

Viperin regulates chondrogenic differentiation via CXCL10 protein secretion

**Mandy M.F. Steinbusch<sup>1</sup>, Marjolein M.J. Caron<sup>1</sup>, Don A.M. Surtel<sup>1</sup>, Guus G.H. van den Akker<sup>1</sup>, Paul J. van Dijk<sup>2</sup>, Franziska Friedrich<sup>3</sup>, Bernhard Zabel<sup>4</sup>, Lodewijk W. van Rhijn<sup>1</sup>, Mandy J. Peffers<sup>5\*</sup>, Tim J.M. Welting<sup>1\*</sup>**

<sup>1</sup>Laboratory for Experimental Orthopedics, Department of Orthopedic Surgery, Maastricht University Medical Centre, Maastricht, the Netherlands

<sup>2</sup>Department of Anatomy and Embryology, Maastricht University, Maastricht, the Netherlands

<sup>3</sup>University Heart Centre Freiburg, Faculty of Medicine, University of Freiburg, Institute for Experimental Cardiovascular Medicine, Freiburg, Germany

<sup>4</sup>Medical Faculty, Otto-von-Guericke-University of Magdeburg, Magdeburg, Germany

<sup>5</sup>Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, United Kingdom

\*Authors contributed equally

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To whom correspondence should be addressed: Tim J.M. Welting, Ph.D., Department of Orthopaedic Surgery, Maastricht University Medical Centre, P.O. Box 5800, NL-6202 AZ, Maastricht, the Netherlands; [t.welting@maastrichtuniversity.nl](mailto:t.welting@maastrichtuniversity.nl); Tel. +31-43-3884157.

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## ABSTRACT

Viperin participates in the cell's innate immune response against a number of viruses. *Viperin* mRNA is a substrate for endoribonucleolytic cleavage by RNase MRP and mutations in the RMRP snoRNA subunit of the RNase MRP complex cause cartilage-hair hypoplasia (CHH), a human developmental condition characterized by metaphyseal chondrodysplasia and severe dwarfism. It is unknown how CHH-pathogenic mutations in RMRP snoRNA interfere with skeletal development and aberrant processing of RNase MRP substrate RNAs is thought to be involved. We hypothesized that viperin plays a role in chondrogenic differentiation and discovered that viperin is expressed in differentiating chondrocytic cells, regulates their protein secretion and outcome of chondrogenic differentiation through influencing TGF- $\beta$ /SMAD2/3 activity via CXCL10. Disturbances in this viperin-CXCL10-TGF- $\beta$ /SMAD2/3 axis were also found in CHH chondrocytic cells. Our data demonstrates that the anti-viral protein viperin controls chondrogenic differentiation by

influencing secretion of soluble proteins and for the first time identifies a molecular route that may clarify impaired chondrogenic differentiation of CHH patient cells.

## INTRODUCTION

Viperin, also known as RSAD2, is an interferon-inducible and evolutionary conserved protein that participates in the cell's innate immune response against a number of viruses. Viperin localizes to the cytosolic face of the endoplasmic reticulum (ER), mitochondria and lipid droplets. It is described to exert its antiviral properties via several pathways, including inhibition of soluble protein secretion, alterations of mitochondrial energy metabolism, inhibition of virus replication in lipid droplets and modulation of cellular signaling events (1). The mRNA of viperin was identified as a substrate for endoribonucleolytic cleavage by the RNase MRP small nucleolar ribonucleoprotein complex (2). Mutations in the RMRP gene, which encodes the essential small nucleolar (snoRNA) subunit of the RNase MRP complex, are known to be the cause of the human genetic disease cartilage-hair

hypoplasia (3,4) (CHH: OMIM #250250). A major phenotypic hallmark of CHH is impaired skeletal development characterized by metaphyseal chondrodysplasia, leading to a severe form of dwarfism (5). Growth plates are central drivers in the formation of the skeleton and their endochondral ossification depends on a tightly orchestrated continuing process of chondrogenic differentiation, ultimately resulting in longitudinal growth of the long bones (6). Although a number of RNase MRP substrates have been identified in man (2,4,7-9), including viperin, there is a major lack of studies investigating the role of RNase MRP substrate RNAs in development, and in chondrogenic differentiation in particular. It is expected that insight into the involvement of RNase MRP substrates in chondrogenic differentiation will provide insight into the molecular mechanism leading to impaired skeletal development in CHH. In concert with viperin mRNA as a substrate for RNase MRP cleavage, viperin expression levels have been described to be increased after knockdown of protein components of the RNase MRP complex in HEp-2 cells (2), in leukocytes of CHH patients (10) and recently we demonstrated that viperin expression is also upregulated after knockdown of RMRP RNA in chondrogenic differentiating ATDC5 cells(11). Since increased viperin expression as a result of interference with RNase MRP function appears to be conserved amongst several cell types, we hypothesized that viperin regulates the course of chondrogenic differentiation. In this study we examined the expression of viperin, determined its role in chondrocyte protein secretion, and investigated its crosstalk with the TGF- $\beta$ /SMAD2/3 signaling pathway in chondrogenic differentiation. We discovered that viperin is expressed in differentiating chondrocytic cells, regulates their protein secretion and impacts the outcome of the chondrogenic differentiation program through influencing TGF- $\beta$ /SMAD2/3 activity via CXCL10. Our data for the first time shows that the anti-viral protein viperin regulates chondrogenic differentiation by influencing the secretion of soluble proteins and highlights its involvement in impaired chondrogenic differentiation in CHH patient cells.

## RESULTS

### ***Viperin protein is expressed throughout chondrocyte differentiation***

To investigate whether viperin is expressed during early chondrogenic differentiation, tissue sections were prepared from E15.5 mouse embryos. Expression of viperin was detected in growth plate chondrocytes throughout the developing growth plate, with highest expression levels in proliferating and terminally differentiating hypertrophic chondrocytes (Figure 1A). Cells in the upper zone of the growth plate displayed dispersed viperin positivity; viperin expression was either barely detectable in individual cells, was weakly expressed, or was relatively high expressed in individual cells (Figure 1B). We further interrogated potential viperin expression during chondrogenic differentiation of the ATDC5 cell line (12,13) and of primary human bone marrow stem cells (hBMSCs) (14). Chondrogenic differentiation of ATDC5 follows a well-defined cellular differentiation program with temporally increasing Sox9 and Col2a1 levels (Figure 2A/B) and increasing levels of Runx2 and Col10a1 (Figure 2C/D). During the first 3 days of ATDC5 chondrogenic differentiation viperin mRNA expression was hardly detectable (Figure 2E). From day 4 through day 14 (the latest time point tested), viperin mRNA expression was induced as compared to undifferentiated ATDC5 cells (t=0), and with a prominent peak expression at day 5 and 6 in differentiation. Similar expression dynamics were observed for viperin protein expression in these chondrogenic cultures (Figure 2F). During chondrogenic differentiation of hBMSCs (COL2A1 and COL10A1 mRNA expression in Figure 2G/H), peak induction of viperin mRNA expression was detected at day 7 of differentiation (Figure 2I), which is in agreement with viperin expression during ATDC5 chondrogenic differentiation. Collectively these data show that developing chondrocytes express viperin *in vivo* and *in vitro*; with undetectable to low expression in the early differentiation phase and a profoundly increased expression later in differentiation.

***Viperin knockdown reduces, while viperin overexpression increases protein secretion***

Previous work by Cresswell and colleagues (15) showed that viperin regulates protein secretion from the ER and it has been suggested that this is one of the mechanisms by which viperin confers its cellular anti-viral activity. Since there is no other cell biological role identified for viperin so far that could explain its expression in differentiating chondrocytes, we postulated that viperin regulates protein secretion in differentiating chondrocytes. To investigate total protein secretion in relation to viperin levels we introduced a secretable Gaussia-luciferase (pGluc-CMV) at either day 3 or 5 in ATDC5 differentiation, followed by transfection of either a viperin siRNA duplex or a p3xFLAG-viperin plasmid at day 4 or 6 in differentiation, followed by sampling at day 5 or 7, respectively. Reduced expression of viperin at day 5 or 7 in differentiation was demonstrated upon siRNA-mediated knockdown (Figure 3A) and ectopic expression of FLAG-viperin was confirmed at same time points (Figure 3A). Bioluminescent analyses showed that the amount of secreted Gaussia-luciferase was reduced following viperin knockdown and increased with viperin overexpression (Figure 3B). This was the case at both day 5 and 7 of differentiation. These data indicate that overall cellular protein secretion responds to changing viperin levels during chondrogenic differentiation.

***The viperin-controlled secretome influences chondrogenic differentiation***

Considering the central role secreted signaling molecules play during chondrogenic differentiating (6,16), we next questioned whether the observed viperin-controlled protein secretion plays a role in determining the chondrogenic outcome of the differentiation process via control over the chondrocyte's secretome. We investigated this possibility by reducing endogenous viperin levels or overexpressing viperin in differentiating ATDC5 chondrocytes and collecting conditioned media (CM) from these donor cultures. These CM were subsequently used to differentiate new ATDC5 cultures. Altered chondrogenic capacity of these cultures would reveal whether reduced or increased viperin levels during chondrogenic

differentiation differentially influence the chondrocyte's secretome with downstream consequences for chondrogenic differentiation. Knockdown or over-expression of viperin was confirmed in the donor cultures from which CM were obtained (Figure 4A). Differentiation of ATDC5 cells in CM obtained from donor cultures in which viperin levels were reduced led to an overall increase in chondrogenic capacity, evidenced by increased expression of Sox9, Col2a1, Runx2, Col10a1 and Alpl (Figure 4B). Reciprocally, CM obtained from differentiating ATDC5 donor cultures in which viperin levels were over-expressed, displayed an inhibitory effect on the chondrogenic differentiation capacity, evidenced by reduced Sox9, Col2a1, Runx2, Col10a1 and Alpl expression (Figure 4C). The stimulatory and inhibitory actions of these CM on chondrogenic differentiation were further confirmed by determination of the glycosaminoglycan (GAG) content of these cultures. Differentiation of ATDC5 in CM obtained from donor cultures with reduced viperin levels caused an increased GAG content, while differentiation of ATDC5 in CM obtained from donor cultures in which viperin was over-expressed led to a reduced GAG content (Figure 4D). These results together indicate that alterations in viperin levels during chondrogenic differentiation change the cell's secretome with important functional consequences for differentiation.

***Differential CXCL10 levels in viperin knockdown and overexpression secretomes***

Since media obtained from donor cultures with reduced or over-expressed viperin levels enhance or inhibit chondrogenic differentiation (respectively), we postulated that this was caused by a differential protein composition of their secretomes. To determine the differential proteome of the conditioned culture supernatants obtained from differentiating ATDC5 cultures with reduced or over-expressed viperin levels, we undertook a label-free mass-spectrometry proteomics approach using liquid chromatography tandem mass spectrometry (LC-MS/MS). When comparing the conditioned culture supernatants of differentiating ATDC5 cultures in which a scrambled siRNA was transfected or in which viperin levels were

reduced using the viperin siRNA (Figure 5A), we identified six protein species that were differentially expressed in the CM (Figure 5C). When comparing the conditioned culture supernatants of differentiating ATDC5 cultures in which an empty FLAG-vector was transfected or in which viperin levels were over-expressed using the FLAG-viperin plasmid (Figure 5B), we identified eight differentially expressed proteins in the CM (Figure 5C). Interestingly, CXCL10 (grey highlighted in Figure 5C) was the only protein species that was detected in both differential secretome proteomes. CXCL10 was decreased in CM obtained from cultures in which viperin levels were reduced and was increased in conditioned medium obtained from cultures in which viperin levels were over-expressed. Differential CXCL10 protein levels in these culture supernatants were independently confirmed by ELISA (Figure 5D). Since expression of both viperin and CXCL10 are interferon-dependent (1,17) (supplementary Figure 1), we next verified CXCL10 secretion dynamics during chondrogenic differentiation of ATDC5. Using an ELISA, supernatant samples from cultures in Figure 2 were analyzed for CXCL10 protein levels and we found that CXCL10 levels in culture supernatant (Figure 5E) during ATDC5 chondrogenic differentiation mirrored viperin expression dynamics (Figure 2E/F). Data together demonstrates that alterations in viperin levels during chondrogenic differentiation lead to specific differences in the protein composition of the chondrocyte's secretome, with CXCL10 a shared factor between the differential viperin-dependent secretomes.

#### ***CXCL10 inhibits chondrogenic differentiation***

Conditioned culture medium obtained from differentiating ATDC5 cultures in which viperin levels were over-expressed was found to inhibit chondrogenic differentiation. Besides a limited number of other DE proteins, CXCL10 was the only shared protein species that was differentially present in the viperin-dependent secretomes and was increased in viperin over-expression culture supernatant. We therefore postulated that CXCL10 contributes to the inhibitory action on chondrogenic differentiation of the viperin over-expression culture supernatant (Figure 4C). To determine whether CXCL10

inhibits chondrogenic differentiation, differentiating ATDC5 cultures were exposed to exogenously added mCXCL10 from day 5 of differentiation until day 7 of differentiation (the main time frame in which we observed peak expression of viperin and peak CXCL10 concentrations; Figure 2E/F and Figure 5E). Analysis showed that exogenously added mCXCL10 attenuated chondrogenic differentiation, evidenced by decreased expression of Sox9, Col2a1, Runx2, Col10a1 and Alpl (Figure 6A). To verify the inhibitory action of CXCL10 in an independent model for chondrogenic differentiation, primary hBMSCs were differentiated into the chondrogenic lineage and exposed to increasing concentrations hCXCL10 from day 7 (peak viperin expression in hBMSC chondrogenic differentiation; Figure 2I) in chondrogenic differentiation onward. CXCL10 inhibited chondrogenic differentiation of hBMSCs, evidenced by a decline of COL2A1 and COL10A1 mRNA expression (Figure 6B). These data indicate that viperin-dependent alterations in secreted CXCL10 levels influence chondrogenic differentiation capacity.

#### ***Viperin levels and CXCL10 change SMAD2/3-dependent TGF- $\beta$ activity during chondrogenic differentiation of ATDC5***

The chondro-promotive and -inhibitory actions of CM obtained from donor cultures in which viperin expression was altered, as well as the chondro-inhibitory action of CXCL10, leads to an overall inhibited or increased expression of chondrogenic as well as hypertrophic marker genes. This indicates that the activity of a basic molecular pathway responsible for chondrogenic differentiation is altered and we therefore postulated the involvement of the major chondrogenic TGF- $\beta$ /SMAD2/3 pathway. Viperin levels were either reduced or over-expressed in differentiating ATDC5 cultures from day 5 to day 7 (Figure 7A), and CM were collected. Subsequently, proliferating ATDC5 cells were used as a bioassay for SMAD2/3-dependent TGF- $\beta$  activity by transfection of a CAGA12-luciferase reporter plasmid (18,19). This TGF- $\beta$ /SMAD2/3 reporter bioassay was then exposed to the above CM to determine their TGF- $\beta$ /SMAD2/3-activity modulating action. We found that the CM obtained from

differentiating ATDC5 cultures in which viperin levels were reduced displayed a TGF- $\beta$ /SMAD2/3-activity promoting action (Figure 7B). In contrast, CM obtained from differentiating ATDC5 cultures in which viperin levels were over-expressed displayed a TGF- $\beta$ /SMAD2/3-activity attenuating action (Figure 7B). In concert with these findings Pail and SMAD7 mRNA levels (TGF- $\beta$ /SMAD2/3 target genes(18,20)) were increased or decreased when ATDC5 was differentiated in CM (same as utilized in experiments shown in Figure 4B/C) from viperin knock-down or over-expression cultures, respectively (Figure 7C/D). Subsequently, we determined whether differential CXCL10 levels directly alter TGF- $\beta$ /SMAD2/3 activity during chondrogenic differentiation. ATDC5 cells were transfected with the CAGA12-luciferase reporter plasmid at day 5 in chondrogenic differentiation. Then, starting at day 6, cells were exposed to increasing concentrations recombinant murine CXCL10 and sampled for bioluminescent analyses 24 hours later. Data show that CXCL10 dose-dependently reduces TGF- $\beta$ /SMAD2/3 reporter activity in these cultures (Figure 7E). Immunoblotting for SMAD2 phosphorylation (pSMAD2C) further showed that CXCL10 inhibits SMAD2 phosphorylation during ATDC5 chondrogenic differentiation (Figure 7F), which is in concert with the observed reduction of TGF- $\beta$ /SMAD2/3 reporter activity. Together these results demonstrate that alterations in viperin expression levels and CXCL10 impact SMAD2/3-dependent TGF- $\beta$  activity during chondrogenic differentiation. This is in concert with the observed impact of these conditions on chondrogenic differentiation capacity (Figure 4B/C/D and Figure 6).

***Impaired chondrogenic trans-differentiation of fibroblasts from CHH patients is associated with increased viperin and CXCL10 levels***

Mutations in the RMRP gene, encoding the essential RMRP snoRNA present in the RNase MRP macromolecular protein-RNA complex (4), are the cause of cartilage-hair hypoplasia-type human skeletal dysplasias (3,5). Viperin mRNA is a substrate for endoribonucleolytic degradation by RNase MRP (2). Taking into account the here identified

chondrogenic regulatory role of a viperin-CXCL10-TGF- $\beta$ /SMAD2/3 axis, we postulated that a similar mechanism is active during the impaired chondrogenic trans-differentiation that we recently discovered in CHH fibroblasts (11). In ATDC5, reduction of RMRP snoRNA levels by transfection of an RMRP-specific siRNA (Figure 8A) indeed led to the induction of viperin expression ((11) and Figure 8B), recapitulating the endoribonucleolytic relationship between RNase MRP and its viperin mRNA substrate. A trans-differentiation protocol was used that drives dermal fibroblasts toward a chondrocyte-like phenotype (21). As described before (11), RMRP expression and chondrocytic trans-differentiation of CHH fibroblasts was impaired, as evidenced by inhibited expression of RMRP, SOX9, COL2A1, RUNX2, COL10A1 and ALPL (Figure 8C). In concert with a CHH-associated pathological defective RNase MRP endoribonucleolytic activity, we found that viperin expression in chondrogenic trans-differentiated CHH fibroblasts was increased (Figure 8D). This was accompanied with increased levels of secreted CXCL10 protein in these culture supernatants (Figure 8E) and decreased expression of the TGF- $\beta$  target genes PAIL and SMAD7 (Figure 8F). These data suggest that defective chondrogenic trans-differentiation of CHH fibroblasts is caused by changes in TGF- $\beta$  activity, induced by alterations in the viperin-CXCL10-TGF- $\beta$ /SMAD2/3 axis.

**DISCUSSION**

Viperin expression was detectable early in ATDC5 and hBMSC chondrogenic differentiation and was highly induced at day 5-6-7 of differentiation. In the majority of embryonal growth plate resting zone progenitor cells viperin expression was weak, representing the first days of *in vitro* chondrogenic differentiation. Interestingly, a sub-population of resting zone cells displayed high levels of viperin expression. Chondrogenic differentiation is synchronously initiated in cell culture, while this is not expected to be synchronous in the growth plate's resting zone. We speculate that high viperin-expressing cells in the resting zone are a representation of asynchronous initiation of chondrogenic differentiation and that these cells

are in a similar differentiation phase as ATDC5 and hBMSC cells during their early differentiation, when peak expression of viperin was observed. At later stages in chondrogenic differentiation viperin expression remained increased. This was also observed in growth plates, where proliferating and pre-hypertrophic chondrocytes are positive for viperin expression. Viperin expression is remarkably high in terminally differentiating growth plate chondrocytes. This was not observed in late ATDC5 and hBMSC differentiation time points tested. ATDC5 and hBMSCs are excellent *in vitro* models for chondrogenic differentiation (13). However these models do not enter apoptosis to fully terminally differentiate, as hypertrophic chondrocytes in the growth plate do. The spatiotemporal orchestration of cell differentiation in growth plates and the lack of such spatiotemporal cues *in vitro* may lead to the absence of this differentiation phase in *in vitro* chondrogenesis models and explain the observed difference in viperin expression compared to growth plates.

Viperin was originally discovered as an interferon-inducible protein, suggesting that induction of viperin expression during chondrogenic differentiation may be driven by an intrinsic interferon-related signaling activity (further supported by data in supplementary Figure 1). Interferon-related signaling during chondrogenic differentiation is almost unexplored. Interferon-inducible proteins p202 (22), p204 (23) and PKR (24) have been described to be important in chondrogenic differentiation. Inhibition of JAK rescues chondrogenic differentiation in osteoarthritis-like conditions (25) and IFN- $\gamma$  has been demonstrated to inhibit transcription of the COL2A1 gene (26,27) in mature chondrocytes. STAT1 expression was previously found specifically induced at day 7 in ATDC5 chondrogenic differentiation (24), supporting an intrinsically activated interferon signaling response as explanation for viperin induction at day 5-6-7. Different potential mechanisms to explain viperin's antiviral actions include an Fe-S cluster-dependent virus inhibitory action on lipid droplets (15,28), the inhibition of farnesyl diphosphate synthase leading to important changes in plasma membrane fluidity (29), the

induction of a crystalloid ER (15), and the inhibition of protein secretion from the ER (15). Viperin has been shown to recruit IRAK1 and TRAF6 to lipid droplets in pDCs, leading to nuclear translocation of IRF7 and the production of type I interferons (30). These actions are specifically activated in response to viral infection and it is therefore surprising to find that in a non-viral context viperin is active as a regulator of cellular differentiation. Previously, alterations in viperin expression have been detected during adipogenic differentiation (31) and podocyte differentiation (32). Middle zone articular cartilage chondrocytes express higher amounts of viperin as compared to superficial zone chondrocytes (33) and viperin expression has been described in osteocytes (34). It is therefore likely that the consequences of viperin expression are not limited to differentiating chondrocytes. Until now it remained unexplored whether alterations in viperin levels could influence cellular differentiation processes. Following the findings by Cresswell and colleagues that viperin overexpression inhibits protein secretion from the ER in HepG2 and 293T cells (15), we examined whether this was also the case during chondrogenic differentiation. We found that also in chondrogenic differentiation viperin influences the rate of protein secretion, however in the opposite direction as compared to the findings reported by the Cresswell group. We speculate that due to different cell lineages, differentiation stages and the non-viral context, important differences occur in the viperin interactome (30) that change the manner by which viperin regulates protein secretion from the ER.

We observed that total protein secretion alters in response to viperin levels, and until now no specificity has been reported for viperin-regulated protein secretion. Therefore we expect that the protein species that we found differentially secreted following manipulation of viperin levels are either relatively unstable and inhibition of cellular protein secretion would thus cause a rapid decrease in the level of this species within the secretome, or are secreted in low abundance and increased protein secretion would rapidly lead to detectable differences in the level of this protein species in the secretome. In line with a viperin-mediated intrinsic interferon

response during chondrogenic differentiation, the CXCL10 we detected in our LC-MS/MS analyses is an interferon-inducible chemokine (IP-10). Interestingly, platelet-derived growth factor subunit A (PDGFA) was also found differentially expressed in the viperin over-expression secretome and PDGF has been described to synergistically act with IFN- $\gamma$  to induce CXCL10 expression in blood-derived macrophages (17). CXCL10 has previously been described to be expressed during ATDC5 chondrogenic differentiation (35), as well as during hBMSC chondrogenic differentiation (36). Our data demonstrate that CXCL10 inhibits chondrogenic differentiation and TGF- $\beta$  signaling, which is fully in line with the inhibitory action of viperin-overexpression CM on chondrogenic differentiation.

Since we utilized conditioned media as a means to study the secretomic consequences of alterations in viperin expression during chondrogenic differentiation, we cannot distinguish paracrine from autocrine secreted signals. However, it is currently not clear why chondrogenic differentiation is regulated via viperin. Control over the activity of differentiation signals in the early differentiation phase may be required to enable the cell to undertake chromatin remodeling and coordinated transcriptomic reprogramming, before it can adopt a fully differentiated phenotype. We expect that temporal para- and/or autocrine viperin-dependent CXCL10 secretion may aid in this by antagonizing TGF- $\beta$  signaling and pacing cellular differentiation. Binding of CXCL10 with its CXCR3 receptor is known to activate PI3K in human airway epithelial cells (37) and recently, PI3K activity has been shown to be involved in attenuation of SMAD2/3 activity (38). Indeed we observed that CXCL10 inhibits phosphorylation of SMAD2, providing a potential mechanistic link between viperin-dependent CXCL10 secretion and TGF- $\beta$ -driven chondrogenic differentiation. Alternatively, crosstalk has been identified between IFN- $\gamma$  and TGF- $\beta$  signaling in which IFN- $\gamma$ -dependent STAT1 activity antagonizes SMAD3-dependent TGF- $\beta$  signaling (39).

With a number of varying RNase MRP substrate RNAs it would not be likely that all aspects of CHH pathobiology are caused by

defective processing of one specific substrate. Instead, defective processing of one or the other specific RNase MRP substrate RNA will have different implications for different tissue/cell types. CHH-related defective processing of viperin mRNA leads to aberrant viperin levels in chondrocytic cells as shown in this study. Viperin responses (40) and CXCL10 (41) have been implicated in T cell function and in a microarray mRNA profiling it was found that viperin was one of the highest differentially expressed genes upregulated in CHH leukocytes (10), together with an enrichment of other interferon-related genes. Since CHH also presents with defective immunity caused by T-cell deficiency (42) and together with our findings that viperin and CXCL10 regulate chondrogenic differentiation, is thus tempting to speculate that interferon-related signaling through viperin, and via CXCL10, is an important aspect of the molecular mechanism leading to growth plate and T-cell defects observed in CHH.

In conclusion our data link the antiviral protein viperin to chondrogenic development via a viperin-CXCL10-TGF- $\beta$ /SMAD2/3 axis (Figure 9), and show that a similar molecular mechanism is deregulated in CHH chondrocytic cells. For the first time we identified a molecular route that may clarify impaired chondrogenic differentiation of CHH patient cells.

## EXPERIMENTAL PROCEDURES

### *Immunohistochemistry (IHC)*

Five micrometer-thick formalin-fixed paraffin-embedded tissue sections were prepared from E15.5 NMRI mouse embryos. Use of the embryos was approved by the University of Freiburg (number X-14/10H), according to German law; and methods utilized to obtain the embryos were carried out in accordance with German law. Sections were deparaffinized in a xylene / ethanol series ending in PBS (136 mM NaCl (Merck Millipore, Darmstadt, Germany), 2.7 mM KCl (Merck Millipore), 9.0 mM Na<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O (Merck Millipore), 1.8 mM KH<sub>2</sub>PO<sub>4</sub> (Merck Millipore)). For antigen retrieval, sections were incubated in hot citrate buffer (1.8 mM citric acid (Sigma-Aldrich, St. Louis, MO, USA) and 8.2 mM tri-sodium citrate (VWR

Prolabo, Amsterdam, the Netherlands)) for 30 minutes. Endogenous peroxidase activity was inactivated using peroxidase-blocking solution (Dako, Troy, MI, USA). Slides were blocked with 10% normal sheep serum in PBS-T (0.1% Tween 20; Sigma-Aldrich). Primary antibody was incubated for 2 hours at room temperature. Mouse monoclonal anti-Viperin (Merck Millipore #MABF106) was used at a 1:200 dilution. IgG2a (Dako) was used as an isotype control. Bound antibodies were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse secondary antibodies (Dako EnVision) for 30 minutes at room temperature. DAB chromogen substrate (Dako) was used for detection. Sections were counterstained with haematoxylin (Dako), dehydrated and mounted with histomount (Thermo Shandon, Waltham, MA, USA). Microphotographs were acquired using a Zeiss AxioScope A1.

#### ***Cell culture and differentiation of ATDC5***

ATDC5 cells (12) (RIKEN BRC, Japan) were cultured in a humidified atmosphere at 37°C, 5% CO<sub>2</sub> in proliferation medium (DMEM/F12 (Invitrogen, Carlsbad, CA, USA), 5% fetal calf serum (FCS) (Sigma-Aldrich), 1% antibiotic/antimycotic (Invitrogen), 1% non-essential amino acids (NEAA) (Invitrogen)). Cells were tested negative for potential mycoplasma infection. To induce chondrogenic differentiation, cells were plated at 6,400 cells/cm<sup>2</sup>. After 24 hours, chondrogenic differentiation was initiated by changing the medium to differentiation medium (proliferation medium supplemented with 10 µg/ml insulin (Sigma-Aldrich), 10 µg/ml transferrin (Roche, Basel, Switzerland), 30 nM sodium selenite (Sigma-Aldrich)). Differentiation medium was refreshed every two days for the first 10 days, and each day after day 10. In addition, ATDC5 cells were differentiated for 5 days and then exposed to 0.5 or 5 ng/ml recombinant murine CXCL10 (Peprotech, Rocky Hill, NJ, USA, #250-16) until day 7 in differentiation. When ATDC5 cells were differentiated using conditioned media (CM), the CM was refreshed every other day.

#### ***Cell culture and differentiation of hBMSCs***

Human bone marrow stem cells (hBMSCs) were obtained from residual iliac crest

bone marrow aspirate from young, genetically healthy, individuals undergoing spinal surgery. Approval from the Maastricht University Medical Center Medical Ethical Committee (MEC) for the use of this material was obtained and assigned approval ID: MEC 08-4-056. Human BMSCs from four individual donors were isolated from the aspirate using Ficoll Paque (Amersham). Proliferation medium consisted of DMEM high-glucose (Invitrogen), 10% FCS (ES-grade), 1% antibiotic/antimycotic and 1% NEAA. Passage 3 cells were plated at 30,000 cells/cm<sup>2</sup> in quadruplicates and chondrogenic differentiation was initiated the next day by changing to differentiation medium (proliferation medium supplemented with 1% ITS (Invitrogen), 50 µg/ml L-ascorbic acid-2-phosphate (Sigma) and 1 ng/ml TGFβ3 (R&D)) (14). Medium was changed every 2 days. Human CXCL10 (Peprotech, Rocky Hill, NJ, USA) was used at indicated concentrations (0.5, 5, 50, 200 ng/ml). Cells were harvested in TRIzol at day 0, 2, 7, 14 and 21 in differentiation for RNA isolation.

#### ***Real time quantitative PCR (RT-qPCR)***

RNA was extracted from cells with TRIzol reagent (Invitrogen) and isolated by collecting the aqueous phase after phase separation. RNA was precipitated with isopropanol (30 minutes, -80°C) and centrifuged for 30 minutes at 15,000 RPM, 4°C. RNA pellets were washed with 80% ethanol and potential DNA contamination was removed by DNase I (Roche) treatment (1 hour, 37°C). After subsequent ethanol precipitation, RNA was dissolved in 15 µL DNase/RNase free water (Eurogentec, Seraing, Belgium). RNA quantity and purity were determined spectrophotometrically (Biodrop, Isogen Life Sciences, Utrecht, the Netherlands). Reverse transcription of RNA and RT-qPCR were performed as described previously (11). Housekeeping genes were β-Actin for ATDC5 and CYCLOPHILIN for hBMSCs and human fibroblasts. Primer sequences are provided in Table 1.

#### ***Immunoblotting***

Cells were washed three times with 0.9% NaCl. The 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 EDTA pH 8.0, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF) and phosphatase inhibitor (PhosSTOP; Roche). Samples were homogenized by sonication (Soniprep 150 MSE) using the following protocol: 14 cycles of 1 second sonication followed by a 1 second interval, amplitude 10. Cell debris was removed by centrifugation. Total protein concentration was determined with a BCA assay (Sigma-Aldrich). Samples were separated by gel electrophoresis and transferred to nitrocellulose membranes by electro-blotting. The following primary antibodies were used for immuno-detection: Mouse monoclonal anti-Viperin (Merck Millipore #MABF106; 1:500 dilution) and rabbit monoclonal anti-pSMAD2C (Cell Signaling Technologies #3108; 1:1000 dilution). As controls mouse monoclonal anti-Histone H3 (Abcam #24834; 1:1000 dilution), mouse monoclonal anti-alpha-Tubulin (Sigma-Aldrich #T6074; 1:10,000 dilution) and mouse monoclonal anti-GAPDH (Fitzgerald #10R-G109b; 1:5000 dilution) were used. HRP-conjugated polyclonal rabbit anti-mouse (Dako) or HRP-conjugated swine anti-rabbit (Dako) was applied as a secondary antibody and the bound antibodies were detected by enhanced chemiluminescence (ECL).

#### ***Transfection of siRNAs***

Chondrogenic differentiating ATDC5 cells were transfected (Pepmute; SignaGen Laboratories, Rockville, MD, USA; according to the manufacturer's protocol) with 100 nM siRNA duplexes (custom synthesized by Eurogentec, see Table 2) targeting Viperin (on day 3 or day 5 of chondrogenic differentiation) or Rmrp RNA (on day -1, 2, and 5 of chondrogenic differentiation). Scrambled siRNA duplex was purchased from Eurogentec; REF: SR-CL000-005.

#### ***Plasmids and transfection***

The viperin coding sequence, flanked by *NotI* - *XbaI* restriction sites, was custom-made synthesized by GeneCust (Ellange, Luxembourg) and originally cloned in pUC57 vector and sequence verified. The insert was then sub-cloned into the p3xFLAG plasmid (Promega, Southampton, UK) using the *NotI* - *XbaI* restriction sites, yielding the viperin

overexpression plasmid (FLAG-viperin). On day 4, 5 or 6 of ATDC5 chondrogenic differentiation, viperin expression was further induced in 6-well plates by transfection of 625 ng FLAG-viperin plasmid or an empty plasmid (FLAG-empty) as a control, using polyethylenimine (PEI; Polysciences, Warrington, PA, USA) transfection reagent. DNA and PEI were complexed for 10 minutes at room temperature in DMEM/F12 (1.88  $\mu$ l PEI (1  $\mu$ g/ $\mu$ l) per 625 ng construct per well) and added to the ATDC5 cells. To determine protein secretion 250 ng CMV promoter-driven secreted Gaussia luciferase plasmid (pGluc-CMV(43)) was co-transfected with 200 ng CMV promoter-driven firefly luciferase pGL4.20-CMV (control; Promega) on day 3 or day 5 of ATDC5 chondrogenic differentiation (6-well format), using PEI transfection reagent. Culture supernatant and cells were collected for bioluminescence analyses and Gaussia bioluminescence was normalized to the firefly signal. Viperin levels were either reduced or overexpressed in differentiating ATDC5 cultures from day 5 to day 7 and conditioned culture supernatants were collected. Subsequently, proliferating ATDC5 cells were used as a bioassay for SMAD3-dependent TGF $\beta$  activity by transfection with 500 ng CAGA12-luciferase reporter plasmid (18) (kind gift of Dr. Dalmay (19)) and 125 ng pGluc-CMV (43) plasmid as a transfection control, using PEI transfection reagent. The proliferating ATDC5 cells were then exposed to the above mentioned conditioned culture supernatants for 24 hours to determine the TGF $\beta$ /SMAD3-activity, where firefly bioluminescence was normalized to the Gaussia signal. The action of exogenously added CXCL10 on SMAD3-dependent TGF- $\beta$  activity during chondrogenic differentiation was determined by transfecting day 5-differentiating ATDC5 cells in 6-well format with 500 ng CAGA12-luciferase reporter and 125 ng pGluc-CMV plasmid as a transfection control, using PEI transfection reagent. Twenty-four hours later cells were exposed to 0.5, 5, 20 or 200 ng/ml recombinant murine CXCL10 (Peprotech #250-16). At day 7 in differentiation, culture supernatant and cells were then collected for bioluminescence analyses and firefly bioluminescence was normalized to the Gaussia signal. pGluc-CMV (Gaussia luciferase),

pGL4.20-CMV (firefly luciferase) and CAGA-12 (firefly luciferase) samples were all harvested for bioluminescence detection using the Dual Luciferase reporter assay system (Promega) as described by the manufacturer and measured on a Fluostar Omega plate reader (BMG Labtech, Ortenberg, Germany).

#### ***Alcian Blue and Crystal Violet staining and quantification***

Cells were washed twice with 0.9% NaCl and fixated with 4% paraformaldehyde in PBS for 10 minutes at room temperature. Fixated cells were washed 6 times with distilled water and air-dried. Dried fixated cells were incubated for 30 minutes at room temperature with respectively 1% (m/v) Alcian Blue (Acros Organics, Geel, Belgium) in 0.1 M HCl or 0.1% (m/v) Crystal Violet (Sigma-Aldrich). Cells were washed six times with distilled water and allowed to air dry. Alcian Blue was extracted by incubation with 6 M Guanidine-HCl (Sigma-Aldrich) for 2 hours on a plate shaker (IKA HS 260 Basic, IKA, Staufen, Germany). Crystal Violet was extracted by incubation with 10% acetic acid (VWR) for 15 minutes on a plate shaker (IKA). Extracted Alcian Blue and Crystal Violet were quantified spectrophotometrically at 645 nm or 590 nm respectively using a plate reader (ThermoScientific Multiskan FC, Waltham, MA, USA). Crystal Violet (DNA content) was used as normalization for Alcian Blue glycosaminoglycan (GAG) content.

#### ***In-solution tryptic digestion and mass spectrometry proteomics of media following viperin knockdown or overexpression***

At day 5 in ATDC5 chondrogenic differentiation, viperin expression was either reduced by transfection of a viperin-specific siRNA duplex or a scrambled control siRNA duplex; or viperin expression was further induced by transfection of the FLAG-viperin plasmid or the empty FLAG plasmid. Cells were then further differentiated until day 7 and conditioned medium (CM) was collected. During differentiation phenol-red free DMEM-F12 (Invitrogen) was used in order to accommodate mass spectrometry analysis. To obtain sufficient amounts of CM, the experiment was simultaneously performed in three triplicates for

each condition. Triplicates were then pooled to obtain three times scrambled siRNA duplex, three times viperin siRNA duplex, three times empty FLAG and three times FLAG-viperin overexpression CM samples. Two representative triplicates from each condition were utilized to confirm viperin knockdown and overexpression by means of immunoblotting. The CM was centrifuged for 5 minutes at 1200 rpm, to remove dead cells, and subsequently for 5 minutes at 13,000 rpm to remove remaining debris. Then, Complete Ultra protease inhibitors (EDTA-free; Roche) were added. Samples were stored at -80°C prior to downstream analysis. Protein concentrations of CM were calculated using the Bradford assay with Coomassie Plus™ protein assay reagent (Thermo Scientific, Rockford, IL, USA), read at 660 nm. In solution tryptic digestion was undertaken as previously described (44). Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed on trypsin digests using an Ultimate 3000 Nano system (Dionex, ThermoFisher Scientific, Waltham, MA, USA) on line to a Q-Exactive Quadrupole-Orbitrap instrument (Thermo Scientific) (45). Proteins were identified using Peaks® 7 PTM software (Version 7, Bioinformatics Solutions, Waterloo, Canada), searching against the UniMouse database, with a false discovery rate (FDR) of 1%, a minimum of two unique peptides per protein and a confidence score > 50.

#### ***Label-free quantification of mass spectrometry proteomics data***

Progenesis QI™ software (Version 4, Waters, Manchester, UK) was used to identify fold changes in protein abundance between scrambled siRNA duplex and viperin knockdown siRNA duplex and between the FLAG-empty and FLAG-viperin overexpression constructs as described previously(46). Only unique peptides were used for quantification, and with p-values <0.05, were considered to be differentially expressed (DE).

#### ***CXCL10 ELISA***

To determine CXCL10 protein concentrations in media, the mouse CXCL10/IP-10/CRG-2 DuoSet ELISA (R&D #DY466) and the human CXCL10/IP-10 DuoSet ELISA (R&D

#DY266) were utilized according to the manufacturer's protocol. Plates were colorimetrically measured on a ThermoScientific Multiskan FC plate reader (Waltham, MA, USA).

### ***Chondrogenic trans-differentiation of human dermal fibroblasts***

Human dermal fibroblasts (passage 7-9) from 3 CHH patients (RMRP alleles of CHH patients carried the following mutations: 127G>A and 261 C>G; 4 C>T and 77C>T; 70 A>G and 70A>G) and 3 healthy controls (Ethical permission was obtained from the medical ethical Institutional Review Board of Freiburg University Hospital and methods and experimental protocols to obtain dermal fibroblasts were carried out in accordance with the Freiburg University Hospital medical ethical Institutional Review Board, according to German law. Informed consent was obtained from all subjects) were hyperconfluently plated (100,000 cells/cm<sup>2</sup>) in aggrecan-coated (Sigma-Aldrich; 2.5 µg/cm<sup>2</sup>) wells as previously described(21). Cells were directly plated in trans-differentiation medium which consisted of DMEM/F12+Glutamax (Invitrogen), 10% FCS (PAA), 1% antibiotic/antimycotic (Invitrogen), 1% NEAA (Invitrogen) + 1% ITS (Insulin, Transferrin, Selenium-Sodium Pyruvate; Life Technologies), 50 µg/ml ascorbic acid 2-phosphate (Sigma-Aldrich) and 1 ng/ml human recombinant TGF-β3 (Life Technologies). Trans-differentiation medium was refreshed on day 2

and trans-differentiated cartilaginous nodules were harvested in TRIzol on day 3 of trans-differentiation for RNA isolation.

### ***Statistics***

Gene expression, ELISA, colorimetric and bioluminescence data were statistically analyzed using GraphPad Prism 5 (La Jolla, CA, USA). A two-tailed independent samples t-test was performed relative to the corresponding control condition. Individual values are presented in graphs in dot plots and the p-values and mean ± SEM are indicated in the figures. The number of biological replicates and number of repeated experiments are indicated in the figure legends. Statistical significance was defined as p<0.05.

### ***Data availability***

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (47) with the dataset identifier PXD006999 and 10.6019/PXD006999. All other relevant data are available from the authors.

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## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

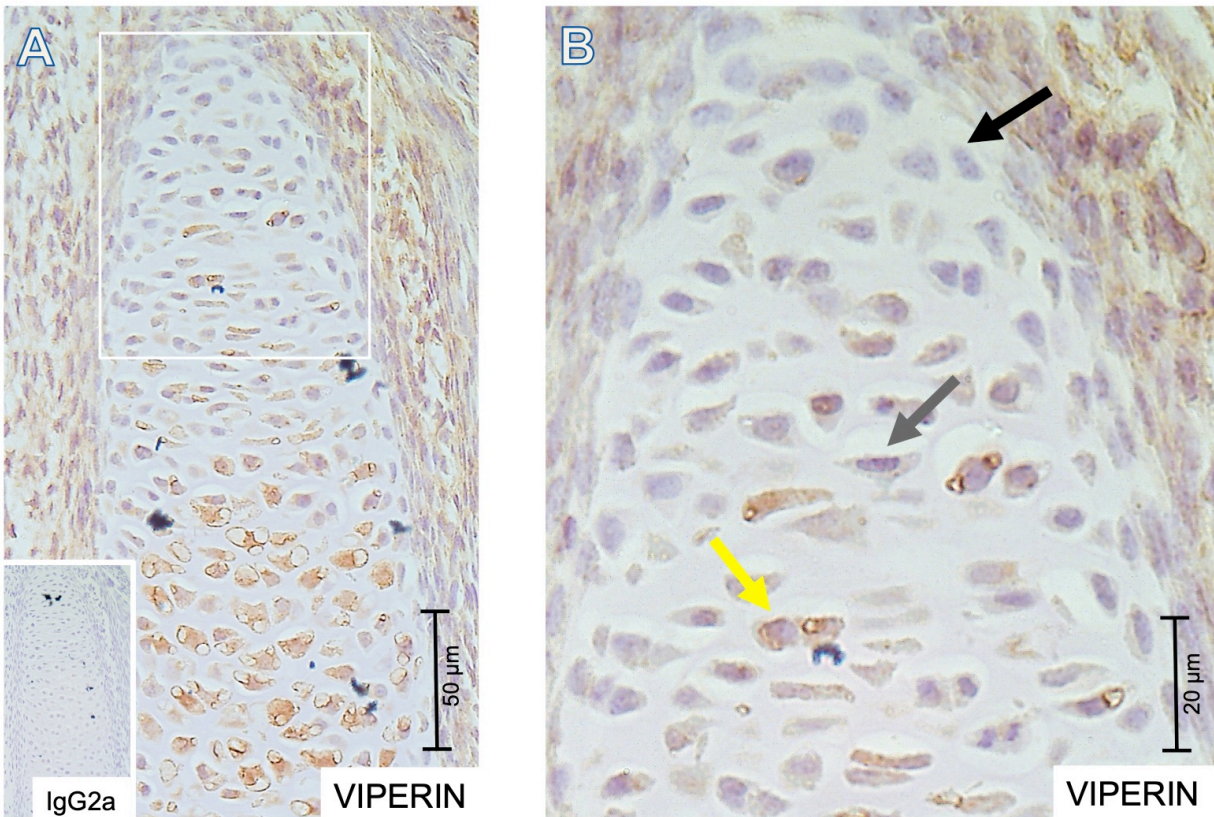
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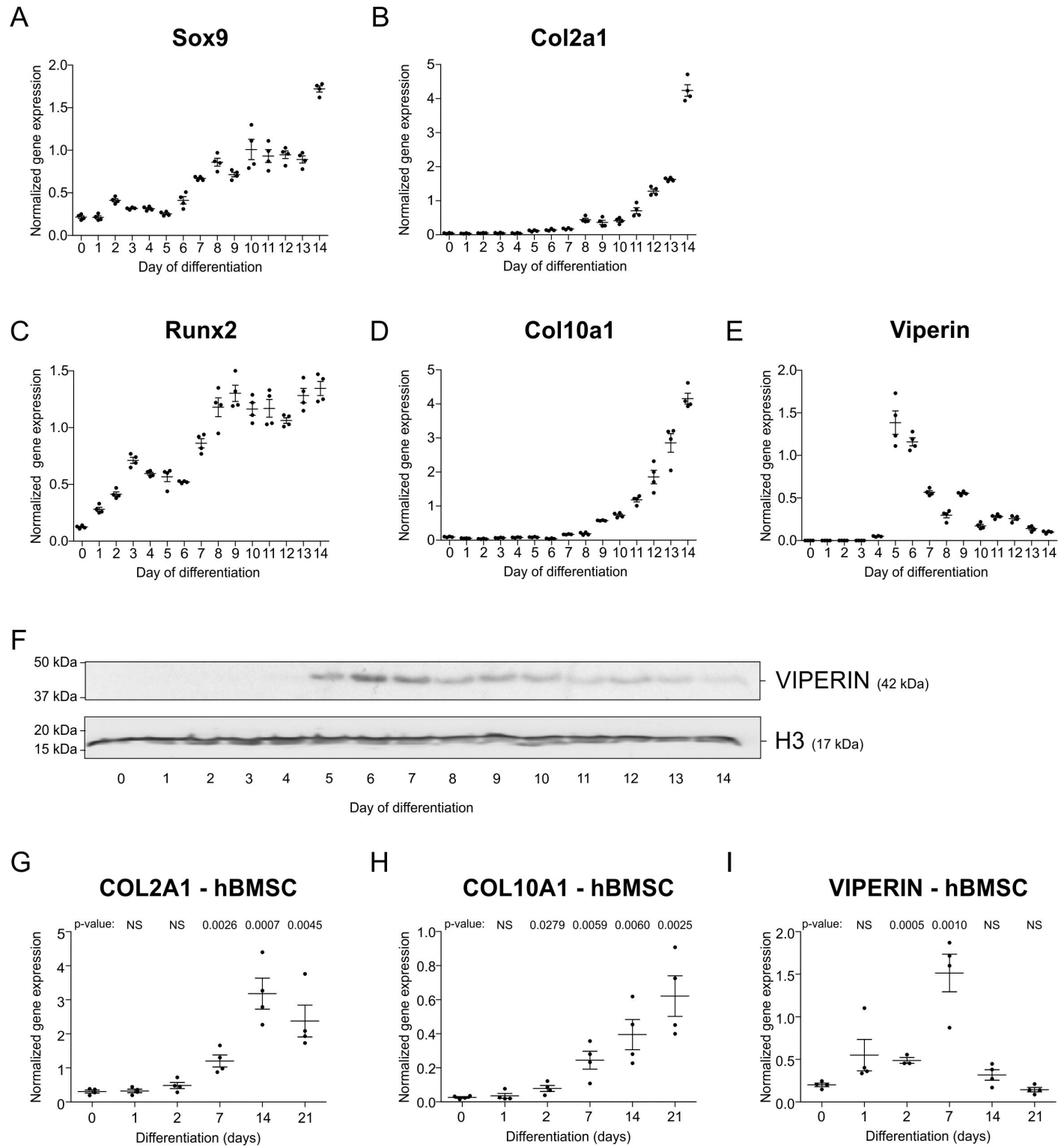
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**FIGURES AND FIGURE LEGENDS**



***Figure 1. Viperin expression in the developing embryonal growth plate***

Five micrometer-thick formalin-fixed paraffin-embedded tissue sections were prepared from E15.5 NMRI mouse embryos. Spatiotemporal expression of viperin was detected immunohistochemically. IgG2a was used as an isotype control. Overview of a representative immunohistochemically stained growth plate with IgG2a negative control in the insert (A). Indicated area from (A) enlarged. Black arrow indicates a representative cell with barely detectable viperin expression; grey arrow indicates a representative cell with weak viperin expression; yellow arrow indicates a representative cell with high viperin expression (B). Scale bars are indicated for magnification reference.

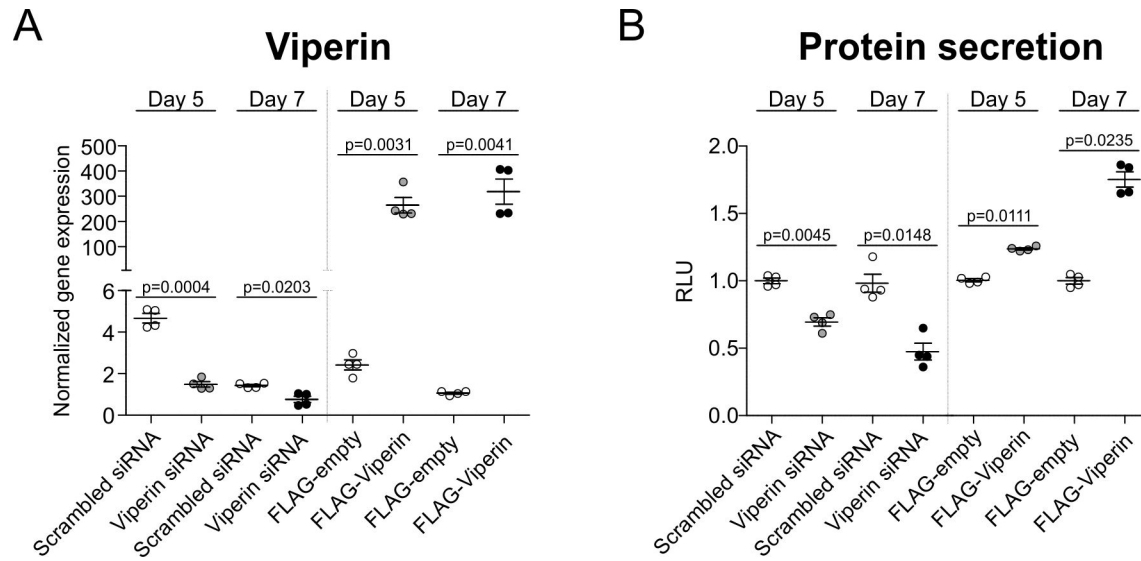


**Figure 2. Viperin is expressed during chondrogenic differentiation of ATDC5 and hBMSCs**

ATDC5 cells were differentiated in the chondrogenic lineage for 14 days. Different stages of chondrogenic differentiation were confirmed by measuring mRNA expression of Sox9 (A), Col2a1 (B), Runx2 (C) and Col10a1 (D) by RT-qPCR. Expression of viperin mRNA during chondrogenic

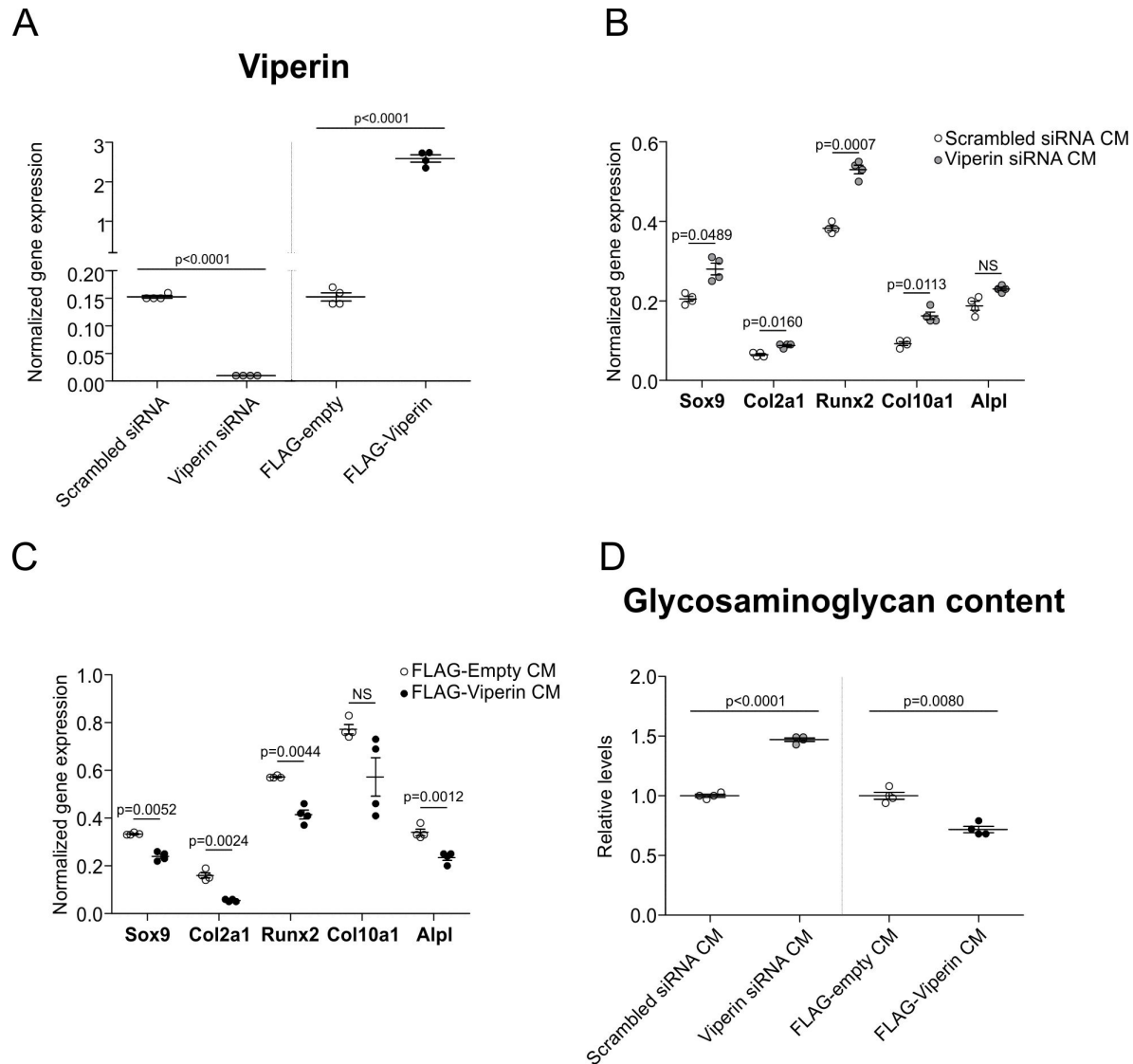


differentiation was determined by RT-qPCR (**E**) and immunoblotting (**F**). Gene expression data (**A-E**) was normalized to  $\beta$ -actin mRNA levels and individual normalized values are presented in dot plots. For Viperin immunoblotting (**F**), Histone 3 (H3) was used as loading control. Primary human bone marrow mesenchymal stem cells (hBMSCs) of 4 human subjects were differentiated in the chondrogenic lineage for 1, 2, 7, 14 and 21 days. Chondrogenic differentiation was confirmed by measuring COL2A1 and COL10A1 mRNA expression (**G/H**). In same samples expression of VIPERIN mRNA was measured (**I**). hBMSC gene expression data (**G/H**) was normalized to CYCLOPHILIN mRNA levels. Individual data points in dot plots represent the average value of 4 biological replicates of one hBMSC donor. The p-values are indicated and NS = not significant. Presented graphs are representative examples of three independent experiments. Error bars represent mean  $\pm$  SEM.



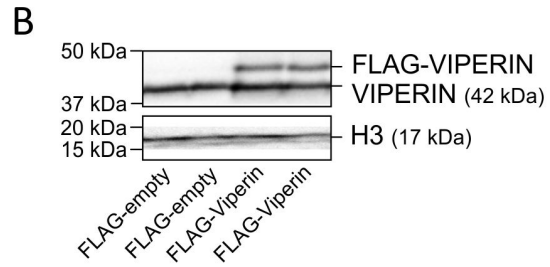
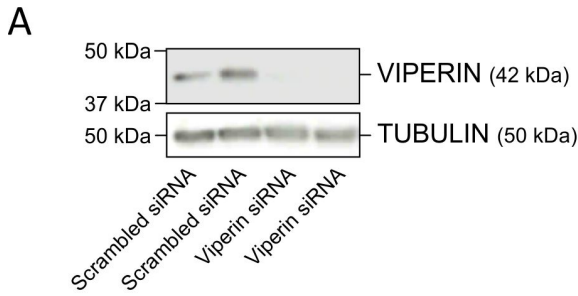
**Figure 3. Viperin knockdown reduces, while viperin overexpression increases overall protein secretion**

A CMV promoter-driven secreted Gaussia luciferase plasmid (pGluc-CMV) was co-transfected with a CMV promoter-driven firefly luciferase pGL4.20-CMV on day 3 or day 5 of ATDC5 chondrogenic differentiation. Then, on day 4 or 6, viperin expression was either reduced by transfection of a viperin-specific siRNA duplex (Viperin siRNA) or a scrambled control siRNA duplex (Scrambled siRNA); or viperin expression was further induced by transfection of a p3xFLAG-viperin plasmid (FLAG-Viperin) or the empty p3xFLAG plasmid (FLAG-empty). Reduced or induced viperin expression (normalized to  $\beta$ -actin) was confirmed by RT-qPCR at day 5 or day 7 in differentiation (A) and individually presented in dot plots. Bioluminescence was assessed at day 5 or 7 in differentiation in culture supernatant (Gaussia luciferase) and cells (firefly luciferase). Gaussia bioluminescence was normalized to the firefly signal. Normalized relative light units (RLU) of controls were set at 1 and condition RLUs were calculated relative to the control RLUs and individually presented in dot plots (B). For statistical evaluation an independent samples t-test was performed relative to the corresponding controls using GraphPad Prism 5. Data are presented of 4 biological replicates. The p-values are indicated and error bars indicate mean  $\pm$  SEM. Presented graphs are examples of 3 individual experiments.



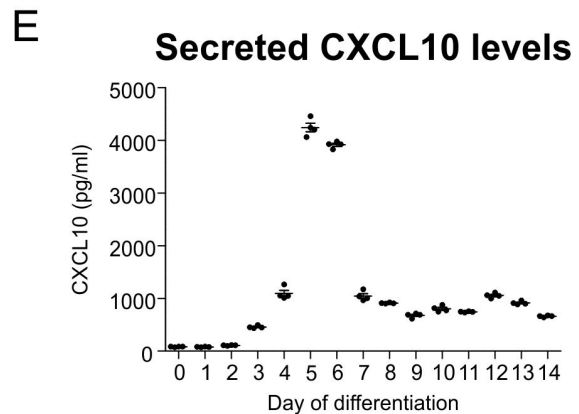
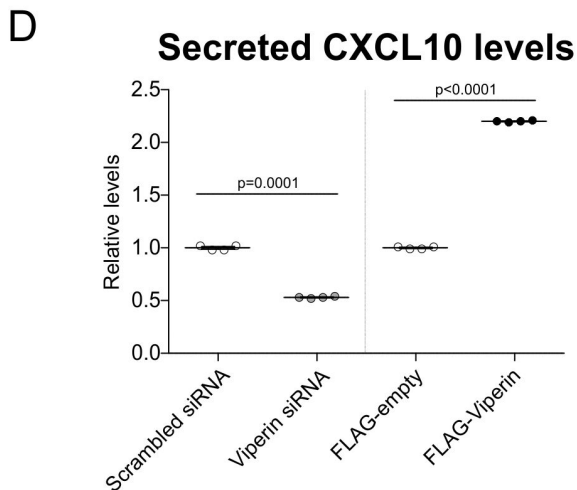
**Figure 4. The viperin secretome influences chondrogenic differentiation**

At day 5 in ATDC5 chondrogenic differentiation, viperin expression was either reduced by transfection of a viperin-specific siRNA duplex (Viperin siRNA) or a scrambled control siRNA duplex (Scrambled siRNA); or viperin expression was further induced by transfection of a p3xFLAG-viperin plasmid (FLAG-Viperin) or the empty p3xFLAG plasmid (FLAG-empty). Cells were then further differentiated until day 7 and viperin expression levels were determined (A). Conditioned culture supernatant (CM) from these donor cultures was harvested at day 7 and newly seeded ATDC5 cells were then differentiated with the CM supernatants for 7 days. RNA was isolated and Sox9, Col2a1, Runx2, Col10a1 and Alpl gene expression was determined in these samples (B/C). Glycosaminoglycan content (Alcian blue assay) was determined in ATDC5 cultures differentiated in CM supernatant from donor cultures in which viperin expression was reduced or further induced (D). RT-qPCR data was normalized to  $\beta$ -actin mRNA levels and individual normalized values are presented as dot plots. Glycosaminoglycan content data is presented as fold change relative to the corresponding control condition. For statistical evaluation an independent samples t-test was performed relative to the corresponding control condition using GraphPad Prism 5. The p-values are indicated and NS = not significant. All presented data were acquired from 4 biological replicates and error bars indicate mean  $\pm$  SEM. Graphs are representative examples of 3 individual experiments.



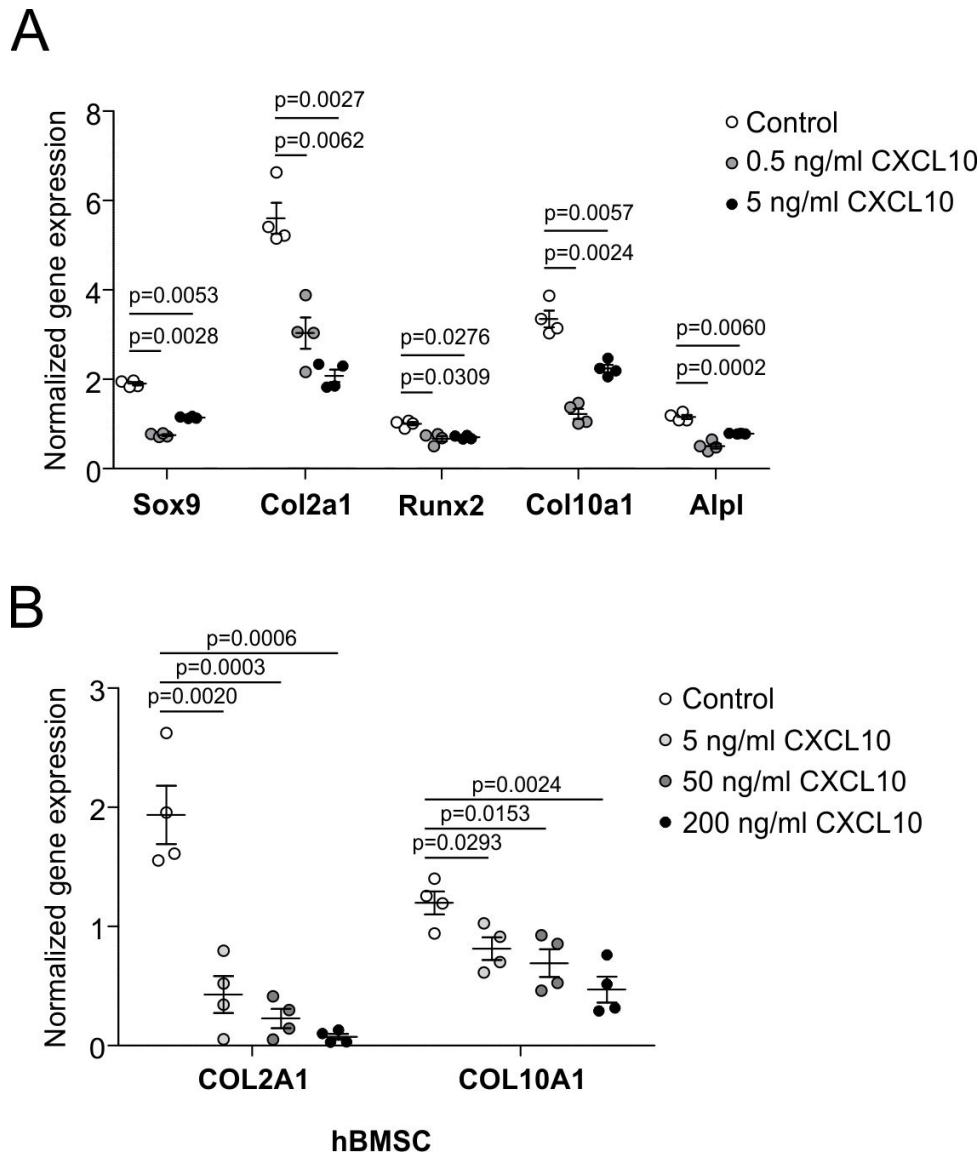
**C**

Differentially secreted extracellular proteins					
VIPERIN knockdown	FC	p-value	VIPERIN overexpression	FC	p-value
Platelet-derived growth factor a (PDGFA)	-2.18	0.033	Granulin (GRN)	2.18	0.002
Collagen 6a3 (COL6A3)	-1.63	0.042	WNT1-inducible-signaling pathway protein 2 (WISP2)	2.57	0.002
Plasminogen (PLG)	-1.37	0.043	Insulin-like growth factor binding protein 5 (IGFBP5)	-1.42	0.004
SPARC-related modular calcium-binding 1 (SMOC1)	1.27	0.045	C-X-C motif chemokine 10 (CXCL10)	2.16	0.014
C-C-C motif chemokine 10 (CXCL10)	-1.44	0.048	Collagen 6a1 (COL6A1)	-1.42	0.017
Insulin-like growth factor binding protein 4 (IGFBP4)	-1.52	0.048	Collagen 12a1 (COL12A1)	-1.32	0.025
			Fibrillin-2 (FBN2)	-2.37	0.029
			C-C motif chemokine 2 (CCL2)	1.53	0.032



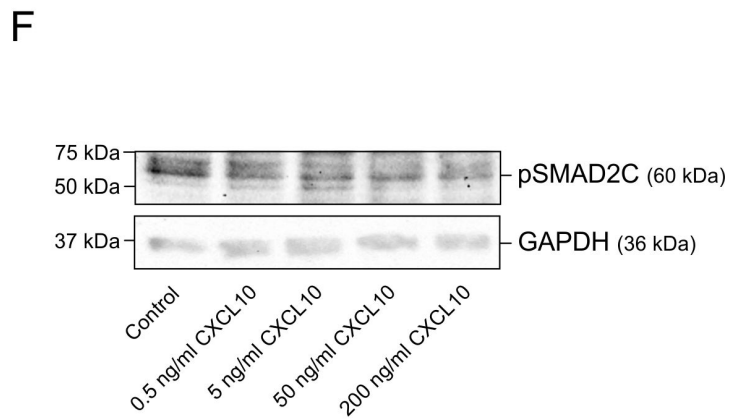
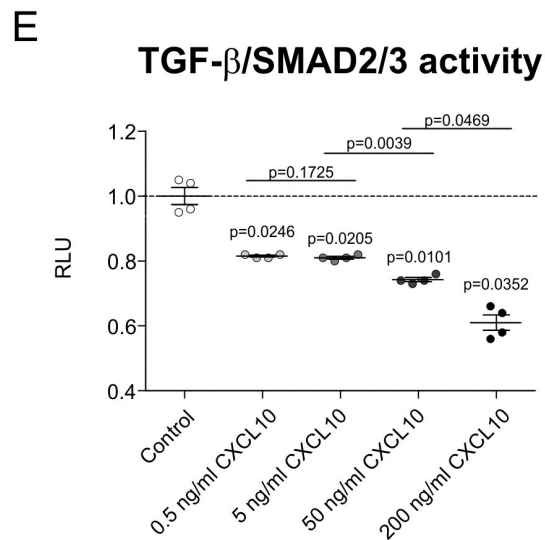
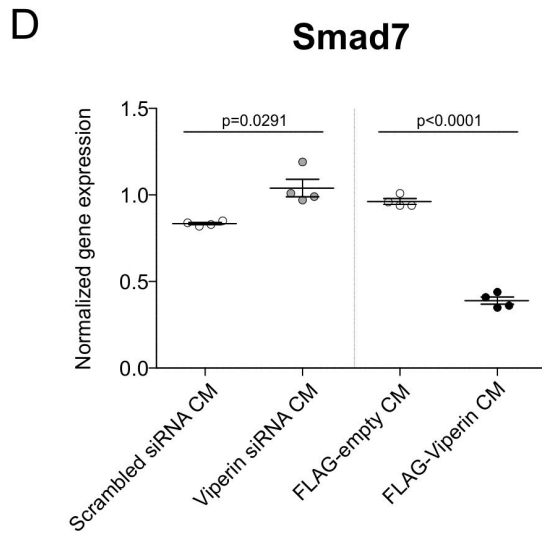
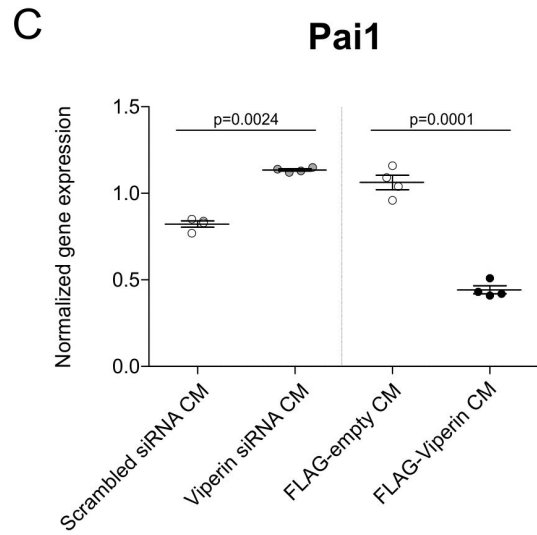
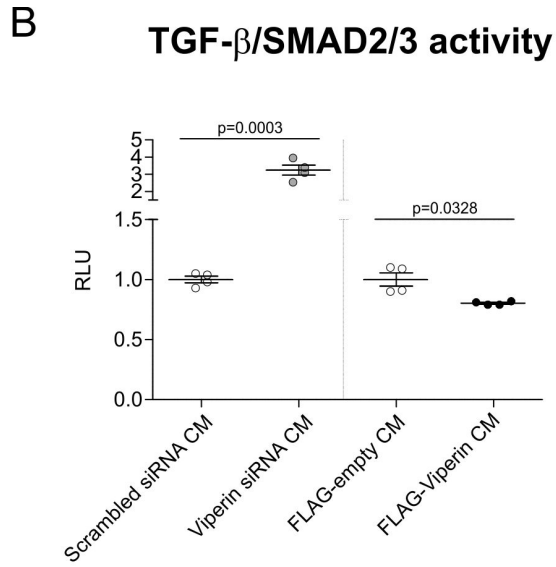
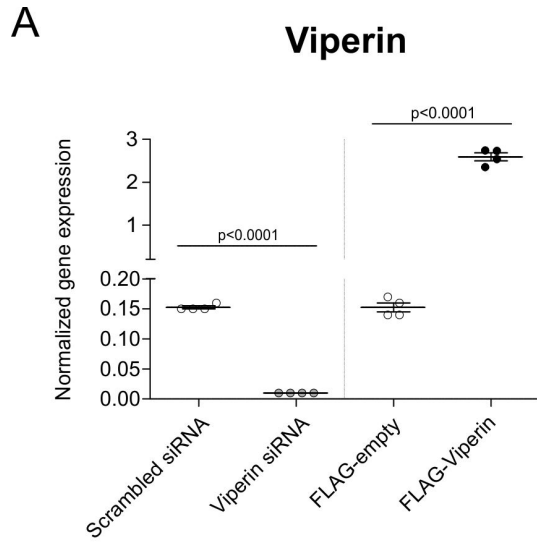
**Figure 5. The viperin secretome and CXCL10**

At day 5 in ATDC5 chondrogenic differentiation, viperin expression was either reduced by transfection of a viperin-specific siRNA duplex (Viperin siRNA) or a scrambled control siRNA duplex (Scrambled siRNA); or viperin expression was further induced by transfection of a p3xFLAG-viperin plasmid (FLAG-Viperin) or the empty p3xFLAG plasmid (FLAG-empty). Cells were then further differentiated until day 7 and viperin expression levels were determined (**A/B**). Immunoblots are shown for two independent representative examples. Culture supernatants from these day-7 cultures were collected and the protein secretome was determined by label-free LC-MS/MS. Differentially secreted extracellular protein species (control *versus* condition) with  $p < 0.05$  are shown (**C**). FC = Fold Change. In culture supernatants analyzed with LC-MS/MS, secreted CXCL10 levels were determined (**D**). In ATDC5 culture supernatants from Figure 2, secreted CXCL10 levels were determined (**E**). The p-values are indicated and error bars indicate mean  $\pm$  SEM.



**Figure 6. CXCL10 attenuates chondrogenic differentiation**

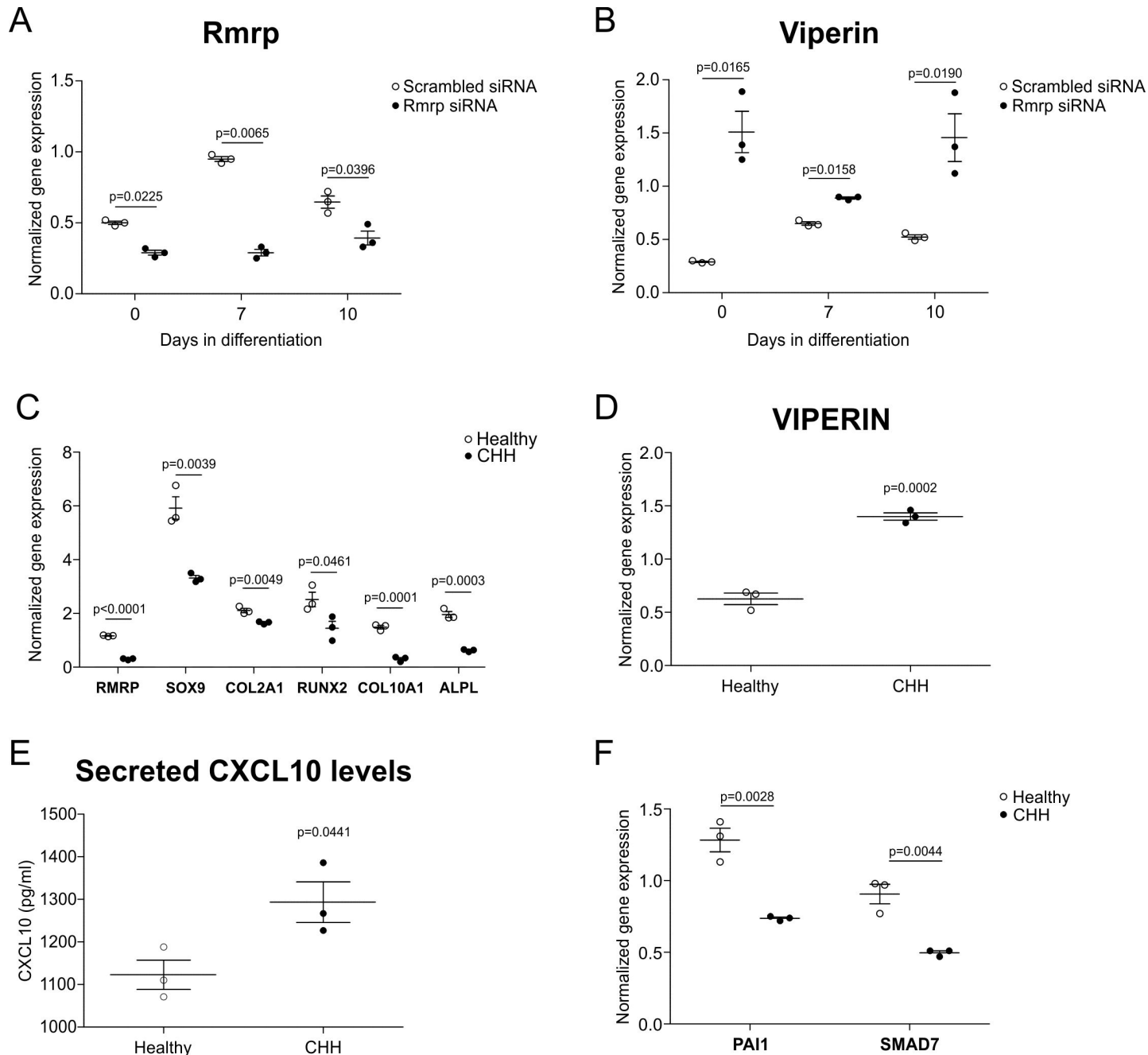
ATDC5 cells were differentiated and exposed to mCXCL10 from day 5 until day 7. RNA was isolated and expression of Sox9, Col2a1, Runx2, Col10a1 and Alpl mRNAs was determined (A). Human bone marrow mesenchymal stem cells (hBMSCs) were differentiated in the chondrogenic lineage for 7 days and exposed to hCXCL10 until day 21. RNA was isolated and expression of COL2A1 and COL10A1 mRNAs was determined (B). ATDC5 data were acquired from 4 biological replicates, normalized to  $\beta$ -actin mRNA levels and individual normalized values are presented in dot plots. hBMSC gene expression data was normalized to CYCLOPHILIN mRNA levels. Individual data points in dot plots represent the average value of 4 biological replicates of one hBMSC donor. For statistical evaluation an independent samples t-test was performed relative to the corresponding controls using GraphPad Prism 5. The p-values are indicated and error bars show mean  $\pm$  SEM. The graphs are a representative example of 3 individual experiments.



**Figure 7. TGF- $\beta$ /SMAD2/3 activity is controlled by the viperin secretome and CXCL10**

At day 5 in ATDC5 chondrogenic differentiation, viperin expression was either reduced by transfection of a viperin-specific siRNA duplex (Viperin siRNA) or a scrambled control siRNA duplex (Scrambled siRNA); or viperin expression was further induced by transfection of a p3xFLAG-viperin plasmid (FLAG-Viperin) or the empty p3xFLAG plasmid (FLAG-empty). Cells were then further differentiated until day 7 and viperin expression levels were determined (A). Conditioned culture supernatants (CM) from these day-7 cultures were collected. Proliferating ATDC5 were used as a TGF- $\beta$ /SMAD2/3 bioassay by co-transfecting a CAGA-12 firefly luciferase TGF- $\beta$ /SMAD3 reporter and pGluc-CMV plasmid. The TGF- $\beta$ /SMAD2/3 bioassay was then exposed to the CM supernatants for 24 hours and supernatant and cells were collected for bioluminescence analyses. Firefly bioluminescence was normalized to the Gaussia signal (B). Gene expression of downstream TGF- $\beta$  target genes *Pai1* and *Smad7* was determined on samples from Figure 4B/C (C/D). ATDC5 cells were differentiated and co-transfected with a CAGA-12 firefly luciferase TGF- $\beta$ /SMAD3 reporter and pGluc-CMV plasmid on day 5 in chondrogenic differentiation. At day 6 in differentiation cells were exposed to 0.5, 5, 50 or 200 ng/ml recombinant mouse CXCL10 for 24 hours until day 7. Culture supernatant and cells were collected for bioluminescence analyses. Firefly bioluminescence was normalized to the Gaussia signal (E). ATDC5 cells were differentiated until day 5 and then exposed to 0.5, 5, 50 or 200 ng/ml mouse CXCL10 until day 7. Protein extracts were separated by SDS-PAGE and electro-blotted on membranes, followed by pSMAD2C immuno detection. GAPDH was used as a loading control (F). All quantitative data were acquired from 4 biological replicates. RT-qPCR data were normalized to  $\beta$ -actin mRNA levels and individual normalized values are presented in dot plots. Bioluminescence data are presented as normalized RLUs. RLUs of controls were set at 1 and RLUs of conditions were calculated relative to the control RLUs. For statistical evaluation an independent samples t-test was performed relative to the corresponding controls using GraphPad Prism 5. The p-values are indicated and error bars represent mean  $\pm$  SEM. Graphs are representative examples of 3 individual experiments.

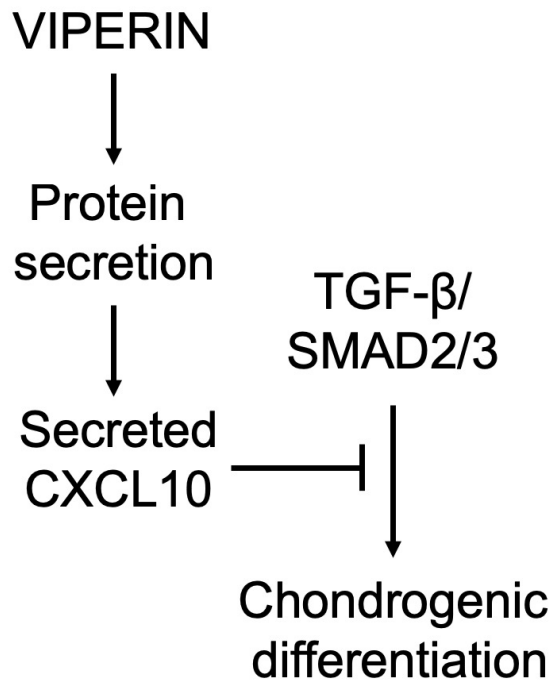




**Figure 8. The viperin-CXCL10-TGF- $\beta$ /SMAD2/3 axis is deregulated in chondrocytic CHH cells**

Rmrp RNA expression was reduced in ATDC5 cells by transfection of a specific siRNA duplex on day -1, day 2, and day 5 during chondrogenic differentiation. A scrambled siRNA was used as control. Samples were harvested for RT-qPCR analysis at day 0, 7 or 10 in differentiation. Expression of Rmrp (A) and viperin (B) was determined at indicated time points. Data was normalized to  $\beta$ -actin mRNA levels and individual normalized values are presented in dot plots. Data were acquired from 3 biological replicates. An independent samples t-test was performed relative to scrambled control using GraphPad Prism 5. The p-values are indicated and error bars represent mean  $\pm$  SEM. Human dermal fibroblasts from three CHH patients (RMRP alleles of CHH patients carried following mutations: 127G>A and 261 C>G; 4 C>T and 77C>T; 70 A>G and 70A>G) and three healthy controls were trans-differentiated into the chondrogenic

lineage by hyperconfluent plating in wells coated with Aggrecan (21,48). RNA was isolated at day 3 of trans-differentiation and gene expression of RMRP, SOX9, COL2A1, RUNX2, COL10A1 and ALPL mRNAs was determined (C). Gene expression of VIPERIN (D) and of PAI1 and SMAD7 (F) was determined in samples from (C). Supernatants were collected from these cultures and secreted CXCL10 protein was determined with ELISA (E). Gene expression data from trans-differentiated fibroblasts was normalized to CYCLOPHILIN mRNA levels and individual normalized values are presented in dot plots. Secreted CXCL10 data are absolute concentrations (pg/mL) and presented in dot plots. For statistical evaluation independent samples t-tests were performed relative to healthy controls using GraphPad Prism 5. The p-values are indicated. Error bars are mean  $\pm$  SEM. Graphs are representative examples of three independent experiments.



***Figure 9. Model of the interactions between viperin and chondrogenic differentiation***

A schematic model of the interactions between viperin and chondrogenic differentiation, suggested by our results. Viperin regulates protein secretion and controls the level of secreted CXCL10. CXCL10 inhibits TGF- $\beta$ /SMAD2/3 activity, which in turn controls the level of chondrogenic differentiation.

**TABLES**

**Table 1. RT-qPCR primer sequences**

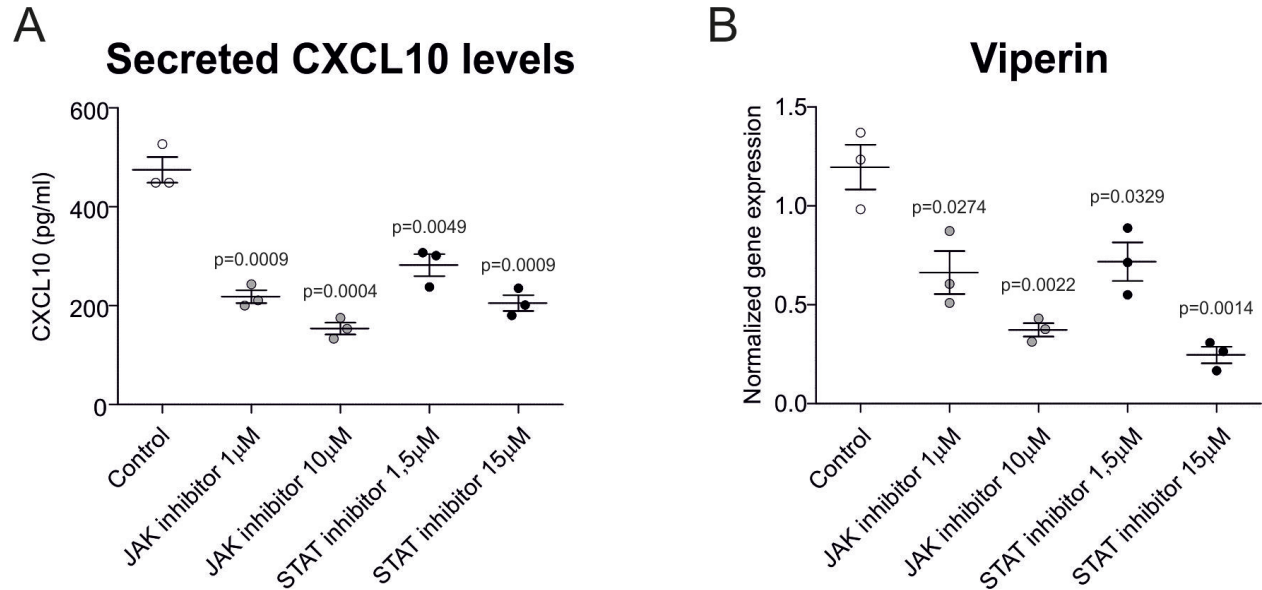
Gene	Forward	Reverse
ALPL ( <i>Hs</i> )	CCGTGGCAACTCTATCTTTGG	CAGGCCCATGCCATACAG
Alpl ( <i>Mm</i> )	CCGATGGCACACCTGCTT	GGAGGCATACGCCATCACAT
$\beta$ -Actin ( <i>Mm</i> )	CCGAGCGCGAGATCGT	TGGCCATCTCGTTCTCGAA
COL2A1 ( <i>Hs</i> )	TGGGTGTTCTATTTATTTATTGTCTTCCT	GCGTTGGACTCACACCAGTTAGT
Col2a1 ( <i>Mm</i> )	TGGGTGTTCTATTTATTTATTGTCTTCCT	GCGTTGGACTCACACCAGTTAGT
COL10A1 ( <i>Hs</i> )	ATGATGAATACACCAAAGGCTACCT	ACGCACACCTGGTCATTTTCTG
Col10a1 ( <i>Mm</i> )	CATGCCTGATGGCTTCATAAA	AAGCAGACACGGGCATACCT
CYCLOPHILIN ( <i>Hs</i> )	TTCCTGCTTTCACAGAATTATTCC	GCCACCAGTGCCATTATGG
PAI1 ( <i>Hs</i> )	GTCTGCTGTGCACCATCCCCATC	TTGTCATCAATCTTGAATCCCATA
Pai1 ( <i>Mm</i> )	ACGTCGTGGAAGTGCCTAC	CAGCGATGAACATGCTGAGG
RMRP ( <i>Hs</i> )	GAGAGTGCCACGTGCATACG	ACGCTTCTTGGCGGACTTT
Rmrp ( <i>Mm</i> )	ATACGAGGGACATGTTTCCTTATCC	TTGGCGGGCTAACAGTGACT
RUNX2 ( <i>Hs</i> )	TGATGACACTGCCACCTCTG	GCACCTGCCTGGCTCTTCT
Runx2 ( <i>Mm</i> )	GACGAGGCAAGAGTTTACC	GGACCGTCCACTGTCACCTT
SMAD7 ( <i>Hs</i> )	CCTTAGCCGACTCTGCGAACTA	CCAGATAATTCGTTCCCCCTGT
Smad7 ( <i>Mm</i> )	GCAACCCCATCACCTTAGTC	GTTTGAGAAAATCCATTGGGTATCTG
SOX9 ( <i>Hs</i> )	AGTACCCGCACCTGCACAAC	CGCTTCTCGCTCTCGTTCAG
Sox9 ( <i>Mm</i> )	AGTACCCGCACCTGCACAAC	TACTTGTAGTCCGGGTGGTCTTTC
VIPERIN ( <i>Hs</i> )	CCAACCAGCGTCAACTATCACTT	GGAAACAGAAGCCGCATTTG
Viperin ( <i>Mm</i> )	TGCTATCTCCTGCGACAGCTT	CCTTGACCACGGCCAATC

Primer sequences were designed for *Mus musculus* (*Mm*) or *Homo sapiens* (*Hs*).

**Table 2. siRNA sequences**

siRNA	Sense	Anti-sense
Viperin siRNA	GGAAGCAGAAAGAUUUCUdTdT	AGAAAUCUUUCUGCUUCCdTdT
Rmrp siRNA	CAUGUCCUUAUCCUUUCGdTdT	CGAAAGGAUAAGGAACAUGdTdT

SUPPLEMENTARY FIGURE



**Supplementary Figure 1. Inhibition of interferon signaling activity during ATDC5 chondrogenic differentiation inhibits secreted CXCL10 levels in culture supernatant and attenuates viperin expression**

ATDC5 cells were differentiated into the chondrogenic lineage until day 6. Downstream interferon signaling activity was then inhibited for 24 hours until day 7. The JAK inhibitor Ruxolitinib was used at 1 µM or 10 µM. The STAT inhibitor Fludarabine was used at 1.5 µM or 15 µM. Control was treatment with vehicle. At day 7 culture supernatant was collected and cells were harvested for total RNA isolation, followed by cDNA synthesis. In culture supernatants secreted CXCL10 protein levels were determined (A). Expression of viperin was determined by RT-qPCR Sox9 (B). Secreted CXCL10 data are absolute concentrations (pg/mL) and presented in dot plots. RT-qPCR data was normalized to β-actin mRNA levels and individual normalized values are presented in dot plots. All data were acquired from 3 biological replicates. An independent samples t-test was performed relative to control using GraphPad Prism 5. The p-values are indicated and error bars represent mean ± SEM.