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Epigenetics and Rheumatoid Arthritis: the role of SENP1 in the regulation of MMP-1 expression

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Abstract

The aggressive phenotype of RA synovial fibroblasts (RASF) is characterised by the increased expression of matrix metalloproteinase (MMP)-1 as well as the small ubiquitin like modifier (SUMO)-1 and decreased expression of SUMO-specific protease SENP1. Since we showed an increased activity of acetyltransferases in this autoimmune disease, we wanted to analyse whether this affects the expression of MMP-1 and can be reversed by the reconstitution of SENP1.

In RASF, the acetylation of histone H4 was significantly increased in the distal region of the MMP-1 promoter by 274 ± 36% compared to OASF. Most interestingly, overexpression of SENP1 in RASF decreased acetylation specifically in this region by 51 ± 0.5% and globally by 73 ± 11%. Furthermore, the overexpression of SENP1 resulted in a downregulation of MMP-1 at both the mRNA (58 ± 7%) and protein levels (28 ± 6%), significantly reduced the invasiveness of RASF (from 34 ± 9% to 2 ± 2%) and led to an accumulation of histone deacetylase 4 (HDAC4) on the MMP-1 promoter (197 ± 36%). Interestingly, SENP1 failed to modulate the expression of MMP-1 in the cells silenced for HDAC4. This is the first study linking the SUMOylation pathway and the production of MMP-1 to an epigenetic control mechanism mediated through histone acetylation which has a functional consequence for the invasiveness of RASF.

Keywords:
Rheumatoid arthritis, MMP-1, SENP1, histone acetylation, HDAC4, epigenetics
Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by persistent inflammatory processes in the joints resulting in a progressive articular destruction associated with the loss of joint function and disability (1, 2). RA synovial fibroblasts (RASF) are intrinsically activated and therefore invade into cartilage by excessive expression of matrix degrading enzymes such as matrix metalloproteinases (MMPs) MMP-1, 3, 9, 10 and 13 (3-7). MMP-1 is one of the most important MMPs since it is responsible for cartilage destruction by cleaving collagen type II.

Recently, our group could show that in RA synovial tissues the balance of activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs) is strongly shifted towards histone acetylation (8), which is associated with increased transcription rates (9, 10). This posttranslational modification of histones belongs to epigenetic modifications regulating gene expression and offers us new mechanism for therapeutic strategies. Another posttranslational modification described in RA is SUMOylation (11, 12). We could show that RASF have intrinsically high levels of small ubiquitin like modifier (SUMO) -1 paralleled by decreased levels of its specific protease (SENP1) which renders them apoptosis-resistant.

Whereas posttranslational modifications such as acetylation/deacetylation directly influence gene transcription, SUMO-1 can influence the properties of its substrates such as stability, localization, interaction with other proteins and activity (13-15). Histone deacetylase 4 (HDAC 4) is known to be modified by SUMO-1 and this is thought to modulate its activity as a negative regulator of transcription (16). There is, however, uncertainty regarding the influence of SUMOylation on the activity of HDAC4. Kirsh et al. reported that a SUMOylation-deficient mutant of HDAC4 shows only a slightly impaired ability to repress the transcription while the nuclear transport of HDAC4 results from the modification by SUMO-1 (16). This suggests that sumoylation of HDAC4 could rather contribute to the change in the localization of HDAC4 than influence the activity directly.

Since SUMO-1 is a modifier of a wide range of transcription and epigenetic factors, we studied here to which extent the activated phenotype of RASF characterised by excessive production of MMP-1 can be modified by epigenetic changes driven by desumoylation. This is the first study linking SUMOylation and the production of MMP-1 to an epigenetic control mechanism mediated through histone acetylation, and therefore, it supports the idea that epigenetic modulations are involved in RA (17).
Materials and Methods

Cells and patients

Synovial fibroblasts were obtained from RA (n=8) and OA patients (n=3) undergoing joint replacement surgery and grown in DMEM with 10% FCS until passages 4–6 as described (18). All RA patients fulfilled the American College of Rheumatology criteria for RA (19).

Expression vectors, siRNA, and transfection

A GFP-tagged SENP1 expression vector (SENP1-pEGF-C1) was generated by subcloning SENP1 into pEGF-C1 as described previously (12). HDAC4 siGENOME SMARTpool was purchased from Dharmacon, Lausanne, Switzerland and siRNA HDAC4 from Santa Cruz, Heidelberg, Germany. The mock vector pEGF-C1 or mismatch oligonucleotides (Qiagen, Hombrechtikon, Switzerland) were used as controls. Transfection of the cells with SENP1 and HDAC4 siRNA were performed by AMAXA nucleofection as described previously (20).

Chromatin immunoprecipitation

In a typical experiment, 0.5 million of SF were harvested, chromatin was crosslinked with 1% formaldehyde for 10 min and the reaction was quenched by adding glycine. Cells were washed twice, snap frozen and stored at -80°C or immediately used. Chromatin sonication was performed on ice in SDS lysis buffer containing protease inhibitors (1x protease inhibitors cocktail, Roche, Basel, Switzerland), 5mM sodium butyrate (Sigma, Buchs SG, Switzerland) and 2.5 μM TSA (Sigma) using Bandelin sonopuls UW 2070 sonicator and a program of 4 cycles of 10s at 57% power. The efficiency of the sonication of the chromatin was analysed by gel electrophoresis. 10% of the sample were kept as input. Chromatin was diluted in ChIP dilution buffer and pre-cleaned by incubation with normal rabbit serum and pre-blocked Protein A agarose beads (Upstate/Millipore, Zug, Switzerland) for at least 2 h. Chromatin immunoprecipitation was performed by overnight incubation with anti-acetyl H4 antibodies, rabbit anti-HDAC4 antibodies (Santa Cruz) or isotype IgG as a negative control and followed by at least 4 h incubation with Protein A agarose beads. Subsequently, immunoprecipitates were washed with buffers containing protease inhibitors and sodium butyrate and incubated for 20 min with the elution buffer containing proteinase K. Reverse crosslinking was performed overnight at 64°C and the DNA was purified using the QIAquick gel extraction kit (Qiagen).
**RNA extraction**

Total RNA was isolated and converted into cDNA and gene expression was quantified by Real-time PCR as described (20, 21).

**Real-time polymerase chain reaction**

**Quantification of mRNA**

Expression of MMP-1 was assessed using self designed previously published primers and probe (22) and HDAC4 mRNA was quantified by Real-time PCR using SybrGreen primers: fwd 5’- TGT ACG ACG CCA AAG ATG AC -3’, rev 5’- CGG TTC AGA AGC TGT TTT CC -3’. To confirm specific amplification by SybrGreen PCR, dissociation curve analysis was performed and water controls were included for the primer pair and NRT controls were analysed for all samples. The amounts of loaded cDNA were normalized using a predeveloped 18S assay (PE Applied Biosystems). Differential gene expression was calculated with the threshold cycle (Ct) and the comparative Ct method for relative quantification. All reactions were performed in duplicates.

**Quantification of ChIP**

Immunoprecipitated promoter fragments were quantified by Real-time PCR using SybrGreen primers. The primer sequences used are as follows: for MMP-1 ChIP1 fwd 5’- TGG GAT ATT GGA GCA GCA AG -3’, rev 5’- AGC TGT GCA TAC TGG CCT TT -3’; for MMP-1 ChIP2 fwd 5’- TAA GGG AAG CCA TGG TGC TA -3’, rev 5’- AGG TTC CCT TCT GCC TTT CT -3’; for MMP-1 ChIP3 fwd 5’- TGA CTG GGA AGT GGA AAC CT -3’, rev 5’- GCC TGC AAT GGT GAG TCAT -3’. To confirm specific amplification by SybrGreen PCR, dissociation curve analysis was performed and water controls were included for each primer pair. The amounts of immunoprecipitated fragments of genomic DNA were normalized to the input DNA. Differential immunoprecipitation of promoter fragments was calculated with the threshold cycle (Ct) and the comparative Ct method for relative quantification. All reactions were performed in duplicates.

**Immunofluorescence**

Immediately after transfection, cells were plated on chamber slides and incubated for 48h. Cells were washed with PBS. Fixation was performed using 4% paraformaldehyde in PBS for
10 min at room temperature. Cells were washed with PBS and permeabilized with freshly prepared 0.1% triton X-100 (Beucher & Hobein, AG, Zurich, Switzerland) in PBS for 10 min at room temperature. All the solutions used in the following steps contained 0.1% triton X-100. Cells were blocked for 1 h using 8% BSA in PBS and incubated overnight with rabbit anti-acetyl H4 antibodies (Upstate/Millipore) or an IgG isotype in 3% BSA in PBS. Cells were washed and incubated with Cy^TM^3 conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch, Magden, Switzerland). After washing cells were mounted. The detection of SENP1-pEGF-C1 and the acetylated histone H4 was performed by fluorescence microscopy.

**Flow cytometry analysis (FACS)**

Cells were permeabilised with 0.4% triton X-100 and incubated with rabbit anti-acetyl H4 (Upstate/Millipore) antibodies and followed by incubation with R-Phycoerythrin-labeled donkey anti-rabbit antibodies (Jackson ImmunoResearch). Unbound antibodies were removed by two washing steps. In control experiments primary antibodies were replaced by a mouse IgG isotype in the same concentration. Measurements were performed using 10,000 cells per sample at “low-flow” modus using FACSCalibur (BD Biosciences, Allschwil Switzerland).

**Acid extraction of histones**

Following transfection, cells were split into two portions and the first portion was subjected to acid extraction of histones, while second portion was lysed in Lemmli buffer and used to assess the efficiency of the transfection. The acid extraction was performed as previously described (23). Briefly, nuclear extracts were prepared by cell lysis in cold hypotonic lysis buffer (10mM Tris –Cl pH 8.0, 1mM KCl, 1.5 mM MgCl₂, 1mM DTT, 1mM PMSF, containing 1x protease inhibitors cocktail (Roche), 1.25 mg/ml sodium butyrate (Sigma) and 2μM trichostatin A (Sigma)). The nuclei were resuspended nuclei in 0.4N H₂SO₄ and incubated on a rotator at 4°C for 1h. Next, histones were precipitated overnight by 33% trichloroacetic acid. The histone pellet was washed with ice-cold acetone and resuspended in distilled water.

**Western blot**

Following transfection, lysates were suspended in 2x concentrated Lemmla buffer (100 mM Tris HCl [pH 6.8], 40% glycerol, 10% sodium dodecyl sulfate [SDS], 0.7M β-
mercaptoethanol, and 0.0005% bromphenol blue) and separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes as previously described (18). Membranes were blocked with 5% milk for 1 h at RT and then incubated overnight with rabbit anti-acetylated histone H4 (Upstate/Millipore), rabbit anti-SENP1 (Chemicon, Asperg, Germany) or mouse anti-MMP-1 (R&D Systems). As secondary reagents, HRP-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch) or HRP-conjugated rabbit anti-mouse IgG antibodies (DAKO, Baar, Switzerland) were used, and signals were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Otelfingen, Switzerland). For normalization, membranes were stripped and probed with mouse anti-human α-tubulin (Sigma), mouse anti-paxillin antibodies (Neomarkers, Basel, Switzerland) or rabbit anti-histone H4 (Upstate/Millipore). Evaluation of the expression of specific proteins was performed using the Alpha imager Software system (Alpha Innotech, San Leandro, CA) via pixel quantification of the electronic image.

**Cell invasion assay**

Investigation of synovial fibroblast invasion in vitro was performed using the recently established matrix associated transepithelial resistance invasion (MATRIN) assay as described (24). This is a highly sensitive electrophysiological technique that is based on the measurement of the electrical resistance of a monolayer of the C7 subclone of Madin-Darby canine kidney cells (MDCK-C7). MDCK-C7 cells were grown in MEM on the back of a 0.4-μm filter cup. Pooled RASF from two patients were transfected with SENP1 (SENP1-pEGF-C1) or the empty vector (pEGFP-C1), and 5 x 10^5 cells were seeded onto a collagen matrix consisting of 97% type I collagen and 3% type III collagen that was used to coat the top of the filter cup. The resistance across this monolayer was measured with a STX2 electrode. The invasion was recorded 12, 19 and 23 h after seeding the cells. Measurement for all cells was performed in triplicates.

**Statistical analysis**

All data are expressed as mean ± SEM. Mann–Whitney *U*-test was used for comparison between two groups of data. P values lower or equal to 5% were considered statistically significant (*p* ≤ 0.05).
Results

**RASF have higher intrinsic levels of acetylated histone H4 in the promoter of MMP-1 than OASF**

Since we could show that the acetylation of histones is increased in RA (8) we investigated whether there is hyperacetylation of histones in the promoter of MMP-1 in RASF compared to OASF contributing to its overexpression in RA. We performed chromatin immunoprecipitation in RASF and OASF using anti-acetyl H4 antibodies and analysed 3 different regions spread along the promoter of MMP-1. Most interestingly, we found that in the upstream region -1524 to -1464 from the transcription starting point of the MMP-1 promoter, the levels of acetylation of histone H4 were significantly increased in RASF when compared with OASF (percent change 274 ± SEM 36%, n = 3, p ≤ 0.05, Fig. 1A). The levels of acetylated histone H4 in the -552 to -488 upstream region from the transcription starting point were slightly lower in RA when compared to OA (22 ± 9%, n = 3, p ≤ 0.05). There was no difference in acetylation of histones in the region -179 to -130 upstream from the transcription starting point. Therefore, we hypothesised that hyperacetylation of the MMP-1 promoter could result in an intrinsically upregulated production of MMP-1 in RASF.

**SENP1 overexpression leads to a decrease in acetylation of histone H4 in the promoter of MMP-1**

RASF are characterised by increased SUMOylation due to an upregulation of SUMO-1 and the downregulation of SENP1. To reverse this phenotype we overexpressed SENP1 in RASF using an expression construct of SENP1 (SENP1-pEGF-C1) or the empty vector (mock transfected, pEGF-C1) and analysed the acetylation status of the MMP-1 promoter. We found that the levels of acetylated histone H4 in the region at -1524 to -1464 bp from the transcription starting point were significantly downregulated by the overexpression of SENP1 by 51 ± 0.5% (n = 3, p ≤ 0.05, Fig. 1B). The levels of acetylated histone H4 in the two other analysed regions were not significantly different in cells overexpressing SENP1.

**Desumoylation decreases the global levels of acetylated histone H4**

Since SENP1 overexpression decreased the acetylation level in the promoter of MMP-1 we were interested whether the decrease in acetylation of histones is also a global phenomenon.
Thus, we compared the global levels of acetylated histone H4 in the cells transfected with SENP1-pEGF-C1 or the mock vector. As visualized by immunofluorescence, RASF transfected with SENP1-pEGF-C1 revealed lower global levels of acetylated histone H4 when compared both to nontransfected or GFP transfected cells (n = 3, Fig. 2A). Using flow cytometry analysis we could further confirm that the global levels of H4-acetylation were significantly decreased by 73 ± SEM 11% when compared to pEGF-C1 cells (n = 3, p < 0.05, Fig. 2B). In addition, using Western blot we could confirm that the 3-fold overexpression of SENP1 (Fig. 2C) leads to a decrease in acetylation of histone H4 (by 51 ± SEM 4%, Fig. 2D).

**Overexpression of SENP1 results in a downregulation of MMP-1 in RASF**

After we observed decreased acetylation of histones in the MMP-1 promoter in RASF overexpressing SENP1, we wanted to analyse whether it resulted in a repression of MMP-1 transcription. Indeed, the overexpression of SENP1 downregulated the expression of MMP-1 mRNA by (mean ± SEM) 58 ± 7% (n = 5, p ≤ 0.05, Fig. 3A). Furthermore, the protein levels of MMP-1 were significantly decreased by 28 ± 6% (n = 3, p ≤ 0.05) in RASF overexpressing SENP1 compared to mock transfected cells (Fig. 3B).

**Overexpression of SENP1 in RASF results in a functionally reduced invasiveness**

Next, since we observed that the overexpression of SENP1 downregulated the expression of MMP-1 in RASF, we wanted to investigate whether therefore also the invasiveness of these cells decreased. Indeed, using an in vitro functional invasion assay, we could observe that RASF transfected with SENP1 for 23h were significantly less invasive (2 ± 2 % decrease in TEER) compared to the mock transfected cells (34 ± 9% decrease in TEER, p ≤ 0.05, Fig. 3C and D). At the earlier time points (12 and 19 h after transfection) we could not observe a significant difference in the invasiveness of SENP1 overexpressing RASF compared to the control cells (Fig. 3D). In conclusion, we could show that the overexpression of SENP1 in RASF not only alters the expression of MMP-1 but also functionally interferes with the invasive phenotype of RASF.

**Overexpression of SENP1 leads to an accumulation of histone deacetylase HDAC4 on the MMP-1 promoter**
Since SENP1 overexpression results in a decrease in acetylation of histones at the MMP-1 promoter, we were interested whether this is due to the counterplaying histone deacetylases. Since HDAC4 is a substrate for sumoylation and this modification is connected with a change of localization of HDAC4, we hypothesised that the overexpression of SENP1 could mediate histone deacetylation through localizing HDAC4 to specific regions of chromatin. To investigate whether this is the case, we performed chromatin immunoprecipitation using anti-HDAC4 antibodies in RASF transfected with the SENP1 expression vector or with the mock control. Next, Real-time PCR for the different regions of the MMP-1 promoter was performed. The level of HDAC4 in the region at -1524 to -1464 bp from the transcription starting point was significantly increased in RASF overexpressing SENP1 when compared to the mock transfected cells by (mean ± SEM) 197 ± 36% (n = 3, p ≤ 0.05, Fig. 4A). There was no significant change in HDAC4 detected in the two other analysed regions. Therefore, the overexpression of SENP1 resulted in a significant increase of HDAC4 localized to the MMP-1 promoter and to the significantly decreased acetylation of histone 4 in the same region of the promoter.

**SENP1 requires HDAC4 for downregulation of MMP-1 expression**

Since SENP1 overexpression lead to an increase in the localization of HDAC4 and at the same time to a decrease of histone acetylation in the promoter of MMP-1, we were interested whether HDAC4 mediates the downregulation of MMP-1 expression in SENP1 overexpressing cells. Therefore, we silenced the expression of HDAC4 and next overexpressed SENP1. As expected, SENP1 overexpression in cells transfected with control siRNA lead to a decreased MMP-1 mRNA production. Most interestingly, in the cells silenced for HDAC4 and next transfected with SENP1 the levels of MMP-1 mRNA were significantly higher than in the cells transfected with SENP1 but expressing HDAC4 (percent change 190 ± SEM 20%, n = 3, p ≤ 0.05, Fig. 4B). Also at the protein level, in the cells silenced for the expression of HDAC4 and next transfected with SENP1 we could not observe a downregulation of MMP-1 in contrast to the cells transfected with control siRNA and next SENP1-pEGF-C1 (percent difference 156 ± SEM 9%, p ≤ 0.05, Fig. 4C). Therefore, SENP1 requires HDAC4 for the downregulation of MMP-1 expression.

**Discussion**
We could show here for the first time that the MMP-1 promoter is intrinsically hyperacetylated in RASF when compared to OASF. Most interestingly, the increased cleavage of the intrinsically overexpressed SUMO-1 in RASF by SENP1 re-established the acetylation pattern in the MMP-1 promoter by deacetylation of histones in the distal fragment of the MMP-1 promoter. Furthermore, the overexpression of SENP-1 led to a decrease in the production of MMP-1 and functionally interfered with the invasive phenotype of RASF. The transcriptional repression of MMP-1 was mediated by an epigenetic mechanism and depended on HDAC4.

It has been described by Huber et al that in RA synovial tissues the balance of HAT/HDAC activity is strongly shifted towards histone acetylation (8). In addition, it has been shown that several HDAC inhibitors had rather beneficial effects in the animal models, where they were shown to improve joint swelling, synovial inflammation, bone and cartilage destruction, downregulated the production of VEGF, blocked angiogenesis and promoted cell cycle arrest and apoptosis (25-28). These reports suggest that HDACs could be misplaced in the subcellular compartments for example being localized aberrantly to specific promoter sites, and thereby contribute further to the pathogenesis of RA. Therefore, it should be carefully considered whether inhibition of HDACs or rather their redirection to a proper localization is more feasible for the treatment of RA.

RASF play a crucial role in cartilage destruction by active invasion via producing MMPs (6). Therefore, targeting of the intrinsically activated RASF could lead to the development of new therapeutic strategies for RA. We could previously show that RASF are characterised by high expression levels of SUMO-1 and at the same time low levels of SENP1 (12). Here we demonstrate that reversing this balance by overexpression of SENP1 leads to a decrease in the invasiveness of RASF and downregulates the expression of MMP-1. Most interestingly, this regulation is dependent on a transcription inhibitor, HDAC4, since in cells silenced for HDAC4 the overexpression of SENP1 failed to decrease the expression of MMP-1. Furthermore, we could show that upon the overexpression of SENP1 in RASF, HDAC4, localized especially to the distal region of the MMP-1 promoter. HDAC4 has been shown to be modulated by SUMO-1 by a SUMO ligase RanBP2 in the nuclear pore complex (16). This suggests that sumoylation of HDAC4 promotes nuclear transport of the modified protein. Such a mechanism has been shown for nuclear translocation of the insulin gene regulator Pdx1 as well as the tumor suppressor Smad4 (29-31). We show here that SENP1 overexpression in the nucleus of RASF led further to the specific accumulation of HDAC4 on the DNA. Furthermore, SENP1 driven modulation of HDAC4 localization re-established the
acetylation pattern in the MMP-1 promoter in RASF by deacetylation of histones in the distal fragment of the MMP-1 promoter. This promoter region contains predicted binding sites for acetyltransferases such as C/EBPα and p300. Moreover, this promoter region contains a predicted binding site for IRF-3, which was described to be superactivated in RA synovial tissues (32), to be regulated by HDAC inhibitors (33) and to interact with PIASy, a SUMO ligase (34). It needs to be investigated whether IRF-3 regulates the transcription of MMP-1. On the contrary, no significant changes were observed in both acetylation of histones and HDAC4 levels after the overexpression of SENP1 in the AP-1 binding site proximal to the transcription starting point (-179 to -130 upstream from the transcription starting point) as well as the region -552 to -488 upstream from the transcription starting point to which no histone acetyltransferases have been predicted to bind. Most interestingly, we were able to show that the overexpression of SENP1 leads to a decrease in the global acetylation of chromatin. This is the first study showing that interference with the sumoylation pathway can decrease the invasiveness of RASF and down-modulates the expression of MMP-1 via epigenetic changes in the chromatin. We therefore propose that the sumoylation pathway could be a promising target for therapy in RA, by inhibiting both the resistance to induced apoptosis and the invasiveness of RASF mediated by MMP-1.
Figure 1: Acetylation of histone H4 in the promoter of MMP-1
Chromatin immunoprecipitation assay showing acetylated histone H4 in the promoter regions of MMP-1 (-179 to -130 bp, -552 to -488 bp and -1524 to -1464 bp regions upstream from the transcription starting point). (A) Baseline levels of acetylated histone H4 in three regions of the MMP-1 promoter in RASF compared to OASF (n = 3 each). The levels of acetylated histone H4 are given in % of input ± SEM and shown in white and black bars respectively. IgG serves as a negative control (paned bars). (B) Levels of acetylated histone H4 in three regions of the MMP-1 promoter in RASF following the overexpression of SENP1. The levels of acetylated histone H4 in mock (n = 3) and SENP1 transfected RASF (n = 3) are given in % of input ± SEM and shown in white and black bars respectively. IgG serves as a negative control (paned bars).

Figure 2: Modulation of global acetylation of histone H4 in RASF by overexpression of SENP1
(A) Immunofluorescent staining of acetylated histone H4 (red) in RASF transfected with SENP1–pEGF-C1 (green, upper panel) or mock pEGF-C1 vector (green, lower panel) (B) Levels of acetylated histone H4 in control cells transfected with the empty vector pEGF-C1 (n = 3, white bar) and SENP1 transfected cells (n = 3, black bar) measured by FACS are shown as mean fluorescence intensity ± SEM. Representative FACS picture for acetylated histone H4 in SENP1–pEGF-C1 and pEGF-C1 transfected cells is shown in the inset. (C) Representative Western blot showing levels of SENP1 in mock- and SENP1- transfected RASF. As internal control α-tubulin was used. The levels of SENP1 expression in mock- (white bar) and SENP1- transfected RASF (black bar) were quantified using densitometric analysis software and normalized against α-tubulin. The values from 3 experiments are shown as mean ratio of densospot units ± SEM. (D) Representative Western blot showing levels of acetylated histone H4 in mock- and SENP1- transfected RASF (upper panel). As internal control histone H4 was used (lower panel). The ratio of acetylated histone H4/histone H4 in mock- (white bar) and SENP1- transfected RASF (black bar) were quantified using densitometric analysis software. The values from 3 experiments are shown as mean ratio of densospot units ± SEM.
Figure 3: Modulation of MMP-1 expression and invasiveness of RASF by overexpression of SENP1

(A) Levels of MMP-1 after transfection of RASF with SENP1. Levels of MMP-1 mRNA in the control cells transfected with the empty vector pEGF-C1 were set as 100% (white bar) and respective values for SENP1 transfected cells (n = 5, black bars) are shown as mean fold change ± SEM. Values were normalized for the expression of 18S. (B) Representative Western blot showing levels of MMP-1 expression in mock- and SENP1-transfected RASF (upper panel). SENP1 in mock- and SENP1- transfected cells is shown in the middle panel. As internal control α-tubuline was used. The levels of MMP-1 expression in mock- (white bar) and SENP1-transfected RASF (black bar) were quantified using densitometric analysis software and normalized against α-tubulin. The values from 3 experiments are shown as mean ratio of densospot units for MMP-1/α-tubulin change ± SEM. (C) Invasiveness of RASF transfected with mock vector pEGF-C1 (white bar) or SENP1 (black bar) shown as % reduction of electrical resistance of a monolayer of MDCK-C7 cells. (D) Invasiveness of RASF transfected with mock vector pEGF-C1 or SENP1 shown as % of electrical resistance of a monolayer of MDCK-C7 (TEER) cells at different time points, where the resistance at time 0 was set as 100%.

Figure 4: HDAC4 driven downregulation of MMP-1 expression in RASF overexpressing SENP1

(A) Levels of HDAC4 in three regions of the MMP-1 promoter in RASF overexpressing SENP1. The levels of HDAC4 in mock (n = 3, white bar) and SENP1 transfected RASF (n = 3, black bar) are given as % of input ± SEM. IgG serves as a negative control (paned bars). (B) Levels of MMP-1 mRNA in RASF measured by Taqman. Levels of MMP-1 mRNA in control cells transfected with siRNA/pEGF-C1 were set as 100% (n = 3, white bar). Relative values of MMP-1 mRNA levels are shown as mean fold change ± SEM for cells transfected with siHDAC4/pEGF-C1 (n = 3, white striped bar), control siRNA/SENP1-pEGF-C1 (n = 3, black bar) and siHDAC4/SENP1 (n = 3, black striped bar). (C) Levels of MMP-1 in the cytoplasmic fraction of RASF transfected with a control siRNA/pEGF-C1 (n = 3, white bar), siHDAC4/pEGF-C1 (n = 3, white striped bar), control siRNA/SENP1 (n = 3, black bar) and siHDAC4/SENP1 (n = 3, black striped bar) measured with densitometric analysis software. The values for MMP-1 were normalized for the expression of cytoplasmic protein paxillin. The levels of MMP-1 in control siRNA/pEGF-C1 transfected cells were set as 100%. Values are given as fold change ± SEM. Representative Western blot is shown (n = 3).
Figure 3

Figure 4
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**References**