

MageA2 restrains cellular senescence by targeting the function of PMLIV/p53 axis at the PML-NBs

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MAGE-A genes are a subfamily of the melanoma antigen genes (MAGEs), whose expression is restricted to tumor cells of different origin and normal tissues of the human germline. Although the specific function of individual MAGE-A proteins is being currently explored, compelling evidence suggest their involvement in the regulation of different pathways during tumor progression. We have previously reported that MageA2 binds histone deacetylase (HDAC)3 and represses p53-dependent apoptosis in response to chemotherapeutic drugs. The promyelocytic leukemia (PML) tumor suppressor is a regulator of p53 acetylation and function in cellular senescence. Here, we demonstrate that MageA2 interferes with p53 acetylation at PML-nuclear bodies (NBs) and with PMLIV-dependent activation of p53. Moreover, a fraction of MageA2 colocalizes with PML-NBs through direct association with PML, and decreases PMLIV sumoylation through an HDAC-dependent mechanism. This reduction in PML post-translational modification promotes defects in PML-NBs formation. Remarkably, we show that in human fibroblasts expressing RasV12 oncogene, MageA2 expression decreases cellular senescence and increases proliferation. These results correlate with a reduction in NBs number and an impaired p53 response. All these data suggest that MageA2, in addition to its anti-apoptotic effect, could have a novel role in the early progression to malignancy by interfering with PML/p53 function, thereby blocking the senescence program, a critical barrier against cell transformation.

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Melanoma antigen gene (MAGE)-A proteins belong to the type I MAGE genes subfamily whose expression is restricted to tumor and germinal cells.¹ Expression of MAGE-A genes is an early event in tumorigenesis and correlates with genome-wide hypomethylation, a frequently observed epigenetic event in carcinogenesis.² Owing to their high sequence homology MAGE-A proteins have been considered functionally redundant, and have been largely exploited in the immunotherapy field through cancer vaccine development or as tumor markers.^{3–5} Only during the last few years, their biological function has begun to be investigated. A growing body of evidence indicates that MAGE-A proteins could confer advantages to cancer cells by different mechanisms and with a certain degree of specificity. For instance, MageA1 associates to SKIP and is able to interfere with Notch-IC regulation,⁶ MageA3 is involved in FGFR signaling^{7,8} and MageA11 regulates AR activation.⁹ We have previously demonstrated that MageA2 is a strong inhibitor of the p53 tumor-suppressor transcription factor through histone deacetylase (HDAC)3 recruitment. Thus, in human primary melanoma cells, MageA2 expression confers resistance to chemotherapeutic drugs by interfering with p53 acetylation, which can be reverted by HDAC inhibitor drugs.¹⁰ Subsequently, other groups also described an opposite correlation between MAGE-A gene expression and p53 activity.^{7,11,12}

Interestingly, only MageA4 has been shown to be involved in some potentially anti-tumor functions such as gankyrin oncoprotein inhibition¹³ and apoptosis induction.^{14,15}

It has been demonstrated that escape to cellular senescence is one of the first barriers to be bypassed during transformation.¹⁶ The promyelocytic leukemia (PML) tumor-suppressor protein triggers senescence in normal cells and it has been shown to be involved in oncogenic RasV12-induced senescence.^{17,18} PML is responsible for the formation of nuclear macromolecular complexes, termed PML-nuclear bodies (PML-NBs).^{19,20} p53 tumor suppressor is recruited to PML-NBs where it became acetylated and activated, and participate in the triggering of cellular senescence.^{17,18,21,22} In addition, PML itself is regulated by acetylation and subsequent sumoylation,²³ and PMLIV sumoylation has been shown to be required for full p53 activation at the PML-NBs.^{21,24}

Here, we have analyzed the ability of MageA2 to interfere with cellular senescence as the final readout of PMLIV activity on p53 acetylation and function. We demonstrate that MageA2 expression correlates with decreased p53 acetylation and activation as induced by PMLIV. Moreover, MageA2 accumulates in PML-NBs through direct interaction with PML proteins and, MageA2 expression results in impaired PMLIV sumoylation and aberrant NB formation. Furthermore, we

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Abbreviations: MAGE, melanoma antigen gene; PML, promyelocytic leukemia; PML-NBs, PML-nuclear bodies; NLS, nuclear localization signal; HDAC, histone deacetylase; TSA, Trichostatin A; SA- β -Gal, senescence-associated β -galactosidase activity; SAHF, senescence-associated heterochromatin foci

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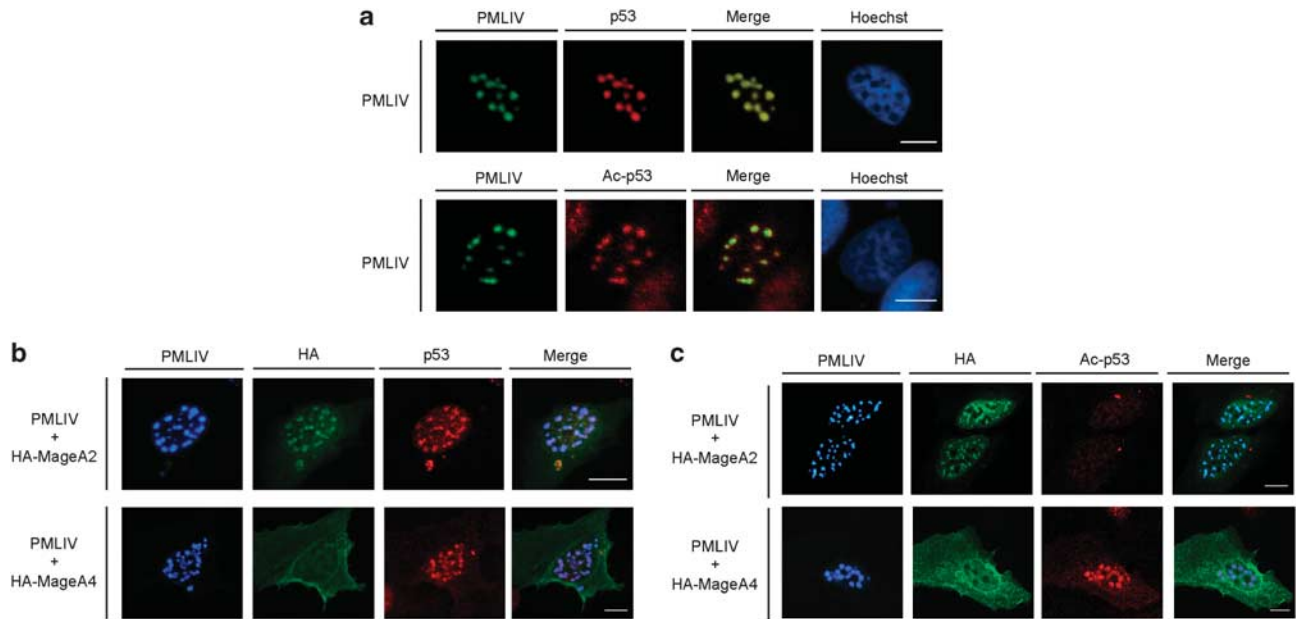


Figure 1 MageA2 represses PMLIV-induced p53 acetylation at PML-NBs. (a) Representative immunofluorescence (IF) images of U2OS cells showing colocalization of transfected PMLIV with endogenous total p53 (upper panel) and acetylated p53 (bottom panel). PMLIV was detected with an anti-PML monoclonal and FITC-conjugated anti-mouse antibodies, endogenous p53 acetylation status was monitored using an anti-Ac-p53Lys382 and TRITC-conjugated anti-rabbit antibodies. (b) Representative IF images of U2OS cells transfected as indicated, showing colocalization of MageA2, PMLIV and endogenous p53 at PML-NBs. PMLIV was detected with an anti-PML monoclonal and Alexa Fluor 350-conjugated anti-mouse IgG1 antibodies, MageA2 with an anti-HA polyclonal and FITC-conjugated anti-rabbit antibodies, and p53 with DO-1 monoclonal and TRITC-conjugated anti-mouse IgG2a antibodies. (c) Panel shows p53 acetylation status at PML-NBs in the same cells of panel B). In this case, PMLIV was detected with an anti-PML monoclonal and Alexa Fluor 350-conjugated anti-mouse IgG1 antibodies, MageA2 with an anti-HA monoclonal and FITC-conjugated anti-mouse IgG2b antibodies, and acetylated p53 with an anti-Ac-p53Lys382 polyclonal and TRITC-conjugated anti-rabbit antibodies. Scale bar: 5 μ m

address the effect of MageA2 in oncogene activated PML-dependent senescence, showing that MageA2 interferes with RasV12-induced cellular senescence and cooperates in cell proliferation, by controlling NBs number and by downregulating the p53-dependent transcriptional activation.

Results

MageA2 impairs p53 acetylation at PML-NBs. Different kinds of stimuli regulate p53 functions by inducing its post-translational modifications thus leading to p53 stabilization and activation.²⁵ We have previously demonstrated that upon DNA damage MageA2 expression hampers the apoptotic response of cells by affecting p53 acetylation and transactivation function.¹⁰ PMLIV is a known regulator of p53; indeed it has been shown that PMLIV by recruiting p53 to PML-NBs facilitates its acetylation inducing its transcriptional activity.^{18,21} In this context, we asked whether MageA2 could also affect p53 acetylation as induced by PMLIV. To address this issue, we co-expressed PMLIV and MageA2 in U2OS cells and evaluated the acetylation status of endogenous p53 at PML-NBs. Immunofluorescence analysis showed that PMLIV re-localized p53 to PML-NBs where p53 is present in its acetylated form (Figure 1a). Of note, MageA2 but not MageA4 colocalized with endogenous p53 at NBs (Figure 1b). As MAGE-A proteins share high level of sequence homology, we used MageA4 as specificity control. Indeed, MageA4 behaves differently respect to MageA2, with respect to p53 regulation (Supplementary Figures S1A and S1C). Importantly, expression of MageA2 correlated with a

strong reduction of p53 acetylation at NBs, while MageA4 did not affect p53 acetylation status (Figure 1c).

In order to confirm the involvement of MageA2 in interfering with PMLIV-induced p53 acetylation, we knocked-down endogenous MAGE-A and analyzed p53 acetylation upon expression of PMLIV. We observed that cells expressing low levels of MAGE-A showed an increased rate of p53 acetylation by PMLIV, with respect to control cells (Figure 2a). In contrast, when MageA2 was co-expressed along with PMLIV we could corroborate its ability to decrease PMLIV-dependent p53 acetylation (Figure 2b). Finally, as p53 acetylation and recruitment to PML-NBs correlate with increased transcriptional activity of p53, we speculated that MageA2 by dampening p53 acetylation at NBs could affect p53 transactivation function induced by PMLIV. We performed reporter gene assays using the *pig3* gene promoter (PIG3-LUC), which was reported to be highly responsive to p53 activation by PMLIV²¹ (Figure 2c, left). Co-expression of MageA2, but not MageA4, resulted in a strong repressive effect on PMLIV-induced p53 activation (Figure 2c, right). It is worth mentioning that the noticed difference between MageA2 and MageA4 on p53 repression seems to be unrelated to their differences in cellular distribution, because a forced nuclear version of MageA4 through nuclear localization signal (NLS) tagging, did not modify its p53 repressing activity or binding capability (Supplementary Figures S1B, S1C and S1D). Altogether, these results suggest that MageA2 localizes to PML-NBs and represses PMLIV-induced p53 acetylation and activation.

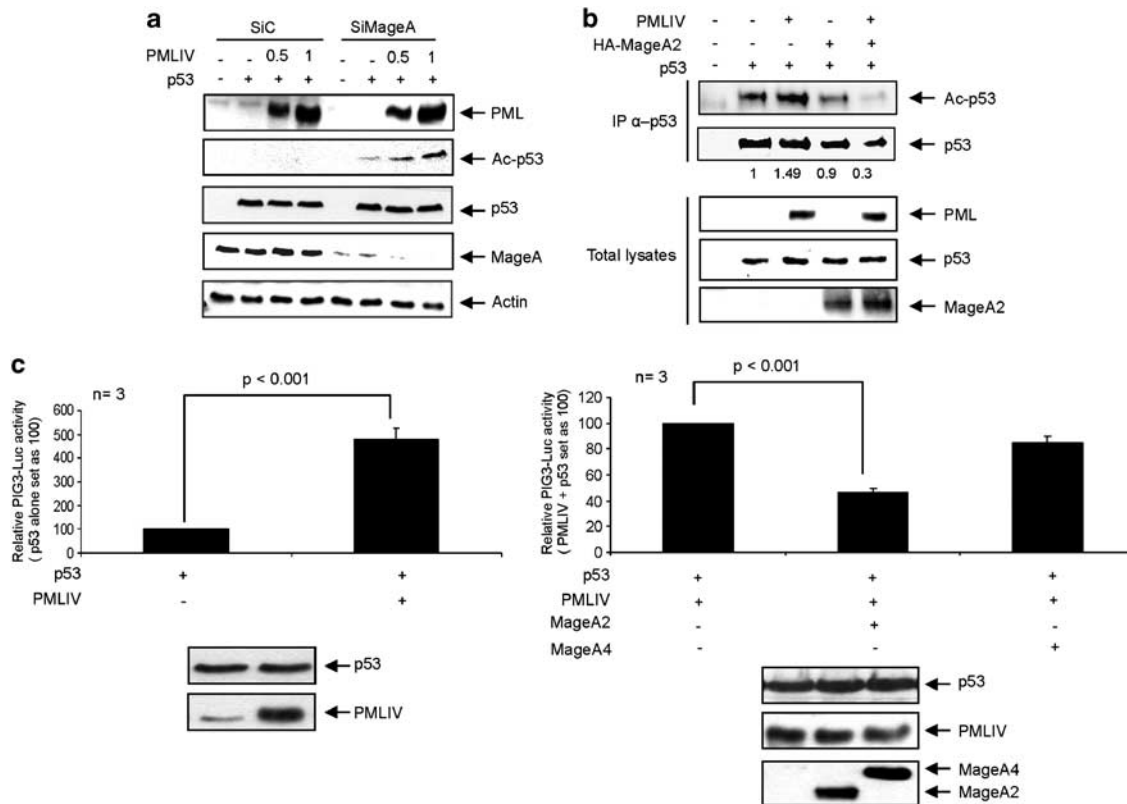


Figure 2 MageA2 represses PMLIV-induced p53 acetylation and function. (a) Analysis of p53 acetylation in H1299 cells transfected as indicated and expressing increasing amounts of PMLIV (0.5 μ g and 1 μ g). Expression of PMLIV, p53 and MAGE-A was analyzed by western blot. Actin levels were used as loading control. (b) H1299 cells were transfected as indicated. p53 was immunoprecipitated with an anti-p53 monoclonal antibody. Total and acetylated levels of immunoprecipitated p53 were analyzed by western blot using anti-p53 and specific anti-Ac-p53Lys382 polyclonal antibodies, respectively. Expression of PMLIV, p53 and MageA2 was evaluated by immunoblotting of total lysates. Numbers below p53 IP blot represent quantification of bands and refer the fold increase in acetylated to total precipitated p53 (acetyl/total p53) ratio, p53 alone was set as 1. (c) Upper: luciferase gene reporter assay in H1299 cells transfected as indicated. Values corresponding to p53 transfection was reported as 100 (left). Values corresponding to p53 + PMLIV transfection was reported as 100 (right). Lower panels show western blot analysis of lysates used in the luciferase assays. *n*, refers to the number of independent experiments carried out; error bars indicates S.D.; a Student's *t*-test was used for statistical analysis

MageA2 interacts with PMLIV. In order to further characterize MageA2–PML functional interaction, we analyzed the subcellular localization of selected MAGE-A subfamily members. Immunofluorescence assays revealed that MageA2, but not other MAGE-A proteins displays punctuate nuclear staining (Figure 3a). Moreover, extraction of soluble proteins with detergent containing buffer evidenced endogenous MageA2 speckled staining and partial colocalization with endogenous PML bodies in 13923M human melanoma cells that express almost exclusively MageA2 protein and in U2OS cells that express different Mage-A proteins including MageA2 (Figure 3b).

We next evaluated whether MageA2 localization to PML-NBs was dependent on MageA2–p53 interaction.¹⁰ To this aim, we knocked-down p53 expression in U2OS cells and analyzed MageA2 localization in PMLIV/MageA2 co-transfected cells. Interestingly, MageA2 efficiently colocalized with PMLIV at NBs independently of p53 expression (Figure 3c). Similar results were obtained by co-expressing PMLIV and MageA2 in H1299 p53 null cells (data not shown).

To verify whether PMLIV directly associates with MageA2, we performed an *in vitro* pull down assay between recombinant GST, GST-MageA2 and GST-MageA4 and *in vitro*

translated PMLIV. Results indicate that MageA2 but not MageA4 interacts directly with PMLIV (Figure 4a).

Interaction between endogenous PML and MAGE-A proteins was evaluated in U2OS cells (Figure 4b, left). Owing to high sequence homology among MAGE-A proteins, antibodies generally recognize more than one MAGE-A subfamily member. Hence, Figure 4b shows that at least one or more endogenous MAGE-A proteins form complexes with PML in U2OS cells. Similar results were obtained by immunoprecipitation assay performed in 13923M melanoma cells (Figure 4b, right), thus strengthening the fact that endogenous MageA2 and PML interacts in cells. Then, we carried out immunoprecipitation assay after overexpressing both PMLIV and HA-MageA2 or HA-MageA4 (Figure 4c), confirming a degree of specificity in MageA2 and PMLIV interaction.

As MageA2 colocalizes with endogenous PML-NBs, and different PML isoforms are expressed in cells as a result of alternative splicing of the *pml* gene,²⁶ we tested whether MageA2 interacts differentially with PML isoforms. We performed co-immunoprecipitation between different Flag-tagged PML isoforms (I to VI) and HA-MageA2. As shown in Figure 4d, MageA2 is able to bind PMLI, II, IV, V and to a

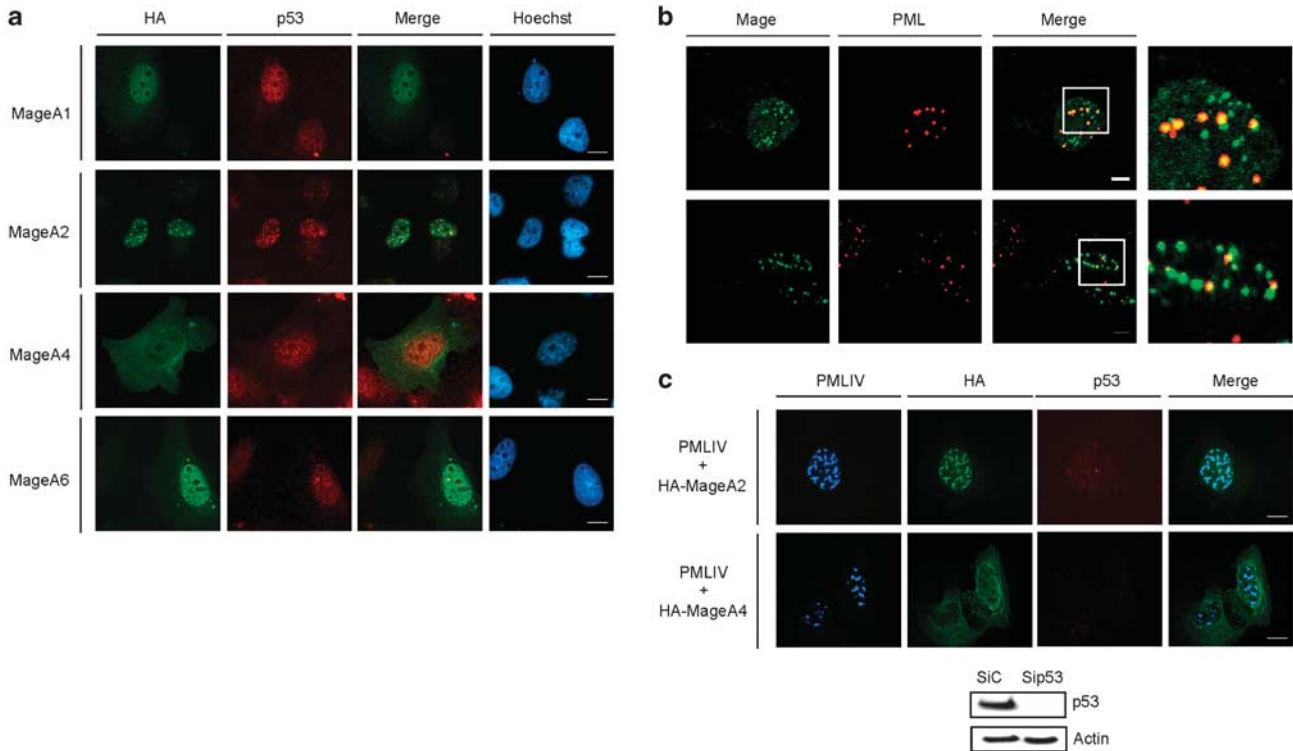


Figure 3 MageA2 specifically localizes at PML-NBs. (a) Representative IF images of U2OS cells transfected with HA-MageA1, -A2, -A4 and -A6, using an anti-HA polyclonal antibody. Endogenous p53 was detected using monoclonal anti-p53 antibody (DO-1). Primary antibodies were recognized with FITC-conjugated anti-rabbit antibody and TRITC-conjugated anti-mouse antibody, respectively. (b) Representative confocal IF images analyzing endogenous MageA2 and PML proteins colocalization in the melanoma cell line 13923M (upper) and HA-MageA2 and endogenous PML in U2OS cells (lower). Cells were treated with CSK buffer before fixation to eliminate insoluble proteins. PML was detected with an anti-PML monoclonal antibody and MageA2 with an anti-MageA polyclonal antibody. Right panels represent higher magnifications of the selected areas in the merge images. (c) Representative IF from U2OS cells transfected with specific siRNA to knock-down p53 and transfected with PMLIV in combination with HA-MageA2 or HA-MageA4. MAGE-A proteins were visualized with an anti-HA monoclonal and FITC-conjugated anti-mouse IgG2b antibodies, PMLIV with an anti-PML polyclonal and Alexa Fluor 350-conjugated anti-rabbit antibodies and p53 with a monoclonal anti-p53 and TRITC-conjugated anti-mouse IgG1 antibodies. Scale bar: 5 μ m

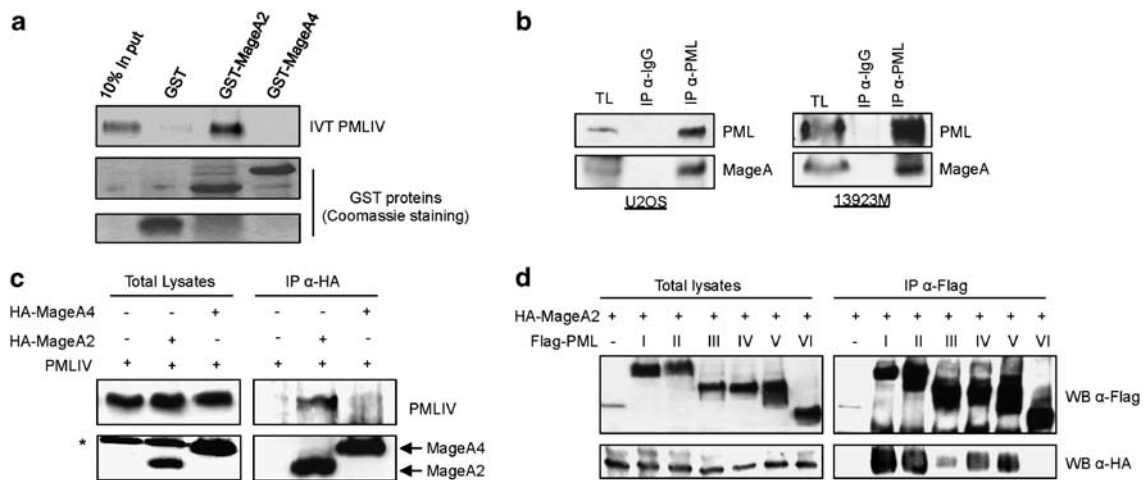


Figure 4 MageA2 binds to PMLIV. (a) *In vitro* binding assay using recombinant/purified GST, GST-MageA2 or GST-MageA4 fusion proteins incubated with *in vitro* translated 35 S-labeled PMLIV (IVT PMLIV). (b) Immunoprecipitation (IP) of endogenous PML/MAGE-A complex in U2OS cells (left) or in the melanoma cell line 13923M (right) using anti-PML monoclonal antibody, control IP was performed with an anti-Flag monoclonal antibody. (c) IP in 293T cells transfected with PMLIV and HA-MageA2 or HA-MageA4 using anti-HA monoclonal antibody. (d) IP in 293T cells transfected with HA-MageA2 and different Flag-tagged-PML isoforms using an anti-Flag monoclonal antibody. TL, total lysate. *Unspecific band

lesser extent PMLIII, but no interaction was observed with PMLVI. All these interactions were confirmed by *in vitro* binding assay (Supplementary Figure S2). In

summary, all these data suggest that MageA2 interacts with PML at the PML-NBs in a p53-independent manner.

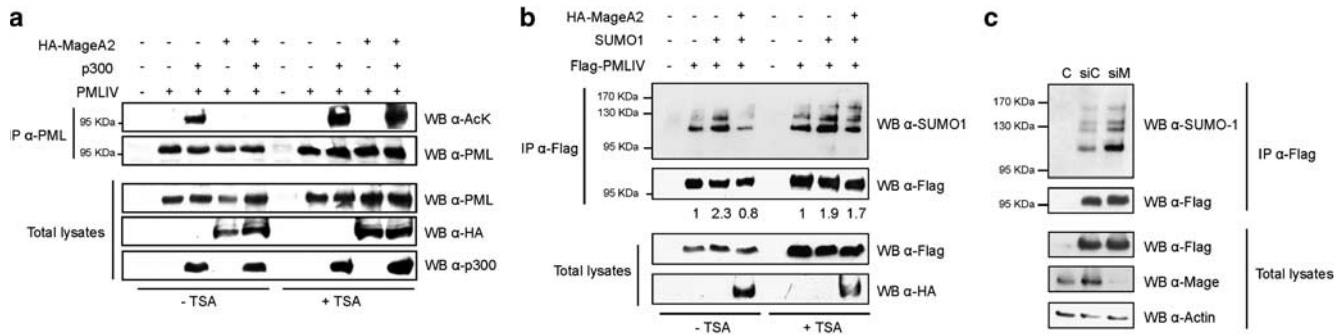


Figure 5 MageA2 impairs PMLIV post-translational modifications. (a) Analysis of PMLIV acetylation in 293T cells transfected as indicated. PMLIV was immunoprecipitated with an anti-PML monoclonal antibody. Acetylated and total PMLIV was detected in western blot with an anti-acetylated lysine and anti-PML polyclonal antibodies, respectively. At 6 h after transfections, cells were treated or not with 1 μ M TSA for 24 h. (b) Analysis of PMLIV sumoylation in 293T cells transfected as indicated. PMLIV was immunoprecipitated with an anti-Flag antibody. The membrane was first incubated with an anti-SUMO1 monoclonal antibody to detect sumoylated PMLIV and subsequently with an anti-PML polyclonal antibody to detect total PMLIV. HA-MageA2 was detected with an anti-HA monoclonal antibody. At 6 h after transfections, cells were treated or not with 1 μ M TSA for 24 h. Numbers below Flag blot in the IP represent quantification of bands and refer the fold increase in sumoylated to total precipitated PMLIV (sumo/total PMLIV) ratio, PMLIV alone was set as 1. (c) Analysis of PMLIV sumoylation in U2OS cells stably expressing Flag-PMLIV and transfected with control (siC) or MageA2-specific (siM) siRNA. PMLIV was immunoprecipitated with an anti-Flag monoclonal antibody. Sumoylated and total PMLIV was detected in western blot with anti-SUMO-1 and anti-PML polyclonal antibodies, respectively. Line C corresponds to U2OS control cell line

Although MageA2 is able to interact with different PML isoforms, subsequent experiments investigating the functional relevance of MageA2–PML interaction have been focused on PMLIV, which mediates acetylation and activation of p53.

MageA2 reduces PMLIV acetylation and sumoylation. PML functionality is highly regulated by post-translational modifications such as phosphorylation, acetylation and sumoylation.²⁷ Along with others, we have demonstrated that MAGE-A proteins can associate to HDAC.^{6,10} As acetylation has been recently reported to be a prerequisite for subsequent PML sumoylation,²³ we decided to investigate whether MageA2–PMLIV interaction could affect PMLIV acetylation. To verify this point we co-transfected cells with PMLIV and p300 to stimulate PMLIV acetylation. Then, PMLIV acetylation was detected using anti-acetyl lysine antibody on immunoprecipitated PMLIV. As shown in Figure 5a, expression of MageA2 together with PMLIV/p300 strongly decreased PMLIV acetylation. Importantly, treatment with the HDAC inhibitor Trichostatin A (TSA) reverted the effect of MageA2 expression on PMLIV acetylation, indicating that endogenous HDACs are involved in such effect (Figure 5a). In support of this data, we observed that MageA2 expression induces the recruitment of HDAC3 to PML-NBs (Supplementary Figure S3A).

Moreover, PMLIV reduced acetylation resulted in PMLIV sumoylation defect: PMLIV was efficiently sumoylated in the presence of SUMO1, whereas MageA2 expression significantly reduced such modification and, accordingly to our previous result, the effect was dependent on endogenous HDAC activity (Figure 5b). Importantly, siRNA-mediated downregulation of MageA2 in U2OS cells stably expressing PMLIV resulted in increased levels of sumoylated PMLIV, demonstrating the involvement of endogenous MageA2 in the regulation of PMLIV sumoylation (Figure 5c). Interestingly, sumoylation of PMLVI isoform was not affected by MageA2 expression (Supplementary Figure S3B), demonstrating the relevance of MageA2–PML interaction. Altogether these

results suggest that MageA2 could affect PMLIV sumoylation through an HDAC-dependent mechanism.

MageA2 impairs PML-NBs formation. It has been demonstrated that PML sumoylation and protein–protein interactions of PML through SUMO are involved in the proper formation of PML-NBs.^{20,28} As our results indicate that MageA2 negatively regulates PMLIV post-translational modifications, in particular sumoylation, we decided to investigate whether MageA2 could affect PML-NB formation. We used PML^{-/-} MEF to analyze PML-dependent neo-formation of NBs. Expression of PMLI, PMLIV or PMLVI in PML^{-/-} MEF leads to the formation of NBs that differ in their number and size (Figure 6a, left). Intriguingly, expression of MageA2 affected the typical morphology and the number of PML-NBs formed by PMLI and PMLIV but not by PMLVI, in agreement with the previously demonstrated capability of MageA2 to interact with specific PML isoforms (Figure 6a, middle). Quantification of the number of cells showing normal number (15–30 NBs per nucleus) and morphology (tiny punctuate) of NBs structures in cells co-expressing MageA2 and PMLIV with respect to PMLIV alone, indicates the degree of impairment that MageA2 expression caused on PMLIV-NBs formation (Figure 6a, right). As a measure of correct formation of PML-NBs, we also evaluated the ability of PML to colocalize with SUMO1 at PML-NBs in cells expressing (Figure 6b, middle) or not MageA2 (Figure 6b, left). Importantly, the ability of PMLI and PMLIV to correctly colocalize with SUMO1 at NBs was significantly impaired by MageA2 expression (Figure 6b, middle and right). Expression of SUMO1 alone displayed a diffused nuclear staining pattern because SUMO1 accumulates mainly in PML-NBs, and in PML^{-/-} cells, PML-NBs do not form (data not shown). In some cases, MageA2 expression blocked colocalization between PML and SUMO1 (Figure 6b, middle), or alternatively it was observed complete colocalization but the overall PML-NB structure was affected by MageA2 expression (Supplementary Figure S4). MageA2 did not affect PMLVI-NBs formation or the

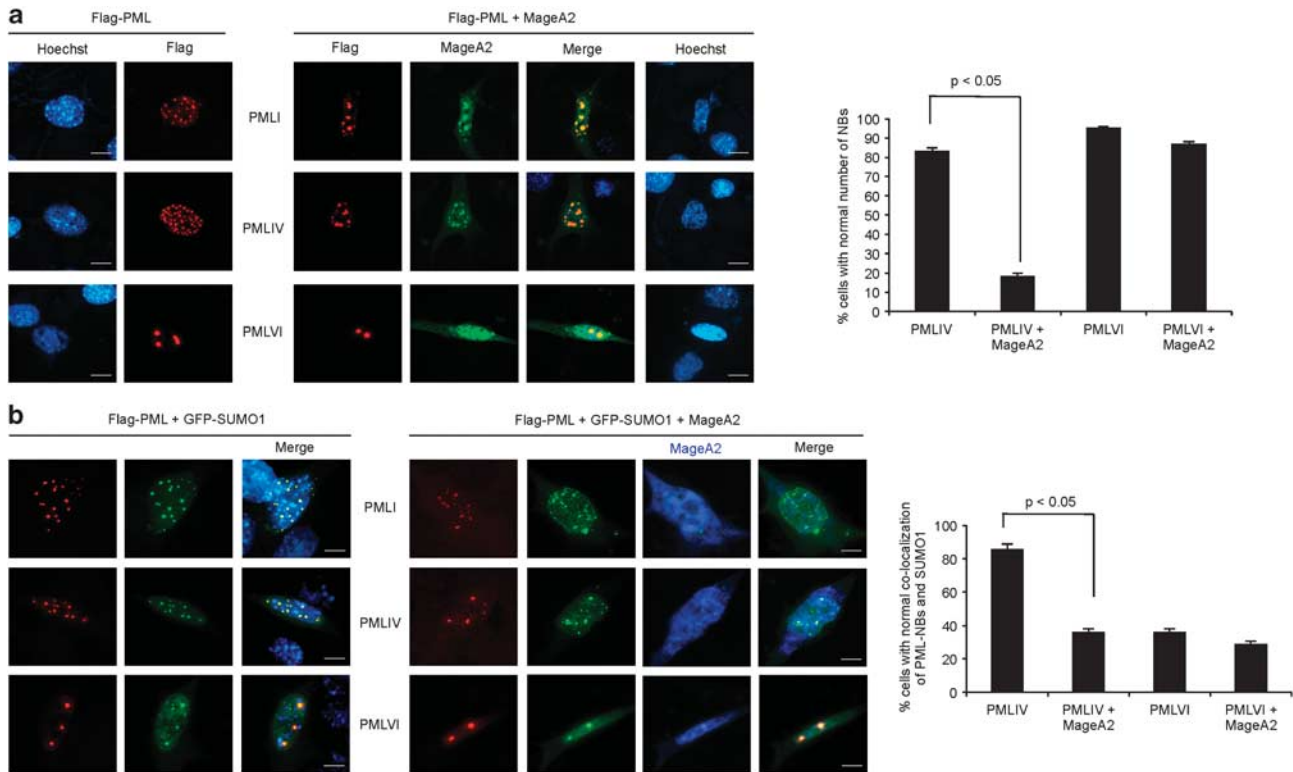


Figure 6 MageA2 impairs nuclear bodies formation. **(a)** Representative IF images from PML^{-/-} MEFs transfected with Flag-PML isoforms alone or in combination with HA-MageA2. PML isoforms were detected with an anti-Flag monoclonal and TRITC-conjugated anti-mouse antibodies, and MageA2 with an anti-HA polyclonal and FITC-conjugated anti-rabbit antibodies. Graphic on the right represents the percentage of cells showing normal number (15–30 NBs per nucleus) and shape (tiny bodies) of NBs for cells expressing PMLIV or PMLVI alone or in combination with MageA2. At least 200 cells were counted in three independent experiments. **(b)** Representative IF images from PML^{-/-} MEFs transfected with Flag-PMLs and GFP-SUMO1 alone or in combination with HA-MageA2. PML isoforms were detected with an anti-Flag monoclonal and TRITC-conjugated anti-mouse antibodies, and MageA2 with an anti-HA polyclonal and Alexa Fluor 350-conjugated anti-rabbit antibody. Graphic on the right represents the percentage of cells showing normal colocalization of PML and SUMO1 in cells expressing PMLIV or PMLVI alone or in combination with MageA2. At least 200 cells were counted in three independent experiments. Error bars indicate S.D.; a Student's *t*-test was used for statistical analysis. Scale bar: 5 μ m

colocalization between PMLVI and SUMO1 (Figure 6b). Importantly, colocalization of MageA2 and PMLIV at NBs in PML knockout cells was not as efficient as shown in PML-expressing cells, suggesting that interaction with multiple PML isoforms could increase MageA2 retention to NBs. Altogether these data indicate that, at least through this over-expression approach, MageA2 could disturb the proper formation of PML-NBs and their overall structure. These effects could be probably attributable to impaired PML post-translational modifications and the consequent interference with partners recruitment to PMLIV-NBs.

MageA2 impairs cellular senescence by affecting PMLIV-induced p53 activity and PML-NBs formation. A connection between PMLIV overexpression, p53 recruitment to NBs, increased p53 acetylation and induction of premature cellular senescence has been already suggested.^{17,18,22} Given that MageA2 colocalizes with p53 and PMLIV in the NBs inducing p53 deacetylation and repressing its trans-activation function, we investigated the impact of MageA2 expression on PMLIV-induced senescence. To this end, normal human fibroblasts were co-transduced using retroviral vectors expressing PMLIV with an empty vector or in combination with MageA2 or alternatively MageA4. After 10 days under selective culture conditions, PMLIV transduced

cells showed all the features of the senescence process, because they ceased to proliferate at sub-confluent densities and became flat and enlarged. Importantly, cells co-expressing PMLIV and MageA2 did not show major morphological changes and behaved similarly to control cells (Figure 7a). Colocalization between MageA2 and PMLIV was observed in these cells (data not shown). Analysis of senescence-associated β -galactosidase activity (SA- β -Gal), a well-known marker of senescence, demonstrated that MageA2 expression significantly reduced SA- β -Gal levels in PMLIV-expressing cells (Figure 7b). Moreover, cells expressing MageA2 and PMLIV showed higher levels of BrdU incorporation with respect to those expressing only PMLIV (Figure 7b). Cells overexpressing MageA2 alone behaved indistinguishably from control or MageA4 transduced cells. Importantly, MageA2 expression correlated with reduced p21 protein levels, probably due to an effect of MageA2 on efficient p53 activation (Figure 7c).

In normal cells, cellular senescence acts as a barrier to prevent transformation triggered by oncogene deregulation.¹⁶ For instance, cells respond to RasV12 oncogene overexpression by activating a senescence program through a functional PML/p53 axis.^{17,18} In fact, PML has been demonstrated to be involved in RasV12-induced senescence by stimulating p53 acetylation and function. Moreover, the number of PML-NBs

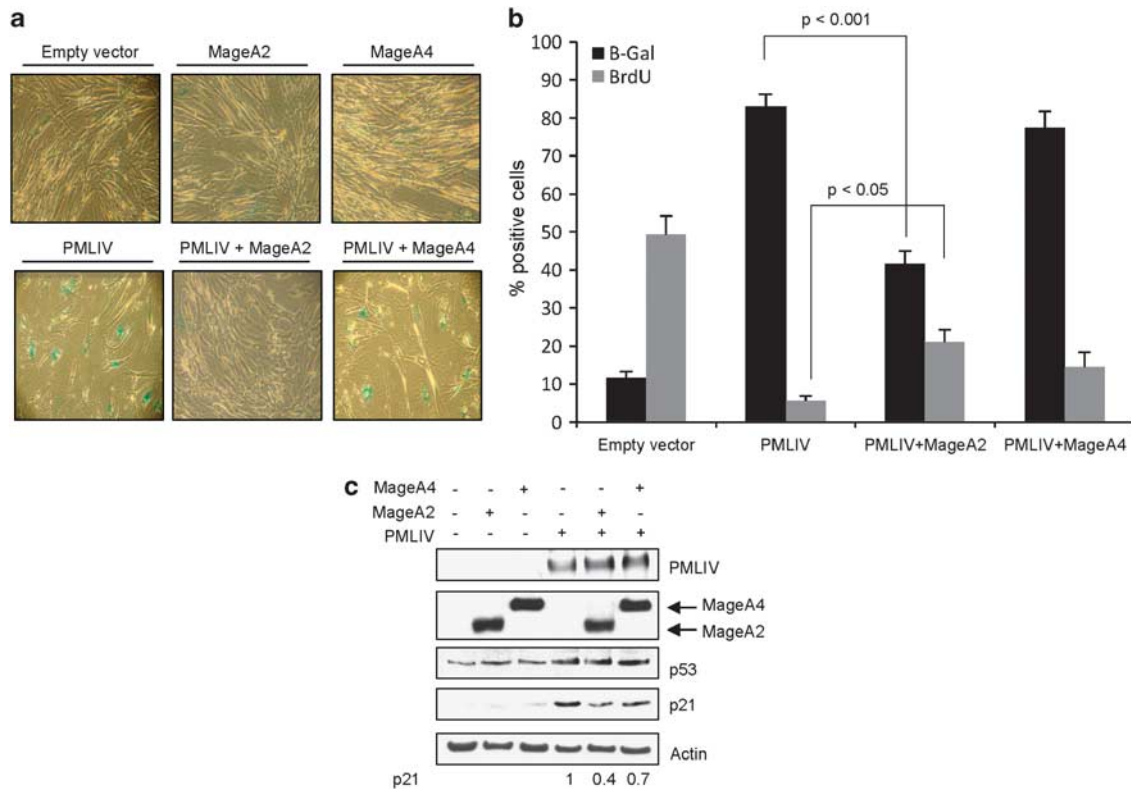


Figure 7 MageA2 antagonizes PMLIV-induced senescence. (a) Wi38 cells were infected with the indicated retroviruses, after 10 days under selection conditions, cells were fixed and stained for SA- β -Gal activity and photographed under a phase contrast microscopy to analyze cell morphology. (b) Analysis of the percentage of cells that stained positive for SA- β -Gal and were positive for BrdU incorporation after counting 200 cells in at least three independent experiments is shown. Error bars indicate S.D.; a Student's *t*-test was used for statistical analysis. (c) Western blot analysis of lysates obtained from Wi38 cells used in (a) and (b). Actin was used as loading control. Numbers below Actin blot represent quantification of p21 bands respect to Actin, the p21 value from PMLIV alone infected cells was set as 1

significantly increases during senescence, implying an enhancement in the PML-NB formation process.^{17,18} In this context, we examined whether MageA2 could regulate RasV12-induced senescence. To address this issue, we used primary human BJ fibroblasts expressing hTERT and 4-OH-tamoxifen (4-OHT)-inducible oncogenic H-RasV12 (BJ/ET/RasV12ER cells)²⁹ co-transduced or not with MageA2. After induction of H-RasV12 in BJ cells, several markers of premature senescence were observed, such as the increase in SA- β -Gal-positive cells and the formation of senescence-associated heterochromatin foci (SAHF) (Figure 8a). Importantly, we observed that MageA2 expression impaired RasV12-induced SA- β -Gal activity and SAHF formation and resulted in higher levels of BrdU incorporation in those cells (Figure 8a). Expression of MageA2 was unable to regulate senescence parameters when PML was knocked-down by shRNA (Supplementary Figure S5). Moreover, MageA2 expression affected p53 activity in this cellular system, as assessed by quantification of p53 target genes (Figure 8b), thus confirming that MageA2 downregulates the p53-dependent response. Similar results were shown at the protein level; in fact, p21 seemed to be highly sensitive to MageA2 expression when compared with other p53 target proteins (data not shown).

Finally, RasV12-induced formation of PML-NBs during senescence was analyzed. As previously reported,^{17,18} after RasV12 induction the number and size of PML-NBs was

increased, nevertheless MageA2 expression significantly blocked this increase (Figure 8c and Supplementary Figures S6A and S6B). Colocalization between MageA2 and PML was also observed in these cells (Supplementary Figures S6A and S6B). Collectively, all these data support the notion that MageA2 impairs the senescence process by targeting the PML/p53 axis, thus blocking a central barrier against cell transformation and tumor progression.

Discussion

MAGE proteins have been discovered as tumor antigens and for many years studies have been mainly focused on their use as immunotherapeutic targets in cancer. Moreover, because of high sequence homology, MAGE genes were considered functionally redundant. Nevertheless, in the last years compelling evidence has accumulated suggesting that individual MAGE proteins could behave in specific pathways critical for tumor progression.^{30–34} We previously demonstrated that MageA2 is a relevant player in favoring chemoresistance to p53 bearing human melanoma cells. Here, we analyze the role of MageA2 as regulator of another important tumor-suppressive mechanism, namely cellular senescence. The relevance of p53 acetylation as a trigger of its function has demonstrated to be highly regulated by the PML tumor suppressor.^{17,18} A model to explain the physiological role of PML-NBs is based on the assumption that they

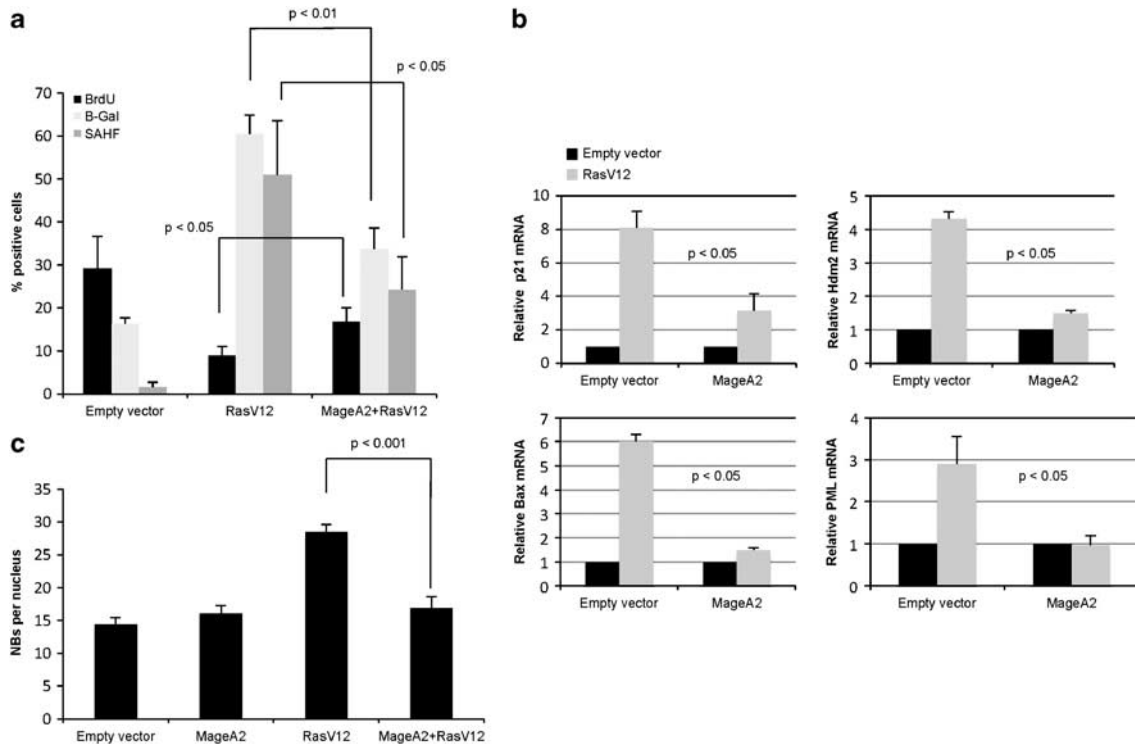


Figure 8 MageA2 antagonizes RasV12-induced senescence. (a) Primary human BJ fibroblasts expressing hTERT and 4-OH-tamoxifen (4-OHT)-inducible oncogenic H-RasV12 (BJ/ET/RasV12ER cells) co-transduced in combination with retroviruses expressing MageA2 were induced for 10 days. Subsequently, BrdU incorporation, B-Gal expression and SAHF number were analyzed. Graphic represents average of cells positive for BrdU, B-Gal or SAHF, after counting 200 cells in at least three independent experiments. (b) Quantitative RT-PCR analysis for the indicated mRNAs in cells from (a). Graphs represent mean from at least three independent experiments. (c) Analysis of average number of NB per nucleus in cells from (a) after counting 200 cells in at least three independent experiments. Error bars indicate S.D.; a Student's *t*-test was used for statistical analysis

function as sites for the recruitment of different proteins and their post-translational modification.¹⁹ Here, we show that a fraction of MageA2 accumulates in PML-NBs and this consequently results in an impairment of p53 acetylation at the NBs and the ability of PMLIV to efficiently induce p53 function. As the colocalization of MageA2 with PML at NBs is not dependent on p53 expression, MageA2 could be able to regulate other PML functions not involving p53 activity. Besides, the ability of MageA2 to interact with different PML isoforms could improve its localization to NBs and suggests a potential role of MageA2 in the regulation of PML isoform-specific functions.

An important consideration raised by our results is that MageA2 behaves in a specific manner considering its distinguishable cellular localization with respect to other MAGE-A proteins. Importantly, MageA2 specificity was emphasized by the fact that despite their high sequence similarities MageA4 does not behave as MageA2 both in terms of p53 and/or PMLIV regulation, even when MageA4 was targeted with a NLS.

PML functionality itself is highly affected by post-translational modifications, such as phosphorylation, sumoylation and as recently demonstrated acetylation.^{23,26,27} In fact, it has been suggested that p300-dependent acetylation of PML allows its subsequent sumoylation and activation.²³ Our data suggest that MageA2 could affect directly PMLIV function by interfering with PML post-translational modifications. We have recently reported that MageA2 forms active complexes with

HDACs to favor deacetylation of p53;¹⁰ here we show that MageA2 could also be found in PML-NBs impairing PML sumoylation through an HDAC-dependent mechanism. Although it has been reported that PMLIV binds slightly to HDAC3,³⁵ MageA2 could enhance HDAC recruitment to PML-NBs, suggesting that MageA2/HDAC3 complexes could be assembled and be functional on different nuclear domains and cellular contexts. This mechanism could explain not only the observed reduction in PML acetylation, but also that of p53 at NBs. Furthermore, although the precise mechanism and final outcome of PML acetylation has not yet been fully addressed, it is possible to hypothesize that different PMLIV functions depending on its acetylation or sumoylation could be impaired in tumor cells expressing MageA2.

PML post-translational modifications, in particular sumoylation, are important in many ways. PML sumoylation is required for proper nucleation of NBs and for recruitment of its partners into NBs.^{20,28} Many oncogenic viral proteins target PML functions by affecting the integrity of the NB through the control of PML sumoylation.²⁶ PMLIV stability is not affected by MageA2 expression, considering the reported involvement of PML sumoylation in the regulation of its stability^{36,37} and recent observations that Mage/RING protein complexes could induce ubiquitin-dependent degradation of targeted proteins.³⁸ Instead, MageA2 seems to affect PML-NBs proper formation and partner's recruitment as we demonstrated in PML^{-/-} MEFs. Moreover, induction of PML by oncogenic RasV12 is not affected by MageA2, nevertheless the overall

structure and the number of NBs upon RasV12 expression is clearly affected by MageA2, suggesting a defect in the assembly of NBs and not on its stability.

Sumoylation-deficient PMLIV is not able to regulate p53 function and SUMO-modified PML results in transactivation and strong recruitment of p53 to NBs.²¹ p53 accumulation at PML-NBs is accompanied by induction of p53 acetylation: a hallmark for PMLIV-induced senescence is the formation of a functional complex between p53, CBP and PMLIV, which results in acetylation and transcriptional activation of p53, followed by growth arrest and senescence.^{17,18} It has been previously reported that factors affecting p53 acetylation at NBs, such as Sirt1³⁹ or viral oncoproteins like E7,⁴⁰ can override PMLIV-induced senescence in human fibroblasts. Here, we present evidence that MageA2 impairs p53 acetylation at NBs and blocks the ability of PMLIV to induce senescence in a normal cellular context. The exact contribution of MageA2 double targeting on p53 and on PMLIV for senescence regulation has not yet been possible to elucidate, because MageA2 deletion mutants losing the ability to bind p53 also lose PMLIV-binding capability probably due to conformational changes. In addition, functional PML and p53 are necessary for senescence induction, thus being impossible to analyze their contribution separately. The biological relevance of MageA2 was highlighted in a more physiological scenario such as the senescence induced by oncogenic signals. In such a context, expression of MageA2 could dampen the RasV12-induced senescence program by targeting primarily the PMLIV/p53 axis and by compromising the assembly of NBs.

PML-NBs have been suggested as important players for the formation of heterochromatin foci (SAHF) in senescence,²⁰ hence it is possible to speculate that MageA2 by disturbing PML-NBs formation could affect in turn the formation of SAHFs.

Potentially oncogenic proliferative signals are able to interfere with a variety of growth-inhibitory processes, such as the induction of apoptosis, differentiation or senescence, each of which restrains neoplastic evolution.¹⁶ *MAGE-A* genes expression is an early event in tumorigenesis, mainly attributable to DNA hypomethylation, a process that accompanies and characterizes cell transformation.^{2,30} Deregulated activity of oncogenes such as *ras* appears to occur during the initial steps toward transformation; in such environment early expression of MageA2 could act in a cooperative functional interaction with oncogenic *ras* to overcome the PMLIV/p53 axis responsible for triggering cellular senescence, which constitutes one of the critical barriers for cellular transformation. Alternatively, in established tumors MageA2 could confer to cells the ability to survive and resist chemotherapy.¹⁰

Evidence presented here suggests that MageA2 expression can regulate critical steps in carcinogenesis and tumor progression. We highlight the potential contribution of MageA2 to override oncogene-induced senescence as an early tumor initiation process that, in addition to its anti-apoptotic effects, reflects the overall relevance of MageA2 in cancer.

Materials and Methods

Cell culture and reagents. U2OS, 293T, H1299 and Wi38 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured as recommended. 13 923 melanoma cells have been described.¹² PML^{-/-}

MEF²¹ and BJ/ET/RasV12ER cells²⁹ were provided by G Del Sal (LNCIB, Trieste, Italy) and have been previously described.

TSA and 4-hydroxytamoxifen have been purchased from Sigma (St. Louis, MO, USA).

Plasmids, siRNA and transfections. Cells were transfected using Calcium Phosphate method for 293T cells, Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) for H1299 and PML^{-/-} MEF, or FuGENE 6 (Roche, Mannheim, Germany) for U2OS cells, according to the manufacturer's instructions. Unless stated otherwise, cells were analyzed 24 h after transfection. HA-MageA1, -A2, -A4 and -A6 were cloned in pCDNA3 (Invitrogen). GST-MageA2, and GST-MageA4 were obtained by subcloning in pGEX-4T1 (GE Healthcare Biosciences, Buckinghamshire, UK). pCDNA3-PMLIV, PIG3-LUC, GFP-SUMO1,²¹ and Flag-HDAC3,¹⁰ have been previously described. pCS2-SUMO1 was from L Collavin (LNCIB, Trieste, Italy). Isoform-specific Flag-PML coding vectors were kindly provided by L Banks (ICGEB, Trieste, Italy). p300 was from B Amati (IFOM, Milan, Italy). MAGE-I silencing was previously described.¹⁰ Control siRNA (siC) targeted the bacterial *LacZ* gene (sequence 5'-GUGACCAGCGAAUACCUGU-3') and was purchased from Eurofins MWG operon (Ebersberg, Germany). Cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen) as recommended by the manufacturer and analyzed after 48 h.

Retroviral infections were performed by standard protocols (for details, see Supplementary Material and Methods). Retroviral plasmids used were: pWZL-Hygro-PMLIV (kindly provided by G Ferbeyre). pLPC-EGF-MageA2 and pLPC-EGF-MageA4 were obtained by subcloning MageA2 and MageA4 cDNAs into pLPC-EGFP (Puromycin resistance).

Antibodies. Western blot analysis was performed according to the standard procedures using the following primary antibodies: for p53: DO1, anti-p53 monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-acetyl p53 (Lys382) (Cell Signaling, Danvers, MA, USA); for Mage detection, affinity purified anti-MageA6 raised against GST-MageA6, anti-MageA monoclonal antibody (6C1-Santa Cruz Biotechnology). Other primary antibodies: anti-PML polyclonal (H-238) and monoclonal (PG-M3) antibodies and anti-p21 polyclonal antibody were from Santa Cruz Biotechnology; anti-SUMO1 monoclonal antibody (Zymed Laboratories, San Francisco, CA, USA), anti-acetylated lysine polyclonal antibody (Cell Signaling) and anti-p300 monoclonal antibody (BD Pharmingen, Franklin Lakes, NJ, USA); and anti-actin polyclonal antibody (Sigma). For tags: anti-HA 12CA5 monoclonal antibody (Roche), anti-HA-7 and anti-Flag M2 monoclonal antibodies were from Sigma.

Immunofluorescence analysis. Cells were plated on glass coverslips in 3 cm culture dishes. After washing with PBS, cells were fixed in 3% paraformaldehyde (PFA) in PBS, treated with 1% glycine in PBS and permeabilized in 0.1% Triton X-100 in PBS. The staining was performed using specific antibodies incubated in 5% bovine serum albumin in PBS at 37 °C followed by fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate-conjugated secondary antibodies (Sigma) as indicated. Treatment with CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes, 3 mM MgCl₂, 1 mM EGTA, 15% glycerol, 1 mM PMSF, plus 0.5% Triton X-100) was performed for 10 min at 4 °C before fixation with PFA. SAHFs were revealed by Hoechst staining. Glass slides were analyzed using a laser scan confocal microscope (Zeiss, Thornwood, NY, USA) or an epifluorescence microscope (Leica, Mannheim, Germany). Images were obtained at × 100 magnification.

Immunoprecipitation and *in vitro* pull-down. Cells were harvested in ice-cold lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.1 mM sodium orthovanadate, 2 mM DTT, 0.1 mM PMSF, 5 mM EDTA and Protease Inhibitor Cocktail (Sigma). After 10 min of rocking at 4 °C, lysates were clarified by centrifugation and pre-cleared with 25 μl of Protein A-Sepharose CL-4B or GammaBind G-Sepharose (GE Healthcare Biosciences). Then, antibody pre-bound to 25 μl of Protein A-Sepharose CL-4B or GammaBind G-Sepharose was added and incubated at 4 °C for 4 h. The resin was then washed and bound proteins were eluted in SDS-PAGE sample buffer. For immunoprecipitation of endogenous PML, cells were harvested in RIPA buffer containing 50 mM Tris-HCl pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 0.2% deoxycholic acid, 0.1 mM sodium orthovanadate, 0.1 mM PMSF and protease inhibitor cocktail. For PML post-translational modifications analysis, RIPA buffer was supplemented with 10 mM *N*-ethylmaleimide (Sigma) for sumoylation analysis, or 5 mM butyric acid (Sigma) for acetylation analysis. Anti-PML monoclonal (PG-M3) antibody was pre-bound to Protein G-Sepharose and incubated with cells lysates for 16 h at 4 °C. For

analysis of endogenous PML sumoylation, U2OS cells stably expressing Flag-PMLIV were lysed in RIPA 200 buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.5% deoxycholic acid, 0.1% SDS, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, Protease inhibitor cocktail and 10 mM *N*-ethylmaleimide). Lysates were sonicated and then centrifugated 1 min at 2000 RPM. Anti-Flag monoclonal antibody was pre-bound to Protein G-Sepharose and incubated with cells lysates for 3 h at 4 °C.

For *in vitro* binding assays ³⁵S-labeled proteins were *in vitro* translated using TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) and incubated with purified GST, GST-MageA2 or GST-MageA4 (immobilized on glutathione-Sepharose 4B beads, GE Healthcare Biosciences) in pull-down buffer (PBS, 0.1% NP-40, 10% glycerol, 0.1 mM PMSF and Protease Inhibitor Cocktail). Bound proteins were eluted and resolved by SDS-PAGE.

Transactivation assays. Cells were co-transfected in a 24-well plate with 100 ng of PIG3LUC reporter constructs together with 35 ng of pCDNA3-p53, either alone or with 150 ng of pCDNA3-HA-MageAs or pCDNA3-PMLIV constructs. For normalization of transfection efficiency, 10 ng of pRL-CMV reporter (Promega), constitutively expressing the *Renilla reniformis* luciferase, was included. After 24 h, cells were lysed and assayed for luciferase activity using the Dual Luciferase kit (Promega).

BrdU incorporation. Cells were pulsed with 30 μM bromodeoxyuridine (BrdU, Sigma) for 3 h, then fixed with 3% PFA in PBS, permeabilized with 0.1% Triton X-100 in PBS, and RNA denatured with 50 mM NaOH for 20 s. BrdU incorporation was measured by immunofluorescence using an anti-BrdU antibody (GE Healthcare Biosciences) and the nuclei were stained with Hoechst. Stained cells were visualized under a fluorescence microscope (Leica) and at least 200 cells were scored for BrdU incorporation in at least three independent experiments.

Analysis of SA-β-Gal. Cells were fixed in 0.5% glutaraldehyde in PBS/2 mM MgCl₂ at pH 6.0 and then stained with staining solution containing 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆ in PBS/2 mM MgCl₂ at pH 6.0 plus 1 mg/ml X-Gal, for 16 h at 37 °C. Cells were then washed extensively with H₂O and observed under a bright field microscope.

Quantitative RT-PCR. Total RNA was extracted with Trizol reagent (Invitrogen) and cDNA was transcribed with a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and StepOnePlus real time PCR machine (Applied Biosystems). Primer sequences are shown in the Supplementary Materials and Methods.

Conflict of Interest

The authors declare no conflict of interest.

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