The helicase HAGE prevents interferon-α-induced PML expression in ABCB5+ malignant melanoma-initiating cells by promoting the expression of SOCS1

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Malignant melanoma-initiating cells (MMICs) are a population of cancer cells that possess the stem cell properties of self-renewal and differentiation.1 This population of cells express the ATP-binding cassette member 5 (ABCB5), a protein that renders malignant melanoma drug-resistant and refractory to therapy. ABCB5+ MMICs also express HAGE (DDX43), a DEAD-Box RNA helicase expressed specifically by tumour cells, that has been shown to have a key role in promoting the proliferation of ABCB5+ MMICs and their survival by enhancing the expression of the small GTPase NRAS.2 As a consequence, this population is thought to be responsible for a highly aggressive type of cancer with a poor prognostic outcome.3,4 Besides drug-based therapy, interferon-α (IFNα) has also been used for the treatment of patients with malignant melanoma and has been shown to result in a modest improvement of relapse-free survival and overall survival.5–7 The mechanisms underlying resistance to IFNα treatment and the involvement of MMICs in this process are unknown.

IFNα signals through the JAK–STAT (janus kinase–signal transducers and activators of transcription) pathway and results in the induction of several genes. This endows IFNα with multiple effects in a variety of malignancies that range from antiangiogenic effects to potent immunoregulatory, differentiation-inducing, pro-apoptotic and anti-proliferative properties.8,9 The gene encoding promyelocytic leukaemia protein (PML) is a well-known downstream target of IFNs signalling and its induction by IFNs results in a significant increase in the expression of PML and the number of PML nuclear bodies (PML-NBs).10–12 PML, a member of the Ring-B Box-Coiled Coil family, is a tumour suppressor which was originally identified at the breakpoint of the t (15;17) translocation found in acute promyelocytic leukaemia (APL).13–15 It is at the heart of many cellular pathways such as cell growth, differentiation, DNA damage, senescence, apoptosis and anti-viral responsiveness.16–18 PML functions by interacting and recruiting different factors that compose these cellular processes into subnuclear structures known as PML-NBs, of which it is the essential component.18 Although the role of PML in IFNs-mediated antiviral responses has been well studied, little is known about its role in the

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Abbreviations: MMIC, malignant melanoma-initiating cell; ABCB5, ATP-binding cassette subfamily B member 5; SOCS1, suppressor of cytokine signalling 1; HAGE, helicase antigen; PML, promyelocytic leukaemia protein; Jak1, janus kinase 1; Jak2, janus kinase 2; STAT1, signal transducer and activator of transcription 1; STAT2, signal transducer and activator of transcription 2; IFNα, interferon α; IFNs, interferons

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anti-tumour properties of IFNs. On the basis of this background, we hypothesised that the helicase HAGE (DDX43) may endow MMICs with a resistance to the anti-tumour effects of IFN-α-induced PML. Here, we reveal a previously unknown role of HAGE, namely that it ensures the survival of MMICs in response to the anti-proliferative and pro-apoptotic effects of IFN-α. Using a stem cell proliferation assay and tumour xenotransplantation assay in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, we show that HAGE promotes tumour initiation and MMIC-dependent growth by preventing the IFN-α-induced inhibition. HAGE expression in malignant melanoma cells prevents the activation of the JAK–STAT signalling pathway which is involved in the induction of PML transcription. Knockdown of HAGE in ABCB5+ MMICs results in increased PML expression at the RNA and protein levels. This event is favoured through an increase in expression of the suppressor of cytokine signalling SOCS1 protein, a known positive regulator of ubiquitination and degradation of JAK proteins. HAGE knockdown in melanoma cell lines expressing ABCB5 decreases SOCS1 protein expression and this is reversed by re-introducing HAGE in these cells. An in vitro unwinding assay provides a mechanistic insight by demonstrating the capacity of the helicase HAGE to unwind SOCS1 RNA complexes and thereby promote the expression of SOCS1 protein. Collectively, these findings support the model by which HAGE promotes the initiation of tumours by ABCB5+ MMICs and their resistance to the anti-proliferative effects of IFN-α by inactivating the JAK–STAT pathway which is necessary for PML expression via a mechanism which involves the SOCS1.

Results

Decreased PML expression in HAGE+−ABCB5+ MMICs. The loss of PML expression has been previously reported in several solid tumours from different tissue origins. To investigate the expression status of PML in HAGE+ and ABCB5+ MMICs, we performed immunostaining on a malignant melanoma tissue microarray (TMA) using antibodies against PML, HAGE and ABCB5. PML expression was significantly decreased in HAGE+ and ABCB5+ tumour cells when compared with the control (normal skin). As a consequence, this decrease correlated with the absence of PML-NBs of which, PML is the essential component. PML has been shown to promote the hypophosphorylation of the retinoblastoma protein (Rb), which regulates the cell cycle progression at the G1−S phase by interacting and sequestering the transcription factor E2F. Immunostaining on a malignant melanoma TMA using an antibody against the phosphorylated forms of Rb showed an increased expression of phosphorylated Rb in malignant melanoma when compared with the normal skin control (Figure 1). Furthermore, PML expression was found in melanocytes of normal skin and in HAGE+−ABCB5+ melanoma cells as shown using an antibody against Tyrosinase, a specific marker of melanin-producing cells. Interestingly, PML was expressed in tissues surrounding ABCB5- and HAGE-expressing cells within the tumours (Figure 1 and Supplementary Figure 1). Similar results were obtained using malignant melanoma cell lines FM82 and FM55, which co-express HAGE and ABCB5. The permanent knockdown of HAGE in these cell lines using small hairpin RNAs (FM82 shRNA 1 and 2, FM55 shRNA 1 and 2) increased PML expression (Figure 2a). The molecular weight of the PML protein (~90 kDa) observed by immunoblotting (IB) suggests that the dominant isoform expressed in these cell lines may corresponds to PML isoforms II, III or both. To further investigate this, we have tested by IB two additional antibodies on whole cell extracts from FM82 control, FM82 shRNA and another melanoma cell line FM6 (Supplementary Figures 2B and C). The first antibody recognises the PML epitope, which maps to a region between 375 and 425 and therefore should recognise all PML isoforms with the exception of isoforms VI and VII. The second antibody recognises the PML epitope, which maps to a region between 575 and 625 and should only recognise PML I and PML IV. The immunoblot using the first antibody showed predominant expression of a band at ~90 kDa, however, the immunoblot with the second antibody showed weak expression of bands at ~80 kDa and ~100 kDa (Supplementary Figures 2B and C). From these results, we concluded that the predominant band at ~90 kDa may represent PML isoforms II or III or both. The decreased expression of PML protein in the presence of HAGE might result from a decrease in PML gene expression. We investigated this possibility by performing a semi-quantitative real-time PCR on mRNAs isolated from FM82 and FM55 controls and FM82 and FM55 shRNAs cell lines. We found that HAGE knockdown resulted in increased PML gene expression when compared with controls (Figure 2b). These results suggest that HAGE negatively regulates the basal expression of PML gene in HAGE+−ABCB5+ MMICs.

Figure 1 Expression of PML in HAGE-positive and ABCB5-positive malignant melanoma. Top panel: representative image of immunohistochemistry with antibodies to PML (green) or ABCB5 (green) or Tyrosinase (red) or HAGE (red) or phospho-Rb (green) on normal skin sections. The arrows indicate PML-NBs in Tyrosinase-positive cells. Lower panel: representative image of immunohistochemistry with antibody to PML (green or red), ABCB5 (green), Tyrosinase (red), HAGE (red) or phospho-Rb (green) in malignant melanoma tissue sections. The negative controls used were an IgG isotype from mouse and rabbit. The blue staining (DAPI) corresponds to the nucleus. Scale bars represent 100 μm (20 ×), 50 μm (40 ×), 20 μm (40 ×).
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FM55 shRNAs were treated with IFNα. PML protein expression upon IFNα treatment was significantly increased in HAGE knockdown cell lines when compared with FM82 and FM55 control (Figures 2d and e). This result suggests that HAGE prevents IFNα-mediated PML gene expression. Analysis of the JAK–STAT pathway in FM82 and FM55 controls and FM82 and FM55 shRNAs following IFNα treatment showed a decrease in Jak1, Tyk2, STAT1 and STAT2 phosphorylation in the presence of HAGE (Figures 2f, g, i and j). This downregulation of the JAK–STAT pathway might be due to a decrease in IFNAR expression on the surface of FM82 and FM55 control cells, thereby resulting in a reduced ligand binding. IB with a specific antibody to IFNAR and using lysates from FM82 and FM55 controls and FM82 and FM55 shRNAs showed no significant difference in IFNAR expression in the presence of HAGE and when HAGE is knocked down (Figure 2i). Finally, IB using antibodies to total Jak1, Tyk2, Stat1 and Stat2 showed no difference in their expression in FM82 and FM55 controls and FM82 and FM55 shRNAs (Figures 2h and k). As the phosphorylation of STAT1 and STAT2, and the dimerisation, nuclear translocation and activation of target genes are dependent on Jak1 and Tyk2 auto-phosphorylation (p-Jak1 and p-Tyk2). These results suggest that HAGE prevents IFNα-mediated PML gene expression via a negative regulation of Jak1 and Tyk2 phosphorylation.

HAGE promotes the expression of SOCS1. The SOCS1 belongs to the SOCS family of negative regulators of the JAK–STAT pathway. SOCS proteins can directly bind to the phosphorylated forms of JAKs via their central domain SH2 and the C-terminal SOCS box region. This binding triggers the ubiquitination and degradation of JAKs through the formation of an E3 ubiquitin ligase complex comprising Culin2 and the elongin B/C20 (Figure 3a). To investigate the potential involvement of SOCS1 in the HAGE-mediated downregulation of p-Jak1 and p-Tyk2, we performed an immunostaining with antibodies against HAGE and SOCS1 on FM82 control and FM82 shRNAs cell lines. SOCS1 was co-expressed together with HAGE in FM82 controls and its expression decreased when HAGE was knocked down (FM82 shRNAs) (Figure 3b). These results were confirmed by IB using cell extracts from FM82 and FM55 controls and FM82 and FM55 shRNAs (Figure 3b). Interestingly, SOCS3, another member of the SOCS family of proteins was also downregulated in FM82 and FM55 shRNAs (Supplementary Figure 2A). However, we were not able to detect SOCS2 another member of this family by IB using a previously described SOCS2 specific antibody (data not shown).23 Furthermore, SOCS1 expression was increased in HAGE-positive malignant melanoma tissue, thereby confirming the in vitro observations above (Figure 3c). To determine the functional relationship between HAGE and SOCS1, we used knockdown experiments using specific small interfering RNAs (siRNAs) for SOCS1 mRNA in FM82 and FM55 controls cell lines which naturally express HAGE. SOCS1 knockdown increased the expression of p-Jak1, p-Tyk2 and PML protein and suggested that SOCS1 has a role in HAGE-mediated downregulation of JAK–STAT–PML pathway (Figure 3d).

RNA helicases of the DEAD-box family unwind RNA duplexes by local strand separation in an ATP-dependent fashion. As a result, RNA helicases are involved in specific processes such as ribosome biogenesis, pre-mRNA splicing and translation.24–26 HAGE may enhance SOCS1 translation and promote its expression by unwinding and stabilising SOCS1 mRNA. To investigate if HAGE promotes the expression of SOCS1 protein, we knocked down SOCS1 mRNA in FM82 control and in FM82 shRNA1 cell lines. SOCS1 protein level was significantly decreased in FM82 shRNA1 transfected with a HAGE-expressing plasmid (Figure 3e). The decrease of SOCS1 expression was not due to a decrease of its mRNA expression upon HAGE knockdown as no significant changes in SOCS1 mRNA expression were observed by semi-quantitative real-time PCR on RNAs isolated from FM82 and FM55 controls and FM82 and FM55 shRNAs cell lines (Figure 3f). HAGE may promote SOCS1 protein expression through its unwinding activity towards SOCS1 mRNA. To determine if HAGE induces the unwinding of SOCS1 RNA, we performed an in vitro RNA unwinding assay in the absence or presence of different concentrations of recombinant HAGE protein and SOCS1 RNA duplexes. Remarkably, HAGE was able to unwind SOCS1 RNA duplexes in a dose-dependent fashion (Figure 3g). Finally, ectopic expression of HAGE promoted the translation of SOCS1 as shown by IB and using a metabolical labelling assay of nascent protein synthesis (Figure 3h).23,27 Taken together, these results suggest that
HAGE enhances the expression of SOCS1 protein expression by promoting SOCS1 RNA unwinding.

SOCS1 promotes p-Jak1 and p-Tyk2 ubiquitination. SOCS1 has been shown to inhibit the JAK–STAT pathway by interacting with the phosphorylated forms of Janus protein kinases. To investigate the potential interaction of SOCS1 with p-Jak1 and p-Tyk2, we performed an immunoprecipitation (IP) of cell extracts from FM82 control using SOCS1 antibody. IB of SOCS1 immunoprecipitates with antibodies against SOCS1, p-Jak1 and p-Tyk2 revealed the presence of SOCS1, p-Jak1 and p-Tyk2 in SOCS1 immunocomplexes (Figure 4a). SOCS1 interactions with p-Jak1 and p-Tyk2 may result in their ubiquitination and thereby their degradation by the ubiquitin proteasome system (Figure 4a). To investigate this possibility, we performed IPs of cell extracts from FM82 control using antibodies against p-Jak1 and p-Tyk2. IB using antibodies against p-Jak1, p-Tyk2 and ubiquitin revealed the presence of p-Jak1 and p-Tyk2 in p-Jak1 and p-Tyk2 immunoprecipitates, respectively. Furthermore, p-Jak1 and p-Tyk2 were highly ubiquitinated in the FM82 control immunoprecipitates when compared with FM82 shRNA immunoprecipitates (Figures 4b and c). Finally, the rescue of HAGE or SOCS1 expression by ectopic expression in FM82 shRNA1 promoted the ubiquitination status of p-Jak1 (Figure 4d). These results demonstrate that SOCS1 interaction with p-Jak1 and p-Tyk2 promotes their ubiquitination and this increases in the presence of HAGE.

HAGE provide ABCB5+ MMICs resistance to the anti-proliferative effect IFNα in vivo. We have previously shown that HAGE is expressed by ABCB5+ MMICs. These cells possess the capacity of self-renewal and differentiation into ABCB5+ or ABCB5− cells. To investigate if HAGE provides resistance to the anti-proliferative effect of IFNα treatment mediated at least through PML, we performed a self-renewal assay using FM82 control and FM82 shRNA cell lines. These melanoma spheres were stained using antibodies against HAGE, ABCB5, SOCS1 and PML.
Interestingly, SOCS1 was highly expressed in FM82 control when compared with FM82 shRNAs. In contrast, PML expression was higher in FM82 shRNAs when compared with FM82 control (Figures 5a, b and c). Furthermore, melanoma spheres generated from FM82 stably expressing PMLII gave rise to smaller spheres when compared with FM82 stably expressing the empty vector (Figures 5d and e). The ectopic expression of HAGE or SOCS1 in FM82 stably expressing PMLII did not affect the anti-proliferative effect of PMLII on the spheres growth (Figure 5e). In addition, PML knockdown by lentiviral expression of PML shRNAs in FM82 shRNA resulted in significantly bigger sized spheres when compared with spheres generated from FM82 shRNA infected with control lentiviruses (Figures 5f and g). Finally, IFNα treatment of growing FM82 and FM55 control and FM82 and FM55 shRNA melanoma spheres resulted in significantly smaller FM82 and FM55 shRNA spheres when compared with the FM82 and FM55 control spheres.
(Figures 6a, b and c). Similar results were observed with tumours derived from FM82 (control) and FM82 shRNA cell lines that had been xenotransplanted into NOD/SCID mice and treated with IFNα (Figures 6d and e). Immunostaining of sections from these tumours using PML antibody revealed an increased expression of PML in FM82 shRNA tumours treated with IFNα when compared with FM82 control, FM82 control treated with IFNα and IFNα untreated FM82 shRNA-derived tumours (Figure 6f). PML increased expression in IFNα-treated FM82 shRNA-derived tumours correlated with
Figure 6  HAGE counteracts the anti-proliferative effect of IFNα in vivo. (a, b and c) FM82 and FM55 controls and FM82 and FM55 shRNA 1 and 2 melanoma sphere formation in the presence or absence of IFNα. Student t-test: **P = < 0.01. (d) Summary of the in vivo experimental procedure. (e) Measurement of tumour growth in mice injected with either FM82 control cells or FM82 shRNA1 cells and treated or untreated with IFNα. ANOVA: **P = < 0.0028; Mann–Whitney U-test: *P = < 0.0108 (when comparing FM82 control + PBS and FM82 shRNA + PBS; Mann–Whitney U-test: **P = < 0.0079 (when comparing FM82 control + IFNα and FM82 shRNA + IFNα treated). (f) Immunohistochemistry/fluorescence staining with antibodies to HAGE (red), ABCB5 (green), SOCS1 (green) and on sections from FM82 control and FM82 shRNA1 NOD/SCID xenotransplanted tumours and treated or untreated with IFNα. Scale bar 100 μm
an increased apoptosis and a decreased cellularity in these
tumours when compared with FM82 control, FM82 control
treated with IFNα and IFNα-untreated FM82 shRNA-derived
tumours (Figures 7a and b). Taken together, these results
suggest that HAGE expression in ABCB5+ MMICs provides
resistance to IFN alpha-PML mediated anti-proliferative and
pro-apoptotic effects by promoting the expression of SOCS1 and the inhibition of PML expression through the inactivation of JAK–STAT pathway (Figure 7c).

Discussion

Chemotherapy and adjuvant IFNα therapy have been used to prevent recurrence of disease in patients with malignant melanoma. Despite important advances, the fact that many patients develop resistance to these therapies highlights the need to identify and further dissect the cellular and molecular mechanisms that are involved in this resistance. MMICs have been shown to offer such resistance to chemotherapy via their expression of ABCB5. This allows these populations to initiate and perpetuate their malignancy. In this regard, the mechanisms implicating ABCB5+ MMICs in chemo-resistance have been well studied. However, it is not known if these populations of melanoma stem cells are implicated in the resistance to IFNα therapy. This study focused on investigating those mechanisms with an emphasis on molecules that are involved in the JAK–STAT signalling pathway. We have previously shown that HAGE provides MMICs with resistance to the anti-proliferative effects of IFNα by enhancing the expression of SOCS1, a negative regulator of JAK–STAT pathway. We have previously shown that HAGE promotes the proliferation and survival of MMICs by promoting the unwinding and the translation of NRAS mRNA. Consistently, a similar mechanism is described here, where we show that HAGE unwinds SOCS1 mRNAs/siRNA duplexes and may therefore promote SOCS1 translation in tumour cells. SOCS1, a ubiquitin E3 ligase, has been shown to bind the phosphorylated forms of JAKs proteins, trigger their ubiquitination and prime them for protein degradation.

We show here that the helicase HAGE provide MMICs with resistance to IFNα. Indeed, we show here that the helicase HAGE promote MMICs with resistance to the anti-proliferative effects of IFNα by enhancing the expression of SOCS1, a negative regulator of JAK–STAT pathway. We have previously shown that HAGE promotes the proliferation and survival of MMICs by promoting the unwinding and the translation of NRAS mRNA. Consistently, a similar mechanism is described here, where we show that HAGE unwinds SOCS1 mRNAs/siRNA duplexes and may therefore promote SOCS1 translation in tumour cells. SOCS1, a ubiquitin E3 ligase, has been shown to bind the phosphorylated forms of JAKs proteins, trigger their ubiquitination and prime them for protein degradation. We show that HAGE knockdown results in a decreased ubiquitination of p-Jak1 and p-Tyk2, which further demonstrates a functional link between HAGE and SOCS1. Furthermore, SOCS1 was found to be highly expressed in HAGE-expressing tumour cells highlighting the importance of this molecule in melanoma progression. SOCS1 has also been shown to have an important role in melanoma progression and in the downregulation of biological responses by endogenous and/or therapeutically administered cytokines. The tumour suppressor PML has an important role in many cellular processes such as cell growth, apoptosis and differentiation. PML exerts its functions by interacting and recruiting different factors into subnuclear structures known as PML-NBs. PML localisation is not restricted to PML-NBs and it is observed to be present in other cellular compartments such as the nucleoplasm, the nucleolus, the nuclear envelope and the cytoplasm. The PML gene encodes a variety of isoforms, which are found in all human cell types, with isofrom I/II having the highest levels of expression. Expression of PML and its isoforms can be induced at the transcriptional level by the JAK–STAT pathway in response to IFNα. Interestingly, PML II, PMLIII or both seem to be predominantly expressed in malignant melanoma cell lines and their level of expression increases following IFNα treatment. Although the localisation of this isoform is mostly nuclear, a cytoplasmic expression can also be observed.

As HAGE prevents the activation of the JAK–STAT pathway in ABCB5+ MMICs, it appears that PML mRNA expression appears to be affected and this leads to the observed decrease in PML protein expression. This is probably the case, as PML expression upon IFNα treatment was also affected by HAGE expression. Moreover, several studies have reported a role for PML in the regulation of stem cell proliferation and differentiation and this effect involving HAGE might be one of the mechanisms by which cancer stem cells avoid the anti-proliferative and pro-apoptotic responses mediated by PML. Interestingly, PML expression is found in the cells surrounding HAGE+ cells, which suggest that HAGE targets specifically PML to prevent its anti-proliferative and pro-apoptotic functions. Furthermore, FM82 HAGE shRNA tumours treated with IFNα have an increased level of PML expression (and PML-NBs), higher levels of apoptosis and lower cellularity. Finally, PML knock down in FM82 HAGE shRNA resulted in increased size of spheres which pointed out to the specific targeting of PML by HAGE.

Collectively, these findings provide evidence for a previously unknown role of HAGE in ABCB5+ MMIC-dependent resistance to PML-mediated anti-proliferative response of IFNα. We clearly demonstrate that HAGE prevents the activation of JAK–STAT pathway responsible for the induction of the expression of the tumour suppressor PML. Finally, as HAGE is expressed only by tumour cells, these results suggest that cancer therapies targeting HAGE helicase may have broad applications for treating ABCB5+ malignant melanoma and for preventing resistance to IFNα treatment.

Materials and Methods

Cell lines and growth conditions. The human melanoma cell lines, FM82, FM55 and FM6, were a kind gift from Professor D. Schadendorff of the Deutsches Krebsforschungszentrum (Heidelberg, Germany). All cells were cultured as previously described in RPMI 1640 media (Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum and 1% (w/v) L-glutamine (Lonza). Cells were incubated at 37 °C in 5% (v/v) CO2 and 100% humidity. IFNα2a (IMR-236, Immunex, San Diego, CA, USA) was used at a concentration of 1000 U/ml.

Stable and transient transfection of cell lines. A stable knockdown cell line was created using shRNA-bearing plasmids specific for HAGE (Sure Silencing shRNA Plasmid for Human DDX43, SABiosciences, Crawley, UK). FM82 and FM55 cells were seeded at 5 × 10^4 cells/well into a 24-well plate and grown overnight. About 1.2 μg of SureSilencing shRNA were transfigested into cells using Lipofectamine 2000 (Invitrogen, Paisley, UK), as described by the manufacturer. Forty-eight hours later, the cells were seeded into a 96-well plate and grown in media treated with 500 U/ml G418 antibiotic to allow selection of plasmid-transfected cells. HAGE expression status was monitored using semi-quantitative real-time PCR, western-blotting and immunofluorescence (IF). The transient knockdown of SOCS1 was carried out using an SOCS1-specific siRNA molecule (Eurogentec, Southamp ton, UK; sense: 5′-GAGAUGAGUCCGUAUGACUGdTdT-3′; anti-sense: 5′-UGUGAAGAGGUAUGACUGGdTdT-3′; and Interferin transfection reagent (Polyplus, Illkirch, France) following the manufacturer’s recommendations. FM82 empty vector (transfected with pcDNA3.1) and FM82 PMLII cell lines (transfected with pcDNA3.1-PMLII, a kind gift from Dr Mounira Chelbi-Alix, Université de Paris 5) were generated using the same procedure as above. HAGE or SOCS1 ectopic expression were carried out using pCMV6-DLX5-HAGE, pCMV6-PMLII-HAGE or SOCS1 ectopic expression were carried out using pCMV6-DLX5-HAGE, pCMV6-PMLII-HAGE or SOCS1 ectopic expression were carried out using pCMV6-DLX5-HAGE, pCMV6-PMLII-HAGE or SOCS1 ectopic expression were carried out using pCMV6-DLX5-HAGE.
The lentiviral particles used to infect FM82hRNA were produced corresponding to the manufacturer recommendations.

Antibodies. For this study, we used monoclonal antibodies to HAGE (1:250 for IB; 1:50 IF, SAB1400618, Sigma), ABCBS (1:500 for IB; 1:100 for IF, HPA026975, Sigma), PML (1:500 for IF, sc-6620), PML (1:250 for IF, 1:250 for IB, sc-966, Santa Cruz Biotechnology, Montgomery, TX, USA), β-actin (1:250 for IB, sc-130857, Santa Cruz Biotechnology), SOCS1 (1:300 for IB, 13950, Cell Signaling Technology, Danvers, MA, USA), SOCS1 (1:100 for IB, 1:100 for IF, sc-9021, Santa Cruz Biotechnology), ubiquitin (1:300 for IB, 3933, Cell Signaling Technology), Phospho-Jak1 (1:300 for IB, 3331, Cell Signaling Technology), Phospho-Jak1 (1:100 for IB; 1:200 for IF, sc-101716, Santa Cruz Biotechnology), Phospho-Stat3 (1:300 for IB, 9171, Cell Signaling Technology), Phospho-Stat6 (1:500 for IB, 07-224, Millipore, Billerica, MA, USA), Phospho-Tyk2 (1:200 for IB; 1:100 for IF, sc-11763, Santa Cruz Biotechnology), IFN α/β R (1:200 for IB, sc-7931, Santa Cruz Biotechnology), Tyrosinase (1:200 for IF, ab112231, Abcam, Cambridge, UK), SOCS3 (1:200 for IB; sc-51699, Santa Cruz Biotechnology), SOCS2 (1:200 for IB; sc-9022, Santa Cruz Biotechnology), Cleaved caspase 3 (1:1000 for IF, 9661, Cell Signaling Technology) and Phospho-Rb (1:200 for IF, 9308, Cell Signaling Technology).

Immunohistochemistry and IF. Paraffin-embedded human melanoma TMAs were purchased from US Biomax (Rockville, MD, USA). The sections were de-waxed, re-hydrated in graded alcohols, rinsed in ddH2O and antigen retrieval was performed for 10 min (0.01 M citrate phosphate buffer at pH 6.0) in a microwave at 1000 W. The tumours excised from NOD/SCID mice were embedded in OCT media, fixed in 2-methyl-butane (Sigma) that had been super-cooled in liquid N2 and sectioned using a CM1900 cryostat (Leica, Wetzlar, Germany). Melanoma cells were fixed in 4% (w/v) paraformaldehyde and then treated as follows: the sections or cancer cells were washed three times in 1X phosphate-buffered saline (PBS) for 10 min each, blocked and permeabilised in 10% (w/v) bovine serum albumin in 0.1% (v/v) PBS-Tween, incubated overnight with primary antibody (in blocking solution), washed three times for 10 min each with 1X PBS, incubated for one hour with secondary antibody (in blocking solution) and washed three times with 1X PBS. Sections and melanoma cells were counterstained and mounted with DAPI fluorescent medium (Vector Laboratories, Peterborough, UK) for IF microscopy.

Genetic analysis. FM82 and FM55 control and FM82 and FM55 shRNAs cells were grown to 80% confluence as described above. Total RNA was extracted from cells using RNA-STAT 60 reagent (AMS Biotechnology, Abingdon, UK) as described by the manufacturer. Extracted RNAs were quantified using a Nanodrop spectrophotometer (Thermo Scientific, Loughborough, UK). Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA), oligo-dT primers (Promega) and 2 g of total RNA were subsequently used for cDNA synthesis following the manufacturer’s protocol. Semi-quantitative real-time PCR reactions were performed for 10 min (0.01 M citrate phosphate buffer at pH 6.0) in a 10% (w/v) Marvel milk/tris-buffered saline (TBS) solution with 0.05% (v/v) Tween-20 (TBST). The extracted RNAs were quantified using a Nanodrop spectrophotometer (Thermo Scientific, Loughborough, UK). The cDNA was amplified using primers (Promega) and subjected to IP with SOCS1 antibody, followed by IB with Protein G Plus-Agarose beads (IP08, Calbiochem) and corresponding IgGs. For the antibody binding, antibodies to SOCS1, Phospho-Jak1 or Phospho-Tyk2 were incubated with agarose beads for 2 h in IP buffer before adding the pre-cleaned extracts for overnight IP. For SOCS1 protein labelling, FM82 control cell line was transfected with SOCS1 cDNA (Origene, SC111081) and with or without HAGE cDNA. 24 h later the cells were divided into four flasks, each containing 1 × 106 cells for each experiment (in the presence or absence of HAGE). Each flask was incubated in a media without methionine for 30 min (RPMI-1640, R7513; Sigma) and 50 µM of protein labelling compound (L-azidohomoalanine) was added for (0, 30, 60 and 120 min) as recommended by the manufacturer (Click-it AHA (L-azidohomoalanine) for nascent protein synthesis, C10102; Invitrogen). The cell extracts from each flask were treated with Biotin alkyne (Click-it Biotin Protein Analysis Detection Kit, C33372, Invitrogen) and subjected to IP with SOCS1 antibody, followed by IB with Streptavidin-HRP.

In vitro transcription and RNA labelling. The plasmid pCMV6-XL4 containing SOCS1 was purchased from Origene (SC111081) and used for in vitro transcription using T7 promoter to generate SOCS1 transcript (Ambion, Paisley, UK). SOCS1 N-terminal RNA complementary sequence 5′-ACAGCCAGGUUG-GACAGCGAGAUGAUUGCUCGAGCAGC-3′ labelled using Pierce RNA 3′ End Biotinylation Kit (Thermo Scientific). The labelling efficiency was determined by dot blotting following the manufacturer’s recommendations.

Native RNA gels and winding/unwinding assay. Biotinylated N-terminal RNA complementary sequence (2 pmol; labelling efficiency higher that 75%); was placed to wind with unlabelled SOCS1 RNA in a solution containing 3.5 mM equimolar of ATP/MgCl2 for 5 min at 95 °C, followed by 1 h at 37 °C. Recombinant HAGE protein was added at two different concentrations (0.6 µg, 1.2 µg) after the RNA winding assay, followed by 1 h incubation at 37 °C. The reactions were stopped by the addition of 50% (v/v) glycerol, 20 mM EDTA, 2% (v/v) SDS and 0.025% (v/v) bromophenol blue. Samples were analysed on 10% (v/v) native acrylamide gels in 1 × TBE buffer followed by blot transfer and visualisation using a Chemiluminescent Detection Module (Thermo Scientific).

Melanoma spheroid cell culture. For the culture of melanoma spheromas, cells from FM82 empty vector, FM82 PMLII, FM82 and FM55 controls and FM82 and FM55 shRNAs were cultured at clonal density (1 × 104 cells/ml in 24-well plates) in DMEM/F12 media (Gibco Invitrogen, Paisley, UK, 32,500) supplemented with N-2 and B5 Media (R&D Systems, Minneapolis, MN, USA; AR0907), and daily addition of 100 ng/ml FGF basic and 100 ng/ml EGF (both from Sigma) for a 10-day culture period.2 The differentiation of melanoma spheromas was induced by the withdrawal of FGF basic and EGF. The cells were stained by IF as described above. For IFNα studies, the melanoma spheromas were cultured for 15 days and IFNα (100 U/ml) was added at day 9 and 12. Sphere diameters were measured using Carl Zeiss AxioVision software.

Tumour transplantation assay. NOD/SCID mice were acquired (Harlan Laboratories, Shannon, UK) and kept in accordance with the Animals (Scientific Procedures) Act 1986. The mice were used for tumour transplantation Assay (xenotransplantation assay) using FM82 control or FM82 shRNA cells, as previously described.2 When tumours reached 3 mm3 in size (monitored using a caliper), PBS or IFNα2a (4 × 107 IU) (PUH107Z, Abd Serotec, Hercules, CA, USA) were injected locally three times a week for a period of 4 weeks. The tumours were collected and measured using a rule. The sections were obtained from three different levels within the tumours (10 sections of 10 µm separating each level). For determining the tumours cellularity and the counting of the number of cells expressing the cleaved caspase 3, three pictures were taken randomly from each sections and from each level and were used for the counting. The sections were stained by immunohistochemistry/IF as described above.

Conflict of Interest
The authors declare no conflict of interest.
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