Expression and function of EZH2 in synovial fibroblasts: epigenetic repression of the Wnt inhibitor SFRP1 in rheumatoid arthritis

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Abstract

OBJECTIVES: To study the expression, regulation and function of the histone methyltransferase enhancer of zeste homologue 2 (EZH2) in synovial fibroblasts (SF) from patients with rheumatoid arthritis (RA) and osteoarthritis (OA). METHODS: SF were obtained from RA and OA patients undergoing joint surgery. Expression levels were assessed by quantitative real-time PCR and western blot. Kinase inhibitors and reporter gene assays were employed to study signalling pathways. Functional analyses included EZH2 overexpression by plasmid transfection and gene silencing by small interfering RNA. Chromatin immunoprecipitation assay was used to analyse histone methylation within distinct promoter regions. RESULTS: By studying the expression and function of EZH2 in SF the authors found that EZH2 is overexpressed in rheumatoid arthritis synovial fibroblasts (RASF) and further induced by tumour necrosis factor alpha through the nuclear factor kappa B and Jun kinase pathways. As a target gene of EZH2 the authors identified secreted frizzled-related protein 1 (SFRP1), an inhibitor of Wnt signalling, which is associated with the activation of RASF, and show that SFRP1 expression correlates with the occupation of its promoter with activating and silencing histone marks. CONCLUSIONS: These data strongly suggest that the chronic inflammatory environment of the RA joint induces EZH2 and thus might cause changes in the epigenetic programmes of SF.
Expression and function of EZH2 in synovial fibroblasts: epigenetic repression of the Wnt inhibitor SFRP1 in rheumatoid arthritis

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epigenetics/rheumatoid arthritis/synovial fibroblast/Wnt signalling
Abstract

Objectives
To study expression, regulation and function of the histone methyltransferase EZH2 in synovial fibroblasts (SF) from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).

Methods
SF were obtained from RA and OA patients undergoing joint surgery. Expression levels were assessed by quantitative real time PCR and Western blot. Kinase inhibitors and reporter gene assays were employed to study signalling pathways. Functional analyses included EZH2 overexpression by plasmid transfection and gene silencing by siRNA. Chromatin immunoprecipitation assay was used to analyse histone methylation within distinct promoter regions.

Results
By studying expression and function of EZH2 in SF we found that EZH2 is overexpressed in RASF and further induced by tumour necrosis factor alpha through the NF-κB and Jun kinase pathways. As a target gene of EZH2 we identified secreted frizzled related protein (SFRP)1, an inhibitor of Wnt signalling, which is associated with the activation of RASF, and show that SFRP1 expression correlates with the occupation of its promoter with activating and silencing histone marks.

Conclusions
Our data strongly suggest that the chronic inflammatory environment of the RA joint induces EZH2 and, thus, might cause changes in the epigenetic programmes of SF.
Introduction

Rheumatoid arthritis (RA) is an inflammatory disorder characterized by the progressive destruction of joints and bones. The pathogenesis of RA is still not completely understood so far; however, within the inflamed and hyperplastic synovium of RA patients, synovial fibroblasts (RASF) have been identified as important mediators that promote ongoing inflammation and destruction. These cells show an aggressive phenotype, which is characterized by the production of inflammatory mediators and matrix-degrading enzymes, activation of signalling pathways, and by the dysregulation of oncogenes and tumour suppressors [1-2].

Efforts to investigate the relationship of human diseases and epigenetics - defined as changes in gene expression that might be inherited over generations of cells but are not due to an alteration of the underlying nucleotide sequence - have been growing exponentially in recent years [3]. Whereas most of these studies have been undertaken in the field of cancer research, epigenetics might also provide an important concept for the pathogenetic understanding of autoimmune disorders such as RA. In RASF, epigenetic alterations have been investigated on the level of histone acetylation [e.g. 4, 5-6] and DNA methylation [7]. In contrast, histone methylation has not been studied yet in RASF. Depending on the methylated position, the methylation of histones can be associated with active or inactive chromatin regions. The trimethyl mark on histone 3 lysine 27 (H3K27me3) is generally connected with transcriptionally silenced genes. It is generated by the histone methyltransferase Enhancer of Zeste Homologue (EZH)2, the catalytic subunit of the polycomb repressor complex (PRC)2 that adds up to three methyl groups to H3K27 of genes designated for silencing [8-9]. Important targets of the PRCs include developmental genes, e.g. the transcription factors of the homeobox (HOX) gene family, or elements of signal transduction pathways such as the wingless-type MMTV integration site (Wnt) signalling [10-11], which plays an important role in joint development and has been implicated in the pathogenesis of inflammatory arthropathies [12].

To address the question whether RASF show epigenetic changes on the level of histone methylation and whether these changes might contribute to their intrinsic activation, we studied the role of EZH2 in RASF.
Methods

A detailed Methods section is provided in a supplementary file, available online only.

Patient samples/Cell culture
Synovial tissue samples were obtained during joint surgery from RA (n=39) and osteoarthritis (OA, n=22) patients. Fibroblast cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). For stimulation experiments, cells were serum-starved and stimulated with 10ng/ml tumour necrosis factor alpha (TNFα; R&D Systems, Abingdon, UK) in presence or absence of kinase inhibitors.

Quantitative real-time PCR (qPCR)
Messenger RNA expression was quantified by TaqMan or SYBR green real-time PCR on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland; primer sequences: see supplementary table S1, available online only). Data were analysed by the comparative Ct method with 18S rRNA employed as endogenous control.

SDS-PAGE and Western blot
For Western blot mouse-anti-EZH2 (Cell Signalling Technology), mouse anti-H3K27me3, rabbit anti-Histone 3, rabbit anti-secreted frizzled-related protein 1 (sFRP-1) (all from Abcam), and mouse anti-α-Tubulin (Sigma-Aldrich, Buchs, Switzerland and Abcam, Cambridge, UK) antibodies were used. Horseradish peroxidase-labelled species specific secondary antibodies (Jackson ImmunoResearch, Newmarket, Suffolk, UK) and enhanced chemiluminescence (GE Healthcare, Glattbrugg, Switzerland) were utilised for detection, and protein expression was quantified with Alpha Imager software.

Immunohistochemistry
To show expression of EZH2 in vivo, formalin-fixed paraffin embedded tissue sections were stained with mouse anti-EZH2 (BD Biosciences, Allschwil, Switzerland) and mouse anti-vimentin or mouse
anti-CD68 (Dako, Baar, Switzerland) antibodies to demonstrate localization to fibroblasts and macrophages.

Plasmid construction

The coding sequence of EZH2 was cloned into the pcDNA3.1(+) vector (Invitrogen, Basel, Switzerland). A part of the EZH2 promoter (-1095 to +48, as described in [13]) and the GAPDH promoter (-1087 to -24) were cloned into pGL3basic and pRL (Promega, Dübendorf, Switzerland).

Reporter gene assay

Using Nucleofector technology (Amaxa, Cologne, Germany) RASF were transfected with 0.5μg pRL_GAPDH and 2μg pGL3basic_EZH2prom (wildtype or mutated), serum-starved and stimulated with TNFα (10ng/ml) for 24h. Firefly luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega) and normalized to the activity of Renilla luciferase.

Overexpression and gene silencing

RASF and OASF were transfected (Amaxa Nucleofection) with 1.5μg pcDNA_EZH2 or 10pmol siRNA (Qiagen, Hombrechtikon, Switzerland). Empty vector or AllStars Negative Control siRNA (Qiagen), respectively, served as controls. After 72h or 96h cells were harvested and gene overexpression or knockdown was analysed by qPCR and Western blot.

sFRP-1 enzyme-linked immunosorbent assay (ELISA)

To measure sFRP-1 in cell culture supernatants a sandwich ELISA was developed utilizing goat anti-sFRP-1 (R&D Systems) as capture antibody, rabbit anti-sFRP-1 (Abcam) as detection antibody and horseradish peroxidase-labelled goat anti-rabbit-IgG antibody (Jackson) together with 3,3',5,5'-Tetramethylbenzidin for colour development.

Chromatin immunoprecipitation assay
SF were fixed with 1% formaldehyde and lysed. Chromatin was sheared by sonication and pre-cleared with normal rabbit serum (Jackson) and Protein A beads (Upstate/Millipore, Zug, Switzerland). For ChIP, 1 to 2 μg of antibody (anti-Histone 3, anti-H3K4me3 (both from Abcam), anti-H3K27me3 (Cell Signaling Technology, Danvers, MA, USA) or normal rabbit IgG (Santa Cruz, Heidelberg, Germany)) was added. Chromatin was precipitated with Protein A beads, washed, eluted, reverse-crosslinked, digested with Proteinase K and analysed by qPCR.

Statistics

For statistical analysis GraphPad Prism 5.0 software was used. Values are presented as mean±SD. A p value less than 0.05 was considered significant.
Results

*EZH2 is constitutively overexpressed in RASF.*

The histone methyltransferase EZH2 is associated with the epigenetic silencing of genes and has been found to be overexpressed in various cancers [14]. Since RASF share many pathogenetic features with malignant cells, including alterations in the regulation of the cell cycle, adhesive properties, invasion to and consecutive destruction of surrounding tissues [15], we investigated the expression of EZH2 both in SF and in tissue sections from RA patients.

*In vitro*, we found a constitutively higher expression of EZH2 mRNA in RASF as compared to OASF (Figure 1A). These findings were confirmed on the protein level, showing an increased expression of EZH2 in RASF as analysed by Western blot (Figure 1B). Furthermore, by using synovial tissue specimen of RA and OA patients, we found that EZH2 is expressed *in vivo* as well, both in fibroblasts (stained for the mesenchymal marker vimentin, Figure 1C) and macrophages (CD68-positive cells, Figure 1D). These data indicate that EZH2 might play a role in the epigenetic regulation of genes in RA.

*Stimulation with TNFα induces EZH2.*

TNFα is a major inflammatory mediator in the pathogenesis of RA and inflammation has already been associated with epigenetic changes [16-17]. We thus addressed whether the addition of TNFα might modulate the expression levels of EZH2 in SF. As shown in Figure 2, stimulation of cells with TNFα increased the expression of EZH2 mRNA both in RASF and in OASF with the strongest effect observed after 48h (Figure 2A). Similar results were obtained by Western blot analysis for EZH2 protein (Figure 2B). As the catalytic subunit within the PRC2, EZH2 exerts its functional activity only in connection with the other PRC2 components [18]. We therefore measured the expression of other PRC2 constituents, i.e. suppressor of zeste homologue 12 (SUZ12) and embryonic ectoderm development (EED) (online Supplementary Figure S1).
The upregulation of EZH2 in SF does not correlate with global H3K27me3.

Due to the primordial function of EZH2 as a distinct methyltransferase that targets lysine 27 on histone 3, its upregulation might lead to a concomitant increase in the methylation of its substrate. By addressing the global state of H3K27 trimethylation in RASF compared to OASF by Western blot, however, no differences in H3K27me3 could be detected between patient groups or after stimulation with TNFα (Figures 2C-D).

TNFα induced upregulation of EZH2 is mediated through JNK and IKK2-dependent pathways.

In a next step we elucidated the intracellular mechanisms involved in the TNFα induced upregulation of EZH2 by performing kinase inhibition experiments for the major downstream signalling cascades of TNFα, i.e. MAP kinases and NF-κB-pathways. The inhibition of IKK-2 (inhibitor of nuclear factor κB kinase subunit beta) completely abrogated the induction of EZH2 mRNA in RASF and OASF (Figure 3A), whereas the inhibition of Jun kinase (JNK) resulted in a partial reduction of EZH2 induction (Figure 3B). Inhibition of the p38 MAP kinase did not show any effects on EZH2 expression (Figure 3C).

E2F acts as direct upstream transcriptional activator of EZH2.

It has already been shown that the transcription factor E2F is a direct upstream regulator of EZH2 in response to serum stimulation [13]. Moreover, it has been reported that its direct inhibitor pRb is constitutively inactivated in RASF [19]. In order to investigate whether the TNFα induced expression of EZH2 might be due to the activation of E2F, we performed reporter gene assays with luciferase vectors containing the EZH2 promoter with the wildtype or a mutated binding site for E2F (Figure 3D). TNFα stimulation resulted in a higher activity of the EZH2 promoter as compared to unstimulated control. This effect was partially inhibited by mutation of the E2F binding site, indicating a direct interaction between E2F and the EZH2 promoter.

sFRP-1 is targeted by EZH2 in SF.
In the rheumatoid synovium the expression of agonists and antagonists of Wnt signalling was found to be dysregulated, among them the Wnt inhibitor sFRP-1 [20]. Since elements of the Wnt pathway comprise known targets of EZH2 [10-11], we investigated whether EZH2 might be involved in the silencing of this specific target gene in RASF. EZH2 overexpression by transfection reduced the levels of SFRP1 mRNA and protein in SF (Figures 4A-B). Conversely, transfection of RASF with siRNA targeting EZH2 resulted in a significant increase of SFRP1 mRNA at 72h (Figure 4C).

*sFRP-1 is differentially expressed in RASF and OASF and is strongly repressed by TNFα.*

To test the hypothesis that SFRP1 might be epigenetically silenced in RASF, its differential expression between RASF and OASF was assessed. As shown in Figure 5A the expression of SFRP1 mRNA was significantly reduced in RASF compared to OASF. Figures 5B and C emphasize that this was also true for the expression of sFRP-1 protein with RASF reaching only half the sFRP-1 expression observed in OASF.

Since EZH2 is upregulated by TNFα, and sFRP-1 appeared to be a target of EZH2-mediated gene silencing, we asked whether TNFα also has an impact on the expression of sFRP-1. As shown in Figures 5D and E stimulation of SF with TNFα strongly decreased the expression of SFRP1 mRNA and protein after 48h. Yet, a direct link between the TNFα induced upregulation of EZH2 and the downregulation of SFRP1 remains presumptive at this moment.

*Activating and silencing chromatin marks of the SFRP1 gene promoter are different between RASF and OASF.*

To prove that SFRP1 is subject to EZH2 mediated silencing we performed chromatin immunoprecipitation (ChIP) analysis. Chromatin precipitated with antibodies against histone 3, H3K4me3 and H3K27me3 was analysed for enrichment in three regions of the SFRP1 promoter (upstream, downstream and at the transcription start site (TSS), Figure 6A). In general, the differences in H3K4me3 and H3K27me3 occupation of the SFRP1 promoter between RASF and OASF were strongest in region 3. Whereas the activating methylation of H3K4 was reduced in RASF, the
repressive H3K27 trimethylation was increased (Figure 6B). The trends observed in promoter regions 1 and 2 were similar but weaker than in region 3.

Since we observed a strong variability of H3K4me3 and H3K27me3 at the SFRP1 promoter between cells from different patients, we analysed whether this might correlate with the expression of SFRP1. As shown in Figure 6C, a strong positive correlation between H3K27me3 and the ΔCt value of SFRP1 was found (Pearson’s r=0.634). On the other hand, a high H3K4me3/H3 ratio correlated with a low SFRP1 ΔCt value and thus higher mRNA expression levels (r=-0.845). These data indicate that SFRP1 expression in RASF is reduced due to decreased H3K4me3 and increased H3K27 methylation in its promoter.

We also analysed other Wnt inhibitors, including Dickkopf-1 (DKK1) and SFRP2-5, but could only detect significant expression differences for SFRP2 (online Supplementary Figure S2). Although there was a general trend towards a correlation between H3K4me3 and H3K27me3 occupation of the promoters and Wnt inhibitor expression levels, there was not such a distinct pattern of expression correlating with both histone methylation marks as for SFRP1. These data suggest that the differential regulation by H3K4/H3K27 methylation in OASF and RASF is unique to SFRP1.
Discussion

In this study we focused on the expression and function of the histone methyltransferase EZH2 in synovial fibroblasts of RA patients and found that a) EZH2 is overexpressed in RASF as compared to OASF; b) expression of EZH2 is induced by TNFα involving NF-κB and MAP-kinase pathways; and c) SFRP1 is a direct target gene of EZH2, whose expression pattern correlates with the occupation of activating and inactivating histone methylation marks in its promoter region.

Whereas the status of histone methylation and its respective mediators has not been assessed in RA so far, EZH2 has been found overexpressed in different cancers and, in malignancies involving the prostate and breast, it has been proposed as a marker of disease progression and metastatic potential [21-22]. Likewise, the behaviour of cells within the rheumatoid synovium has been compared to the growth of an invasive tumour from which synovial fibroblasts actively invade and destroy the articular cartilage [23-25]. Consistent with the findings from cancer tissues, we showed in the present study that the expression levels of EZH2 are increased in RASF and, moreover, that these levels can be further enhanced by TNFα stimulation. We could link this effect to the intracellular activation of NF-κB, JNK and E2F-Rb pathways, all of which represent major signalling cascades involved in the activation of SF.

Despite the induction of EZH2 expression, we could not detect changes of global H3K27me3 in response to TNFα. One thus could speculate that EZH2 is specifically targeted to certain chromatin areas so that its activity is eventually not reflected in global changes of H3K27me3. Such specific targeting of local chromatin areas has already been suggested by others. De Santa et al. could demonstrate that in macrophages the LPS-induced expression of Jmjd3, i.e. the demethylase for H3K27me3, did not cause alterations in global methylation of H3K27 [16]. In a next step we thus focused on potential target genes that might show local methylation changes in their promoters and identified SFRP1 as a direct target of EZH2. Decreased expression of sFRP-1 within the rheumatoid synovium has already been described by Imai et al. [20]; here, we identified the distinct epigenetic mechanisms of this downregulation and unravelled the signalling pathways in RASF in vitro.
sFRP-1 inhibits Wnt signalling by directly binding to Wnts and is thus believed to antagonize both the canonical and non-canonical pathways [26]. Inactivation of SFRP1 by DNA methylation has been described among the early changes occurring in the development of colorectal cancer [27]. In RA, canonical Wnt signalling has been shown to participate in the activation of RASF through the regulation of cytokines, matrix metalloproteinase (MMP)3 and production of extracellular matrix molecules [28-29]. Furthermore, the RA synovium has been found to contain higher levels of Wnt1, Wnt5A, and Wnt10B than OA or normal synovium [20, 30]. It is thus highly suggestive that, by producing considerably less amounts of sFRP-1, the RASF is turned even more responsive to Wnt proteins in the synovial tissue. Inhibition of Wnt agonists or upregulation of Wnt antagonists, e.g. by interfering with epigenetic mechanisms, therefore appears to be favourable in the pathogenesis of RA regarding the attenuation of the invasive and destructive potential of RASF. On the other hand, bone erosions can be inhibited by blocking the Wnt inhibitor DKK1 which was found to enhance osteoclastogenesis and repress bone formation by osteoblasts [31-32]. The question thus arises whether the influence of Wnt signalling on joint destruction is cell type dependent or defined by the different modes of action of DKK1 and SFRPs.

Our data indicate that the epigenetic landscape of the SFRP1 promoter in RASF is changed towards a transcription-inhibiting state. Overexpression of the H3K27-methyltransferase EZH2 led to reduced levels of SFRP1 and, conversely, silencing of EZH2 to increased SFRP1 levels. By comparing RASF with OASF, we could observe increased H3K27me3 with a concomitant decrease of H3K4me3. H3K4me3 and H3K27me3 are known to co-localise in bivalent domains which, depending on the type of cells, cause genes to tend towards a rather repressed or activated state [33-34]. Ke et al. showed that genes carrying H3K4me3 are more expressed than those carrying both H3K4me3 and H3K27me3, which in turn are more highly expressed than those carrying only H3K27me3 [34]. Indeed, we have demonstrated that the SFRP1 promoter in SF contains such a bivalent domain and that the expression of SFRP1 correlates with both methyl marks.

Our experiments suggest the following model of SFRP1 silencing in SF in the chronically inflamed RA joint. Continuous exposure to TNFα and other inflammatory mediators (see online Supplementary
Figure S3) might lead to alterations in epigenetic programmes, e.g. through the induction of EZH2 expression. Trimethylation of lysine 27 of histone 3 and the loss of H3K4 trimethylation within the promoter regions might implement the TNFα induced silencing of SFRP1 so that it could persist even outside the inflammatory environment. Whereas only a weak correlation between the EZH2 expression and SFRP1 expression was found, the levels of SFRP1 mRNA and methylation status of its promoter could be strongly correlated. In cell culture, RASF escape from the ongoing exposure to inflammatory cytokines thus probably leading to a relative normalization of the EZH2 levels. This could explain the proportionally moderate increase of basal EZH2 expression in RASF when compared to OASF as observed in the present study. These basal expression levels of EZH2, however, might already be sufficient to maintain the epigenetic memory of the cells by copying the methyl mark on the SFRP1 gene during replication. Whether the addition of TNF antagonists in patients might have a similar effect and, over time, even reverse the epigenetic phenotype is speculative. Since our data suggest an analogous role of EZH2 in RASF and in cancer cells, it might be of interest for further studies to investigate EZH2 as potential marker for disease activity, in particular for patients with ongoing joint destruction despite treatment with TNF antagonists.

In summary, we addressed here for the first time histone methylation in RASF and found an overexpression of the histone methyltransferase EZH2 thus leading to alterations in the Wnt signalling pathway by downregulation of the tumour suppressor gene SFRP1.
Acknowledgements

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References


Legends to figures

Figure 1 EZH2 expression in RASF and synovial tissue
A. EZH2 mRNA (relative to 18S rRNA) was increased by 1.5-fold in RASF (ΔCt: 7.61±0.63, n=15) as compared to OASF (ΔCt: 8.16±0.5, n=12) with higher ΔCt values representing lower expression levels [35]. B. EZH2 protein was increased by 1.5-fold in RASF (n=14) as compared to OASF (n=10) (ratio EZH2/α-tubulin RASF: 0.47±0.15, OASF: 0.32±0.1, p<0.05). C and D. In vivo, EZH2 (blue) is expressed both by fibroblasts (C, Vimentin+ cells, red, n=2 OA, n=2 RA) as well as by macrophages (D, CD68+ cells, red, n=2 OA, n=4 RA). As a smaller inset the respective IgG control is depicted in 100x magnification.

Figure 2 Effect of TNFα on EZH2 and H3K27me3
A. EZH2 mRNA was significantly induced by TNFα in OASF (2.21±0.55-fold and 3.13±0.94-fold, n=6) and RASF (2.93±1 and 3.17±1.12-fold, n=7) after 24h and 48h, respectively. The dashed line represents the unstimulated control for each time point. B. EZH2 protein was induced by TNFα in OASF (2.2±0.8-fold) and RASF (2.1±1-fold) after 48h (p≤0.0001, n=14 and 15, respectively). C. Constitutive global H3K27me3 is not different in OASF and RASF (n=3 each). D. Global H3K27me3 in TNFα stimulated OASF and RASF (n=4 each) after 48h is not changed. * p<0.05, ** p<0.005, *** p<0.001.

Figure 3 Regulation of TNFα induced EZH2 expression
A. Induction of EZH2 mRNA was abrogated by pretreating SF with the NF-κB pathway inhibitor sc-514 (IKK2i) (n=4 each). B. Induction of EZH2 mRNA was reduced by pretreating SF with the JNK inhibitor II (JNKi) (by 47±17% in RASF and 81±4% in OASF, n=4 each). C. Induction of EZH2 mRNA was not affected by pretreating SF with the p38 inhibitor SB203580 (p38i) (n=4 each). D. In reporter gene assays, the relative activity of the EZH2 promoter (firefly luciferase normalized to Renilla luciferase) in RASF (n=5) was increased after 24h TNFα stimulation by 2.1±0.6-fold.
Mutation of the E2F binding site reduced the EZH2 promoter activation by TNFα to 1.7±0.7-fold. * p<0.05, ** p<0.005, *** p<0.001.

Figure 4 Overexpression of EZH2 in RASF reduces sFRP-1 expression
A. Overexpression of EZH2 decreased SFRP1 mRNA by 15±17% (72h) and 18±9% (96h). B. sFRP-1 protein in cell culture supernatants was reduced from 689±216 pg/ml to 507±56 pg/ml (minus 21.6±22.8%) after 72h and from 1095±101 pg/ml to 836±150 pg/ml (minus 22.9±17.7%) after 96h of EZH2 transfection, as assessed by ELSIA. C. Knockdown of EZH2 resulted in a significant upregulation of SFRP1 mRNA by 15±11.7% at 72h.

Figure 5 Constitutive and TNFα regulated expression of sFRP-1 in RASF and OASF
A. SFRP1 mRNA (relative to 18S rRNA) was reduced by 86% in RASF (ΔCt: 7.1±2, n=15) as compared to OASF (ΔCt: 4.5±1.7, n=12). B. Western blot analysis showed strongly reduced levels of sFRP-1 protein in RASF (n=10) compared to OASF (n=7) (ratio sFRP-1/α-tubulin RASF: 0.31±0.18, OASF: 0.63±0.31, p<0.05). C. sFRP-1 levels in supernatants of RASF (566±354 pg/ml, n=10) were reduced by 56% compared to OASF (1285±845 pg/ml, n=7). D. SFRP1 mRNA levels were reduced by TNFα in RASF (by 85±6%, n=6) and OASF (by 82±7%, n=7) with the strongest effect after 48h. The dashed line represents the respective unstimulated control for each time point. E. sFRP-1 protein expression was decreased after 48h of TNFα stimulation in RASF (by 36±19%) and OASF (by 45±7%) (n=6 each, p≤0.005). * p<0.05, ** p<0.005, *** p<0.001

Figure 6 ChIP analysis of the SFRP1 promoter in RASF and OASF
A. Schematic representation of the SFRP1 promoter. Shaded areas represent CpG islands as calculated by MethPrimer (www.urogene.org/methprimer). “+1” indicates the transcription start site (TSS). Black boxes represent the three different amplicons for ChIP-qPCR (1: -750/-699 bp, 2: -100/-51 bp, 3: +991/+1024 bp relative to TSS) B. qPCR analysis of chromatin from RASF (n=10) and OASF (n=7) ChIPed with rabbit IgG, anti-Histone 3, anti-H3K4me3 and anti-H3K27me3. To compare different patient samples, the ratio between the levels of the methylation mark and total histone 3 at
promoter regions was calculated. RASF showed a decreased trimethylation of H3K4 (ratio to H3: 0.31±0.27, OASF: 0.66±0.32) and increased H3K27me3 (ratio to H3: 0.45±0.36, OASF: 0.22±0.08).

C. ΔCt of SFRP1 mRNA in RASF (■) and OASF (▲) correlated negatively with the H3K4me3/H3 ratio (right) and positively with the H3K27me3/H3 ratio (left) in region 3 of the SFRP1 promoter.
Figure 1

A

\[ \Delta C_{\text{EZH2/18S rRNA}} \]

\[ \text{p}<0.03 \]

B

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<td>α-Tubulin</td>
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C

100x

400x

D

100x

400x
Figure 2

A

x-fold change EZH2 mRNA

TNFα 2h 24h 48h

OASF RASF

** * **

B

EZH2

α-Tubulin

C

H3K27me3

Histone 3

OASF RASF

D

H3K27me3

Histone 3

OASF RASF
Figure 3

A

B

C

D

relative EZH2 induction in %

**

***

DMSO RASF OASF
IKK-2i IKK-2i

relative EZH2 induction in %

**

***

DMSO RASF OASF
JNKi JNKi

relative EZH2 induction in %

relative promoter activity

EZH2 wt EZH2 ΔE2F

TNFα

- + +

ns
Figure 4

A

SFRP1 mRNA in %

<table>
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<tr>
<th>Time</th>
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<td>96h</td>
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p=0.086  p<0.05

B

sFRP-1 in pg/ml

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<tr>
<td>96h</td>
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p=0.11  p<0.05

C

SFRP1 mRNA in %

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<tr>
<td>96h</td>
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p<0.05
Figure 5

A. 

B. 

C. 

D. 

E.
Figure 6

A

-1000

+1 SFRP1

+1000

B

1

-750/-699bp

2

-100/-51bp

3

+991/+1042bp

C

r=-0.845

p<0.0001

r=0.634

p=0.0063
Figure S1

A

x-fold change SUZ12 mRNA

TNF-α 2h 24h 48h 2h 24h 48h

OASF RASF

x-fold change EED mRNA

TNF-α 2h 24h 48h 2h 24h 48h

OASF RASF

B

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SUZ12

EED

α-Tubulin
Figure S2

A

\[ \Delta Ct /18S rRNA \]

\[ \begin{aligned}
&\text{SFRP1} \\
&\text{DKK1} \\
&\text{SFRP2} \\
&\text{SFRP3} \\
&\text{SFRP4} \\
&\text{SFRP5} \\
\end{aligned} \]

n.d.

OASF

RASF

B

\[ \text{ratio H3K27me3/H3} \]

\[ \text{ratio H3K4me3/H3} \]

\[ \begin{aligned}
&\text{SFRP1} \\
&\text{DKK1} \\
&\text{SFRP2} \\
&\text{SFRP3} \\
&\text{SFRP4} \\
&\text{SFRP5} \\
&\text{GAPDH} \\
&\text{MYOD} \\
\end{aligned} \]
Figure S3

A

x-fold change EZH2 mRNA

IL-1β  Pam3  poly(I:C)  LPS

B

x-fold change SFRP1 mRNA

IL-1β  Pam3  poly(I:C)  LPS

*  ***
Supplementary Methods

Patient samples/ Cell culture
Synovial tissue samples were obtained during joint surgery from RA and osteoarthritis (OA) patients after they had given written informed consent for further use of their tissue for research purposes. Work with patient materials was approved by the local ethics committee. For fibroblast cultures, tissue specimen were digested with collagenase and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Fibroblasts from passages 4 to 9 were used.

Stimulation experiments/ Inhibitor studies
SF were starved for 16-24h in DMEM containing 0.5% FCS and stimulated with 10ng/ml TNF-α (R&D Systems). For kinase inhibition studies, SF were pre-incubated with sc-514 (50μM, IKK-2 inhibitor), JNK Inhibitor II (20μM), p38 inhibitor SB203580 (10μM) (all from Sigma Aldrich) or DMSO alone for 1h before adding TNF-α.

SDS-PAGE and Western blot
Whole cell lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose or PVDF membranes. Membranes were blocked with 5% milk or 2% horse serum in TBS-T and incubated with the following antibodies: mouse-anti-EZH2 (Cell Signaling Technology), mouse-anti-H3K27me3, rabbit-anti-Histone 3, rabbit-anti-SUZ12, rabbit-anti-sFRP-1 (all from Abcam), sheep-anti-EED (R&D Systems), mouse-anti-α-Tubulin (Sigma-Aldrich and Abcam). Horseradish peroxidase (HRP)-labelled species specific secondary antibodies (Jackson ImmunoResearch) and enhanced chemiluminescence (GE Healthcare) were utilised for detection, and protein expression was quantified with Alpha Imager software.

Immunohistochemistry
Formalin-fixed paraffin embedded tissue sections were deparaffinised and underwent antigen retrieval with 1mg/ml Trypsin for 25 min at 37°C (only for CD68 staining) and in 10mM citrate buffer (pH 6.0)
for 30 min at 90°C. Unspecific binding was blocked by 5% goat serum in PBS (+1% BSA) before the sections were incubated with mouse-anti-EZH2 (BD Biosciences) or mouse IgG (Dako) (10μg/ml) over night at 4°C. Biotinylated secondary goat-anti-mouse antibody (Jackson ImmunoResearch), Vectastain ABC Kit for alkaline phosphatase and Vector Blue (Vector Laboratories) were used for detection. Subsequently, endogenous peroxidase was blocked with 3% H₂O₂ for 10 min before incubating with 5% goat serum in PBS (+1% BSA) for 1h. To show the expression of EZH2 in cells of mesenchymal origin (fibroblasts) or in macrophages, mouse-anti-vimentin or mouse-anti-CD68 (2μg/ml, Dako) antibodies were applied over night at 4°C and detected using HRP-labelled goat-anti-mouse antibody (for vimentin) or biotinylated goat-anti-mouse antibody and Vectastain Elite ABC Kit for HRP (for CD68) together with aminoethylcarbazole as chromogen.

Plasmid construction

The coding sequence of EZH2 was amplified from human cDNA and inserted into the pcDNA3.1(+) vector (Invitrogen) via the BamHI and XhoI restriction sites. A part of the EZH2 promoter (-1095 to +48, as described in [1]) and the GAPDH promoter (-1087 to -24) were amplified from human genomic DNA and cloned into pGL3basic and pRL (Promega), respectively, via the BglII and HindIII sites. The E2F binding site (+33 to +40) in the EZH2 promoter was mutated by site-directed mutagenesis in four positions.

Transfection

RASF and OASF were transfected with the Basic Nucleofector Kit for Primary Mammalian Fibroblasts (Amaxa) according to the manufacturer’s instructions. The medium was replaced 24h after transfection to remove floating cells.

Reporter gene assay

RASF were transfected with 0.5μg pRL_GAPDH and 2μg pGL3basic_EZH2prom or pGL3basic_EZH2prom_ΔE2F. After 24h in complete medium, cells were starved for 24h (DMEM + 0.5% FCS) and stimulated with TNF-α (10ng/ml) for another 24h. Firefly luciferase activity was
measured with the Dual Luciferase Reporter Assay System (Promega) and normalized to the activity
of Renilla luciferase.

sFRP-1 enzyme-linked immunosorbent assay (ELISA)
To measure sFRP-1 in cell culture supernatants microwell plates were coated with goat-anti-sFRP-1
antibody (0.5mg/ml, R&D Systems) in sodium carbonate buffer (pH 9.5) over night at 4°C (as in [2]).
PBS with 0.05% Tween-20 was used for the washing between incubation steps. After coating, wells
were blocked with 300μl reagent diluent (2% non-fat dry milk in PBS) for 1h at room temperature.
Recombinant sFRP-1 (R&D Systems) was used to generate a standard curve (range: 0-10000 pg/ml)
and incubated together with the undiluted cell culture supernatants for 2h at room temperature. For
detection, rabbit-anti-sFRP-1 antibody (1:1000, Abcam) was added for 2h. HRP-labelled goat-anti-
rabbit-IgG antibody (1:5000, Jackson) was incubated for 1h and detection was performed with
3,3′,5,5′-Tetramethylbenzidin. Colour development was stopped with 2N H2SO4 and absorbance was
measured at 450 nm (with wavelength correction at 560 nm).

Chromatin immunoprecipitation assay
Three million SF were fixed with formaldehyde solution for 10 min (see online Supplementary Table
S2 for details of buffer compositions). Fixation was stopped by adding 200mM glycine. After washing
the cells with PBS three times, lysis buffer was added. Chromatin was sheared by sonication to a size
of 400-800 bp (Bandelin Sonopuls HD2070, 6 cycles of 10s sonication at 50% power and 1 min pause
on ice) and 10% of the sample was removed as input control. Sonicated chromatin was diluted 1:10
and pre-cleared for 4h by adding 10μl/ml normal rabbit serum (Jackson) and 20μl/ml Protein A beads
(Upstate, 50% slurry in TE blocked with 1mg/ml BSA and 0.4mg/ml herring sperm DNA). Pre-
cleared chromatin was parcelled to aliquots, 1 to 2 μg of antibody (anti-Histone 3, anti-H3K4me3
(both from Abcam), anti-H3K27me3 (Cell Signaling Technology) or normal rabbit IgG (Santa Cruz)
was added and rotated over night at 4°C. Chromatin was precipitated with 40μl/ml Protein A beads for
4h, washed with wash buffers I to III and finally two times TE buffer. Precipitated chromatin was
eluted by incubating twice with elution buffer for 20 min, reverse cross-linked and digested with
Proteinase K over night at 65°C and purified with the PCR purification kit (Qiagen). Samples were analysed by qPCR and normalized to the 10% input sample. Occupation of the GAPDH and MYOD promoters was measured as positive controls for H3K4me3 and H3K27me3, respectively.

Statistics
For statistical analysis GraphPad Prism 5.0 software was used. Values are presented as mean ± standard deviation. Data were analysed using Student’s t-test for parametric samples and the Mann-Whitney test or Wilcoxon signed rank test for nonparametric samples, as well as Pearson’s correlation. A p-value < 0.05 was considered significant. Detailed information about which statistical test was used for individual experiments are provided in Supplementary Table S3.

References for Supplementary Methods


Legends to Supplementary Figures

Figure S1 Effect of TNF-α on the PRC2 components EED and SUZ12
A. SUZ12 and EED mRNAs were significantly induced by TNF-α in OASF (n=6) and RASF (n=7) after 24h and 48h. B. EED (n=5 OASF, n=8 RASF) and SUZ12 protein expression after 48h TNF-α stimulation. SUZ12 protein was induced in OASF (2.6±2.2-fold, p=0.001, n=11) and RASF (1.8±0.8-fold, p=0.0002, n=14).

Figure S2 mRNA expression of different Wnt inhibitors and occupation of their promoters with H3K27me3 and H3K4me3
A. Similar to SFRP1, the expression of SFRP2 was significantly reduced in RASF. Also, SFRP4, which showed the highest expression of all Wnt inhibitors measured (ΔCt RASF: 2.43±1.54, OASF: 1.67±1.3), was reduced in RASF; the reduction observed, however, did not reach statistical significance. In contrast, DKK1 showed a trend towards higher expression in RASF. SFRP3 was only weakly expressed and levels of SFRP5 mRNA could not be detected (n.d.). B. The occupation of the SFRP2 promoter with H3K27me3 was comparable to that of SFRP1. The promoters of DKK1 and SFRP4 showed low levels of H3K27me3. The H3K27me3 occupation of the promoters for SFRP2, SFRP3 and SFRP5 tended to be higher in RASF than in OASF; these differences were not statistically significant. Interestingly, the activating H3K4me3 mark was absent at the SFRP3 and SFRP5 promoters in both OASF and RASF, which may – at least in part – explain the low (SFRP3) or missing (SFRP5) mRNA expression of these Wnt inhibitors. In contrast, the DKK1 and SFRP4 gene promoters contained high levels of H3K4me3, most likely accounting for their high mRNA expression levels. However, different to SFRP1, we could not correlate H3K4me3/H3 and H3K27me3/H3 ratios of DKK1 and SFRP2-5 to their respective mRNA expression levels. H3K4me3 and H3K27me3 levels at the promoters of GAPDH and MYOD are shown as positive (GAPDH for H3K4me3, MYOD for H3K27me3) and negative controls (GAPDH for H3K27me3, MYOD for H3K4me3), respectively. (RASF: n=4-15, OASF: n= 4-12)
Figure S3 Effect of interleukin-1β (IL-1β) and toll-like receptor (TLR) ligands on the expression of EZH2 and SFRP1

Similar to stimulation with TNF-α, treatment of RASF (n=5) with the TLR3 ligand poly(I:C) led to upregulation of EZH2 (A) and downregulation of SFRP1 (B) after 48h. Additionally, SFRP1 mRNA expression was reduced by stimulation with the TLR2 ligand Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (Pam3). IL-1β and the TLR4 ligand lipopolysaccharide (LPS) did not have an effect on the mRNA levels of EZH2 or SFRP1. The dashed line represents the unstimulated control. * p<0.05, ** p<0.005.
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Table S2 Buffers for ChIP

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*supplemented with 1x Protease Inhibitor Cocktail (Roche)
Table S3 Statistical tests

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