose expression was significantly downregulated upon Sox9 knockdown at 2 hours versus 7 day condition. **E**: Venn diagram showing the upregulated genes versus proteins at 2 hours in differentiation. **F**: Venn diagram showing the downregulated genes versus proteins at 2 hours in differentiation.

Figure 4: Early Sox9 expression is involved in ribosomal pathways

Top 3 identified enriched pathways from WikiPathway 2019 and KEGG2019 pathway analysis in control RNAi compared to Sox9 RNAi at 2 hours condition in ATDC5 differentiation for transcriptome and proteome data sets.

Figure 5: Early Sox9 regulates ribosomal protein expression

**A**: Schematic representation of significantly differentially expressed ribosomal proteins from transcriptome and proteome datasets based on Wikipathways (68): WP163 using PathVisio 3 (69). **B**: Rpl38, Rps30/Fau, Nop10 and SBDS expression at 2 hours in ATDC5 differentiation in Control and Sox9 RNAi conditions as measured by RT-qPCR. Results were normalized to β-Actin RNA expression and presented relative to t=0. **C**: In similar samples from B; 18S rRNA, 28S rRNA and 5.8S rRNA expression. Bars represent mean±SEM. ns= not significant, *=p<0.05, **=p<0.01, ***=p<0.0001.

Figure 6: Knockdown of early Sox9 expression leads to a reduced translational capacity, lower amount of ribosomes and alters ribosome modus

**A**: Total protein translation measurements based on puromycin incorporation was normalized to total DNA content per well (Mean±SEM, n=6/group) at indicated time points. **B**: Polysome fractionation of control and Sox9 knock-down cells (Mean only, n=3/group) after 2 hours of differentiation. **C**: Ribosome modus was assessed for the CrPv IGR CCGG mutant IRES, the intact CrPv IGR IRES, the HCV IRES and the P53 IRES after 24 hours of differentiation. Bars represent mean±SEM. ns= not significant, *=p<0.05, **=p<0.01, p=<0.0001.

Figure 7: Overexpression of early Sox9 leads to an increased translational capacity

**A**: Sox9 mRNA expression at 2 hours in ATDC5 differentiation in Control and Sox9 overexpression (OE) conditions as measured by RT-qPCR. Results were normalized to β-Actin RNA expression and presented relative to t=0. **B:** Total protein translation measurements based on puromycin incorporation was normalized to total DNA content per well (Mean±SEM, n=6/group) at indicated time points. **C**:

In similar samples from A; 18S rRNA, 28S rRNA and 5.8S rRNA expression at 2 hours in ATDC5 differentiation in Control and Sox9 overexpression conditions. **D:** In similar samples from A; *Rpl38, Rps30/Fau, Nop10* and SBDS expression at 2 hours in ATDC5 differentiation in Control and Sox9 overexpression conditions. Bars represent mean±SEM. ns= not significant, *=p<0.05, **=p<0.01, p=<0.0001.