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Antagonism of p66shc by melanoma inhibitory activity

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The p66shc protein governs oxidant stress and mammalian lifespan. Here, we identify melanoma inhibitory activity (MIA), a protein secreted by melanoma cells, as a novel binding partner and antagonist of p66shc. The N-terminal collagen homology-2 (CH2) domain of p66shc binds to the Src Homology-3 (SH3)-like domain of MIA *in vitro*. In cells, ectopically expressed MIA and p66shc colocalize and co-precipitate. MIA also co-precipitates with the CH2 domain of p66shc *in vivo*. MIA expression *in vivo* suppresses p66shc-stimulated increase in endogenous hydrogen peroxide (H_2O_2), and inhibits basal and H_2O_2-induced phosphorylation of p66shc on serine 36 and H_2O_2-induced death. In human melanoma cells expressing MIA, endogenous MIA and p66shc co-precipitate. Downregulation of MIA in melanoma cells increases basal and ultraviolet radiation (UVR)-induced phosphorylation of p66shc on serine 36, augments endogenous H_2O_2 levels, and increases their susceptibility to UVR-induced death. These findings show that MIA binds to p66shc, and suggest that this interaction antagonizes phosphorylation and function of p66shc.
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P66shc belongs to the shcA family of adapter proteins, and plays a crucial part in governing mammalian lifespan, the only protein thus far shown to do so. In this regard, p66shc-null mice live 30% longer than their wildtype littermates, and are resistant to oxidant stimuli.[1] In response to such stimuli, p66shc is phosphorylated on serine 36, a residue that is not present in other shcA proteins.[2] The phosphorylation of p66shc on serine 36 is essential to its function as a governor of cellular reactive oxygen species levels.[3]

P66shc governs reactive oxygen species levels by regulating mitochondrial oxidative capacity.[4] Recently, we have reported another mechanism through which p66shc increases oxidant stress: by stimulating the activity of the guanine nucleotide exchange factor son of sevenless-1 toward the rac1 GTPase.[5] This mechanism employs interaction between specific proline residues in the unique N-terminal collagen homology-2 (CH2) domain of p66shc to the src homology-3 (SH3) domains of growth factor receptor bound-2 (Grb2). The ability of the proline-rich CH2 domain to bind to SH3 domains of grb2 raised the possibility of other SH3-containing proteins as binding partners of p66shc.

Melanoma Inhibitory Activity (MIA) is a protein that plays a key role in the progression and metastasis of malignant melanoma. Its name, a misnomer, is derived from its initial characterization as a protein secreted by a human melanoma cell line that inhibited attachment and growth of melanoma cells in culture.[6] Subsequently, MIA was shown to bind to specific extracellular matrix proteins, thereby masking the binding sites of integrins to these proteins,[7] as well as directly binding to specific integrins.[8] MIA promotes melanoma metastasis and invasion *in vivo*,[9] partly through these means. MIA is primarily expressed in malignant melanoma cells, though emerging evidence indicates that other cell types, especially chondrocytes, and particularly other tumor cells, also express varying amounts of MIA.[10–15] In humans, serum MIA is useful in detecting progression of localized melanoma to metastatic disease,[16] and also for monitoring therapy of advanced melanomas.[17] MIA belongs to a select group of small proteins that adopt a single SH3 domain-like structure,[7] and was identified as the first secreted protein with a SH3-like domain.

To identify novel binding partners of p66shc, we screened an array of SH3 domains using the CH2 domain of p66shc as bait. We found that the SH3-like domain of MIA bound avidly to the CH2 domain *in vitro*. Furthermore, we show that MIA is a *bona fide* binding partner of p66shc *in vivo*, characterize the molecular interaction that mediates binding between MIA and p66shc, and demonstrate the impact of this binding on the known physiologic functions of p66shc.

Results

P66shc contains a 110 amino-acid stretch at its N-terminus that is not present in other shcA proteins. This N-terminal region, termed the collagen homology-2 domain, is rich in proline and leucine residues, and is a potential binding partner

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Keywords: Melanoma; p66shc; oxidative stress
Abbreviations: Shc, Src homologous and collagen-like; MIA, melanoma inhibitory activity; CH, collagen homology; Sos, son of sevenless; grb, growth factor receptor bound; ROS, reactive oxygen species; MEF, mouse embryonic fibroblasts; WT, wild type; UVR, ultraviolet radiation; JNK, c-jun N-terminal kinase; ERK, extracellular signal-regulated kinase; OTOR, otoraplin; His, histidine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EGFP, enhanced green fluorescent protein; GTP, guanine triphosphate; GDP, guanine diphosphate; OD, optical density; ELISA, enzyme-linked immunsorbent assay; Bcl-2, B-cell chronic lymphocyte leukemia-2; ER, endoplasmic reticulum; BiP, binding protein; GST, glutathione S-transferase
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of proteins with SH3 domains.[5] To identify novel binding partners of p66shc, recombinant CH2 was used as bait in an *in vitro* screening assay. The SH3-like domain of MIA was identified as a strong binding partner of the CH2 domain by far-Western blotting an array of SH3 domains (Figure 1).

Armed with this finding we asked if MIA binds to p66shc *in vivo*. Ectopic MIA and p66shc expressed in COS7 cells colocalized (Figure 2a) primarily in the peri-nuclear region (Figure 2a). To determine if endogenous MIA and p66shc associate with each other, we utilized a human melanoma cell line HMB2 that expresses MIA, and its derivative cell lines, HMB2-5 in which endogenous MIA expression is suppressed with stable expression of a MIA anti-sense construct, and HMB2-LacZ that stably expresses a control LacZ construct.[18] Fractionation of homogenates from MIA-expressing HMB2 cells demonstrated that MIA was principally present in the endoplasmic reticulum, which also had the majority portion of p66shc (Figure 2b). MIA also co-precipitated with p66shc and the isolated CH2 domain of p66shc, when expressed in COS7 cells (Figures 2c–e). Furthermore, immunoprecipitation of endogenous MIA in HMB2 and HMB2-LacZ cells co-precipitated p66shc, and lack of MIA in HMB2-5 cells led to no precipitation of p66shc (Figure 3a and b). Similar to HMB2-5 cells, in HEK 293 cells that do not express MIA, MIA antibody did not pull down endogenous p66shc (Figure 3a).

Specific proline residues in the CH2 domain mediate its binding to the SH3 domain(s) of the growth factor receptor bound protein grb2.[5] Since MIA adopts a SH3 domain-like structure, we assessed whether these proline residues are important in binding of p66shc to MIA. Compared to wild-type p66shc, binding of p66shc mutated at these proline residues (P47A/P50A) was significantly diminished (Figure 2d).

We then wondered if this association between MIA and p66shc has any functional significance. P66shc mediates the generation of the ROS H_2O_2, and cell death induced by oxidative stimuli.[2] We therefore asked if MIA could modulate p66shc-mediated H_2O_2 generation and cell death. In COS7 cells, overexpression of p66shc enhanced H_2O_2-stimulated cell death that was rescued by co-expression of MIA (Figure 4a). Similarly, expression of p66shc in p66shc $-/-$ MEF promoted H_2O_2-stimulated death that was rescued by co-expression of MIA (Figure 4b and c). In contrast to wild-type p66shc, expression of the non-phosphorylatable p66shcS36A mutant in p66shc $-/-$ MEF did not increase H_2O_2-stimulated cell death (Figures 4b and c). Furthermore, MIA did not suppress H_2O_2-induced death in p66shc $-/-$ cells expressing p66shcS36A (Figures 4b and c). In parallel with these findings, expression of p66shc in p66shc $-/-$ MEF led to an increase in endogenous H_2O_2, and co-expression of MIA inhibited this increase (Figure 4d). In contrast, in p66shc $-/-$ MEF in which exogenous p66shc was not expressed, MIA did not have any effect on basal endogenous H_2O_2, whereas in p66shc $+/+$ MEF (WT-MEF), MIA expression markedly reduced endogenous H_2O_2 levels (Figure 4e). These findings show that MIA suppresses H_2O_2 levels only in cells that express p66shc, and the capacity of MIA to suppress p66shc-stimulated H_2O_2, and inhibit oxidative stress-induced cell death, is only apparent in the context of phosphorylatable p66shc—MIA has no effect on H_2O_2 levels and cell viability in the context of phosphorylation-deficient, functionally inactive, p66shc.

Since our previous work had suggested that one mechanism by which p66shc increases H_2O_2 is by activating rac1,[5] we examined whether MIA expression has any impact on rac1 activity. In WT-MEF expressing endogenous rac1, MIA did not suppress active rac1 levels (Figure 4f).

To determine the role of endogenous MIA in regulating cell survival and ROS levels, we compared H_2O_2 levels and susceptibility to oxidative stress-mediated death in HMB2, HMB2-LacZ and HMB2-5 cells. Basal, steady-state endogenous H_2O_2 levels were significantly higher in the MIA-deficient HMB2-5 cells when compared to HMB2 and HMB2-LacZ cells (Figure 5a). In addition, H_2O_2-induced cell death was markedly greater in HMB2-5 cells when compared to the other two cell lines (Figures 5b and c). Forced reconstitution of MIA in MIA-deficient HMB2-5 cells led to a partial rescue against H_2O_2-induced cell death (Figure 5d). Ultraviolet radiation (UVR), another stimulus that leads to cell death through oxidative stress, also resulted in a much higher proportion of death in HMB2-5 cells, as compared to the MIA-expressing HMB-2 and HMB-LacZ cells (Figure 5e).

Next, we looked into a possible mechanism by which binding of MIA to p66shc may inhibit the function of the latter. Guided by the finding that MIA associates with the CH2 domain, and that MIA has no effect on death in cells expressing the phosphorylation-deficient p66shcS36A mutant, we hypothesized that the association of MIA and wild-type p66shc inhibits the phosphorylation of serine 36 in the CH2 domain, which is essential for the pro-oxidative and pro-apoptotic functions of p66shc.[1,3,19] To test this hypothesis we first examined basal and H_2O_2-stimulated phosphorylation of serine 36 in COS7 cells. Expression of MIA suppressed both basal and H_2O_2-stimulated phosphorylation of serine 36 in endogenous and ectopic p66shc (Figure 6a and b). Since serine 36 is a target of c-jun N-terminal kinases (JNKs),[20] we wondered if MIA suppresses phosphorylation of serine 36 by inhibiting JNK activity. Basal, H_2O_2 and UVR-induced phosphorylated (active) JNKs was not different between MIA-expressing and non-expressing cells (Figure 6c and d), suggesting that MIA-induced decrease in phosphorylation of serine 36 is not due to a decrease in JNK activity. We also

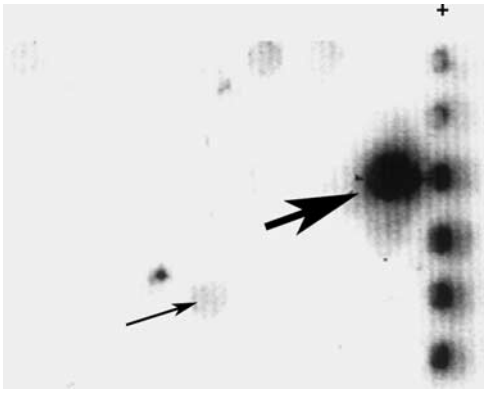

Figure 1 MIA binds to the CH2 domain of p66shc *in vitro*. Far-Western blot of a spotted array of SH3 domains (SH3 domain array III, www.panomics.com) probed with the recombinant CH2 domain of p66shc. Bold arrow indicates position of spotted MIA, and thin arrow the position of the MIA homolog OTOR. + indicates column of positive control spots

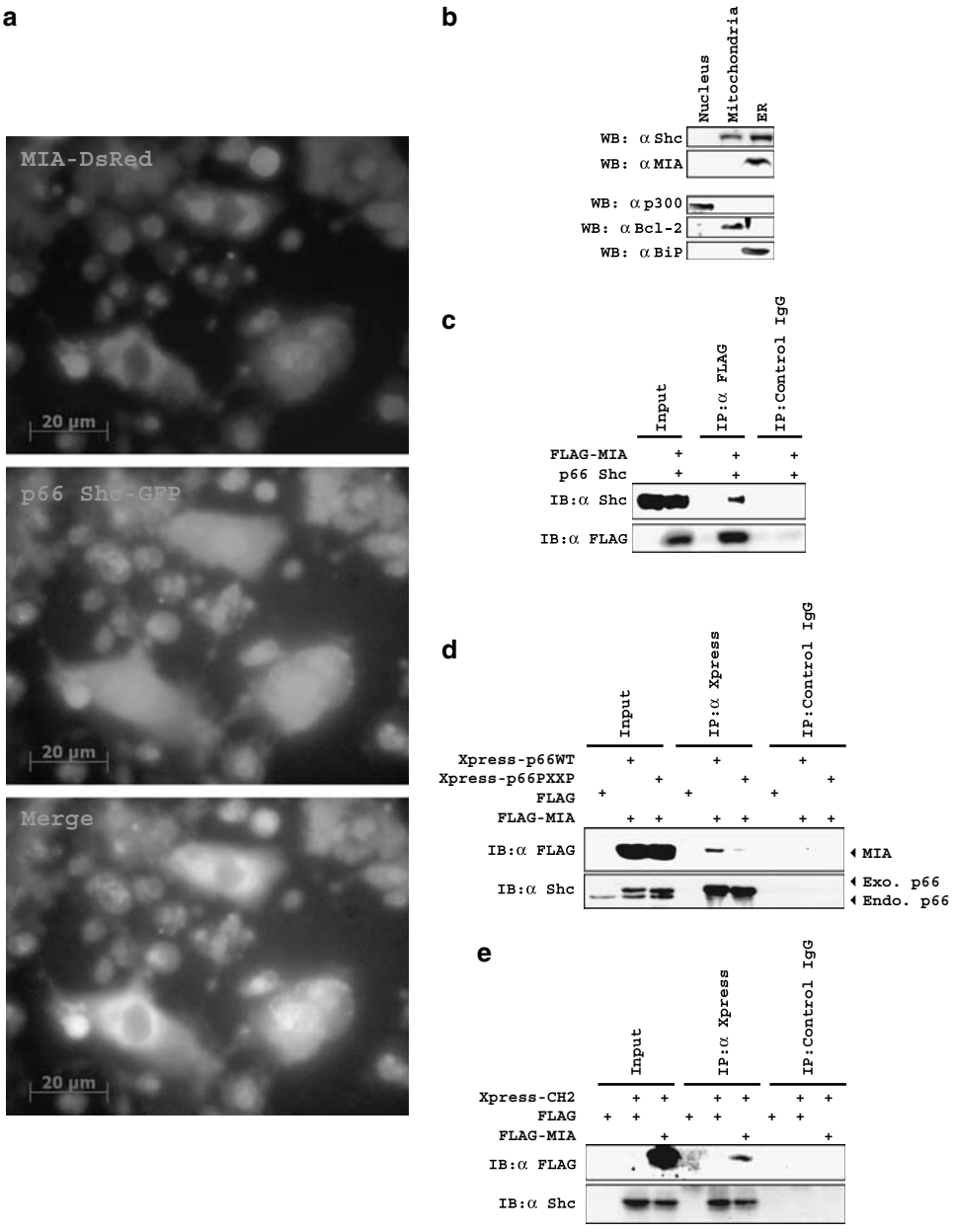

Figure 2 P66shc associates with MIA *in vivo* via proline residue in its CH2 domain. (**a**) Colocalization of MIA and p66 Shc in COS-7 cells. Live red, and green fluorescence images of COS-7 cells co-transfected with MIA-DsRed and pEGFP-p66shc were captured and merged. Nuclei were counterstained with Hoechst 33342. (Original magnification $\times$ 400). (**b**) Co-compartmentalization of MIA and p66shc. Immunoblots of fractionated lysates of MIA-expressing HMB2 melanoma cells. Identity of the fraction was verified by immunoblotting for proteins exclusively expressed in those fractions. Nucleus: p300 histone acetylase; Mitochondria: B-cell chronic lymphocyte leukemia-2 (Bcl-2); Endoplasmic Reticulum (ER): Binding Protein/GRP78 (BiP). (**c**) Co-immunoprecipitation of ectopically expressed FLAG-MIA and endogenous p66shc in COS-7 cells. (**d**) Co-immunoprecipitation of ectopically expressed FLAG-MIA and Xpress-p66shcWT or the mutant p66shcP47A/P50A in COS-7 cells. (**e**) Co-immunoprecipitation of ectopically expressed FLAG-MIA and Xpress-CH2 (the CH2 domain of p66shc) in COS-7 cells

looked at phosphorylation of serine 36 in the HMB2 and HMB2-5 melanoma cell lines. Basal and UVR-induced phosphorylation of p66shc on serine 36 was higher in the MIA-deficient HMB2-5 cells when compared to MIA-expressing HMB2 and HMB2-LacZ cells (Figure 7a and b). Moreover, this higher degree of basal p66shc phosphorylation in HMB2-5 cells was abrogated by forced reconstitution of MIA (Figure 7c). In addition to the HMB2 human melanoma cell line, MIA expression in a murine B16 melanoma-derived cell line was also associated with less phosphorylation of endogenous p66shc on S36, and lower endogenous H_2O_2 levels, when compared to a B16 melanoma cell line that does not express MIA (Figure 7d).

Discussion

Malignant melanoma has high metastatic potential, responds poorly to radiation and chemotherapy, and metastatic

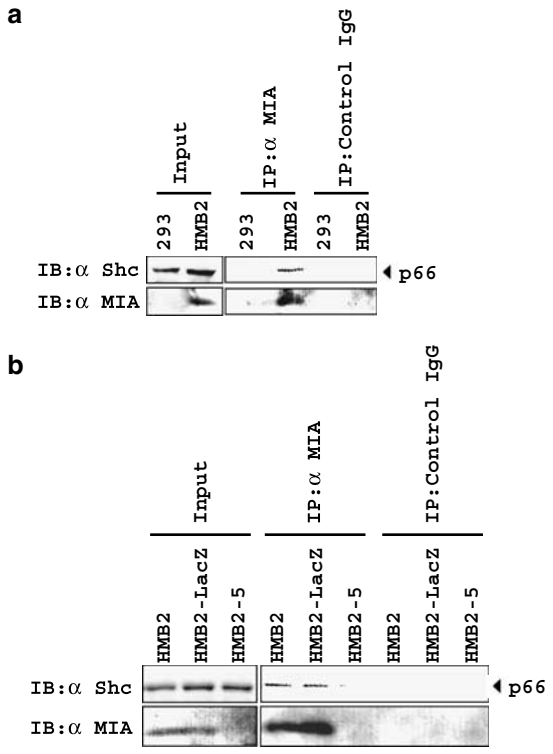

Figure 3 Endogenous MIA and p66shc associate in human melanoma cells. (**a**) Co-immunoprecipitation of endogenous MIA and p66Shc in the human metastatic melanoma cell line HMB2. Lysate from HEK 293 cells that do not express MIA was used as a control. (**b**) Co-immunoprecipitation of endogenous MIA and p66Shc in HMB2 cells, and its derivative cell lines HMB2-LacZ (HMB2 cells stably transfected with the inert *E. coli* LacZ gene), and HMB2-5 (HMB2 cells stably transfected with the antisense cDNA for MIA)

melanoma cells are less susceptible to apoptosis *in vivo* than non-metastatic cells.[21] Moreover, MIA expression correlates with progression of melanocytic tumors, and predicts response of melanoma to traditional therapeutic modalities.[17,22] Our findings, by identifying MIA as a novel binding partner of p66shc, and demonstrating that this binding inhibits p66shc-mediated H_2O_2 generation, suggest that one mechanism by which MIA may confer a survival advantage to melanoma cells expressing it, is by inhibiting p66shc-mediated oxidative stress and cell death induced by irradiation and traditional chemotherapeutic agents. As such, strategies aimed at disrupting MIA-p66shc interaction may offer novel therapies to combat malignant metastatic melanoma.

The functional role of p66shc in cancer cells, much less melanoma cells, is controversial. In prostate cancer cell lines expression of p66shc correlates with proliferation,[23] whereas in other tumor cell lines p66shc plays an apoptotic role.[19] Although we did not directly examine the role of p66shc in proliferative capacity of melanoma cells, comparison of UVR- and H_2O_2-induced death in MIA-expressing and non-expressing cells suggests that in this specific context (in MIA-expressing melanoma cells subjected to these oxidative cytotoxic stimuli) p66shc functions in a pro-apoptotic fashion. In this regard, it should be noted that although initially described as a protein restricted to melanoma cells, it is now appreciated that various malignant tumors express MIA.[10,11,13] Therefore it is tempting to speculate that the antagonistic functional relationship between MIA and p66shc may not be relevant exclusively to melanoma cells.

MIA is secreted by melanoma cells, and its extracellular function as an inhibitor of integrin-mediated cell adhesion has been described.[7,24] MIA has also been reported to localize in the intracellular space,[25] but its function as an intracellular protein is not known. It is known that melanoma cells expressing MIA display alterations in specific intracellular signaling pathways, such as activation of extracellular signal-regulated kinases (ERKs).[18] The mechanism by which MIA leads to activation of the ERK cascade is unclear. However, it is interesting to note that p66shc blunts ERK activation.[19] This reciprocal relationship between p66shc and MIA with regard to ERK activity lends further support to our conclusion that MIA acts as a functional antagonist to p66shc.

MIA is the first member of a family of structurally alike small proteins that assume an SH3-like fold. Other members of this family include OTOR whose expression is limited to the inner ear,[26] MIA2 that is principally expressed in the liver,[27] and TANGO which is expressed in most tissue except that of hematopoetic origin.[28] Although the N-terminal regions of MIA, OTOR, MIA2, and TANGO are quite divergent, many residues important for structural folding are conserved between the four proteins. Our *in vitro* screening assay did detect an interaction between the CH2 domain and OTOR (Figure 1), albeit weaker than between the CH2 domain and MIA. This array does not have MIA2 and TANGO, and therefore we could not screen for an interaction between these two proteins and the CH2 domain. Nevertheless, the possibility that p66shc may bind to structurally conserved MIA homologs that are more broadly expressed than MIA suggests that the physical and functional interaction between MIA and p66shc that we describe in melanoma cells, may have wider implications in regulating oxidative stress, and cell death in other tissues and cells types.

It is important to recognize that MIA may impact only on select mechanisms by which p66shc functions. For example, p66shc-induced oxidative stress is partly mediated by rac1,[5] but MIA expression does not suppress rac1 activity (Figure 4f). This selectivity likely reflects the compartmentalization of MIA within the cell. MIA, which is expressed in the endoplasmic reticulum, would not be expected to interfere with the binding of p66shc to Grb2 (an interaction that is required for p66shc-induced rac1 activation), a protein primarily present at the plasma membrane.

Collectively, these findings identify MIA as a novel and direct binding partner of p66shc, and show that this interaction has a functional consequence *vis-à-vis* the role of p66shc in promoting oxidative stress and cell death. This data also suggests that p66shc through interaction with MIA, which is expressed in a number of malignancies other than melanoma, may counteract the role of MIA in promoting tumor growth and metastases.

Materials and Methods

Cell lines, cDNA, and transfections. The metastatic human melanoma cell line and its derivatives, the murine B16 melanoma cell line and its derivatives, and the cDNA for human MIA, have been described previously.[9,18] The cDNA for

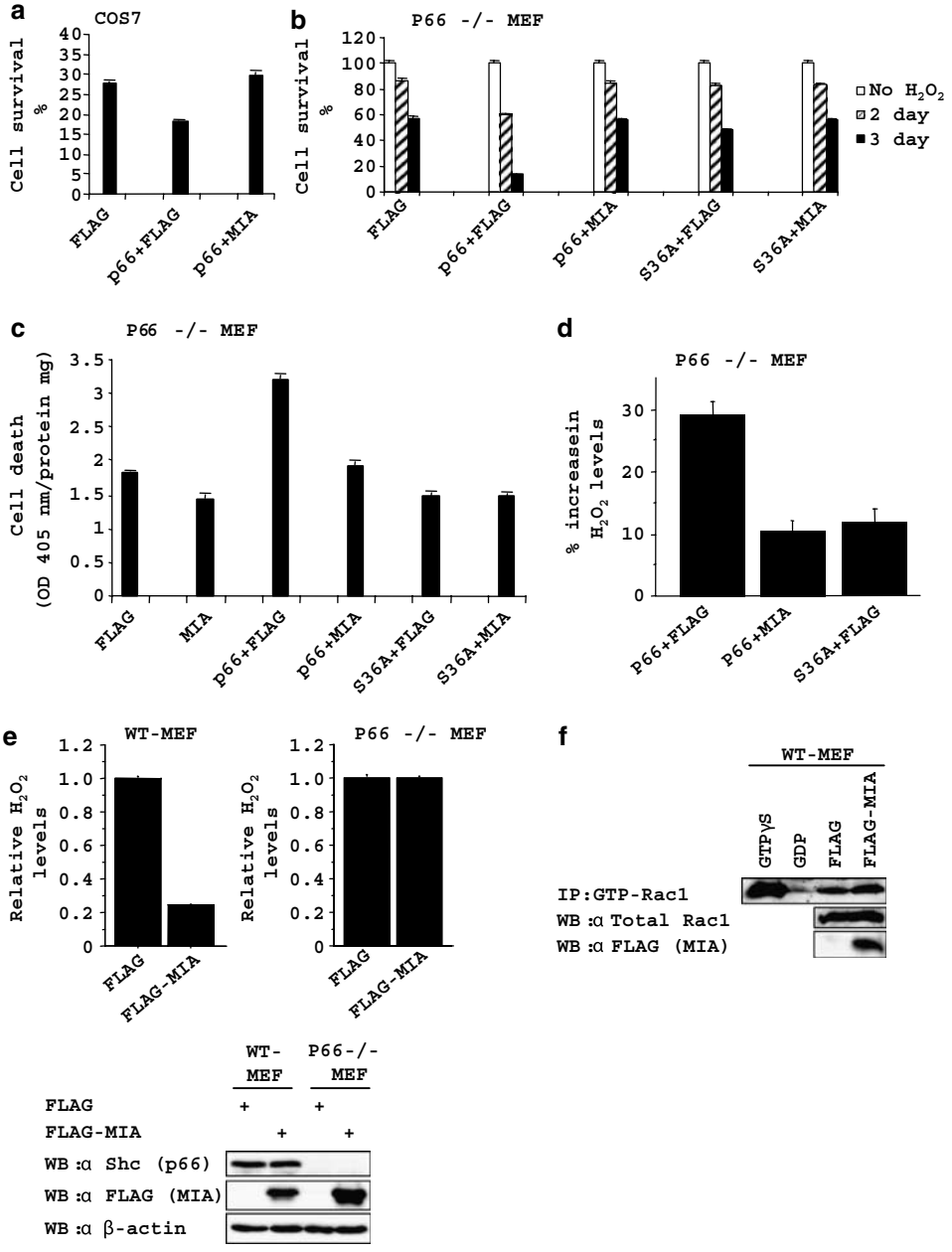

Figure 4 MIA suppresses p66shc-mediated, oxidative stress-induced cell death. (**a**) Cell survival, measured by Trypan blue exclusion, in COS-7 cells overexpressing p66shc with and without MIA. Cells were challenged with 0.4 mM H_2O_2 for 48 h. $N=4$, mean $\pm$ S.E. Importance of serine 36 (S36) in the CH2 domain of p66shc to protection conferred by MIA. Cell survival at 24 and 48 h by Trypan blue exclusion (**b**) and cell death measured at 48 h by ELISA (**c**) in p66shc-null mouse embryonic fibroblasts (p66shc $-/-$ MEF) after challenged with 0.4 mM H_2O_2. $N=4$, mean $\pm$ S.E. Cells were transfected with p66shcWT (p66) or the S36A mutant of p66shc (S36A), with or without MIA. (**d**) MIA inhibits p66shc-induced endogenous H_2O_2 levels. Endogenous hydrogen peroxide levels were measured by Amplex Red fluorescence in p66shc $-/-$ MEF transfected with p66shcWT (p66) or p66shcS36A (S36A), with and without MIA. $N=7$, mean $\pm$ S.E. (**e**) Effect of MIA expression on H_2O_2 levels in p66shc $+/+$ (WT-MEF), and p66shc $-/-$ MEF. Endogenous H_2O_2 levels were measured by Amplex Red fluorescence in p66shc $-/-$ MEF and WT MEF transfected with empty FLAG expression vector or FLAG-MIA. Values are expressed relative to cells transfected with the empty FLAG vector. Expression of FLAG-MIA and endogenous p66shc is shown at bottom. (**f**) Lack of effect of MIA expression on active rac1 levels in WT MEF (p66shc $+/+$). Active GTP-rac1 was pulled down using GST-PAK1 beads, and is shown in top panel. Positive control: cell lysate treated with non-hydrolyzable γGTP. Negative control: cell lysate treated with GDP

MIA was cloned into 3XFLAGpCMV7.1 (Sigma) by standard methods. COS-7 and HEK 293 cells were purchased from ATCC. The Xpress-tagged cDNAs for p66shc, the CH2 domain, p66shcP47A/P50A, p66shcS36A, and p66shc $-/-$ MEF have also been described previously.[5] Cells were transfected using Lipofectamine 2000 (Invitrogen) as per manufacturer's recommendations.

Far-Western blot of SH3 array. An array (TransSignal SH3 Domain Array III) spotted with SH3 domains of several proteins, was purchased (Panomics, Redwood City, CA, USA). Recombinant (His)B_{6B}-tagged CH2 domain was induced and purified from *Escherichia coli* as described previously,[29] and incubated with the SH3 domain as per manufacturer's recommendations. The far-Western blot was

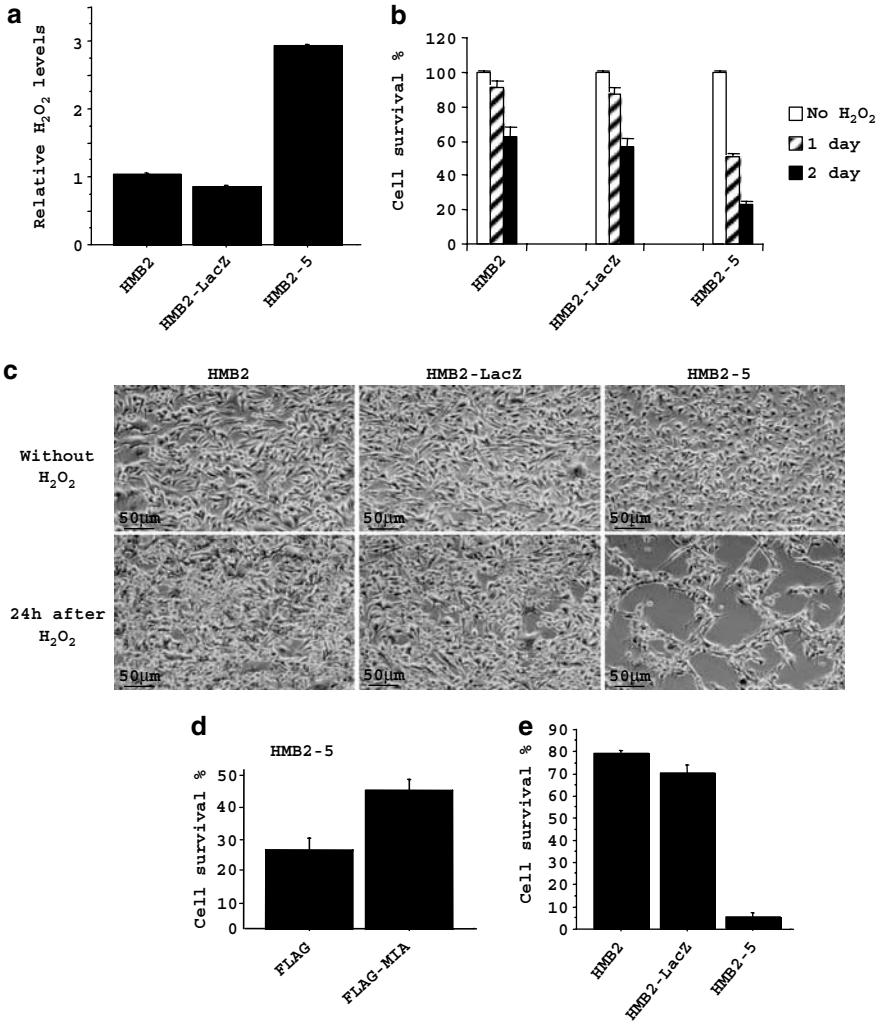

Figure 5 Endogenous MIA suppresses endogenous oxidant levels and protects melanoma cells from oxidative stress-induced death. (**a**) Endogenous H_2O_2 measured by Amplex Red fluorescence, in the human metastatic melanoma cell line HMB2, and its derivatives. Values are expressed relative to HMB2 cells. (**b**) Survival assessed by trypan blue exclusion, in HMB2 cells and its derivatives, 24 and 48 h after 0.4 mM H_2O_2 challenge. (**c**) Phase-contrast microscopic images ($\times$ 100) of HMB2 cells and its derivatives, 24 h after 0.4 mM H_2O_2 challenge. (**d**) Survival, 24 h after H_2O_2 challenge, assessed by trypan blue exclusion, in the MIA-deficient HMB2-5 cell line, with and without re-constitution of MIA. (**e**) Survival assessed by trypan blue exclusion, in HMB2 cells and its derivatives, 24 h after UV irradiation (254 nM UVC at 60 J/m^2 for 10 min^P)

performed using an anti-His antibody (Qiagen), and the appropriate secondary antibody.

Antibodies and immunoblotting. Antibodies to X-press (Invitrogen), FLAG (Stratagene), MIA (R&D Systems, Inc.), shc (BD Biosciences), phospho-S36-p66shc (Axxora, LLC), JNK (Cell Signaling), and phospho-JNK (Cell Signaling) were purchased and used as recommended by the manufacturer. Appropriate species-specific secondary antibodies were used. SDS-PAGE and transfer were carried out by standard protocols. Immunoblots were developed using super Femto substrate (Pierce), and images visualized and digitally captured with a BioRad ChemiDoc apparatus.

Immunoprecipitations. Immunoprecipitations were carried out by standard methods as described previously,[5] typically using 0.5–1.0 mg of protein lysate, 2 μg of antibody, and protein A-sepharose or protein G-sepharose (Amersham). Non-immune IgG was used as a control. Immunoprecipitates were extensively washed before SDS-PAGE and immunoblotting.

Fluorescence microscopy. MIA cloned into the DsRed vector (Clonetech), and p66shc cloned into the EGFP vector (Clonetech) were co-transfected into COS-7 cells. Red and green fluorescence images were captured on a Carl Zeiss Axiovert 200 Fluorescence microscope, and merged. Nuclei were counterstained with Hoechst 33342.

Cell death assays. Cell viability (survival) was determined using a standard Trypan Blue exclusion assay. Cell death (OD 405) was quantified using the Cell Death ELISA (Roche), and normalized for cell number (total protein).

Cell fractionation. HMB-2 cells were homogenized with a glass-Dounce homogenizer, and homogenate was separated into nuclear, mitochondrial, and ER fractions as described previously.[30–32]

Rac1 activity. Active GTP-bound rac1 was measured using a rac1 activity assay (UBI), as described previously.[5]

H_2O_2 measurements. Hydrogen peroxide (H_2O_2) was measured in cell medium with the Amplex Red assay (Molecular Probes) as described previously.[5] Endogenous H_2O_2 levels were measured by fluorescence at 595 nm 15 min after addition of Amplex reagent. Fluorescence intensities were normalized for cell number (total protein).

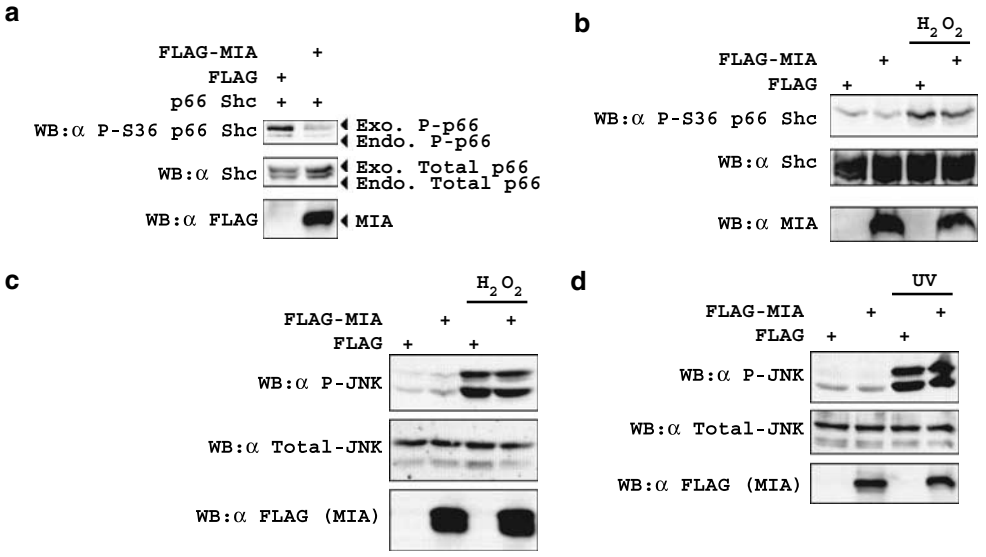

Figure 6 Ectopic MIA inhibits phosphorylation of p66Shc on Serine 36. (**a**) Basal phosphorylation on serine 36 (S36) of endogenous and ectopic p66shc, with and without MIA expression, in COS-7 cells (**b**) H_2O_2-induced phosphorylation on S36 of endogenous p66shc, with and without MIA expression, in COS-7 cells. Cells were challenged with 0.4 mM H_2O_2 for 30 min. (**c**) Lack of effect of MIA on H_2O_2-induced phosphorylation of c-jun N-terminal kinase in COS-7. Cells, with and without MIA, were challenged with 0.4 mM H_2O_2 for 30 min. (**d**) Lack of effect of MIA on UV radiation-induced phosphorylation of c-jun N-terminal kinase in COS-7. Cells, with and without MIA, were challenged with UV irradiation (UVC 254 nM at 60 J/m^2 for10 min)

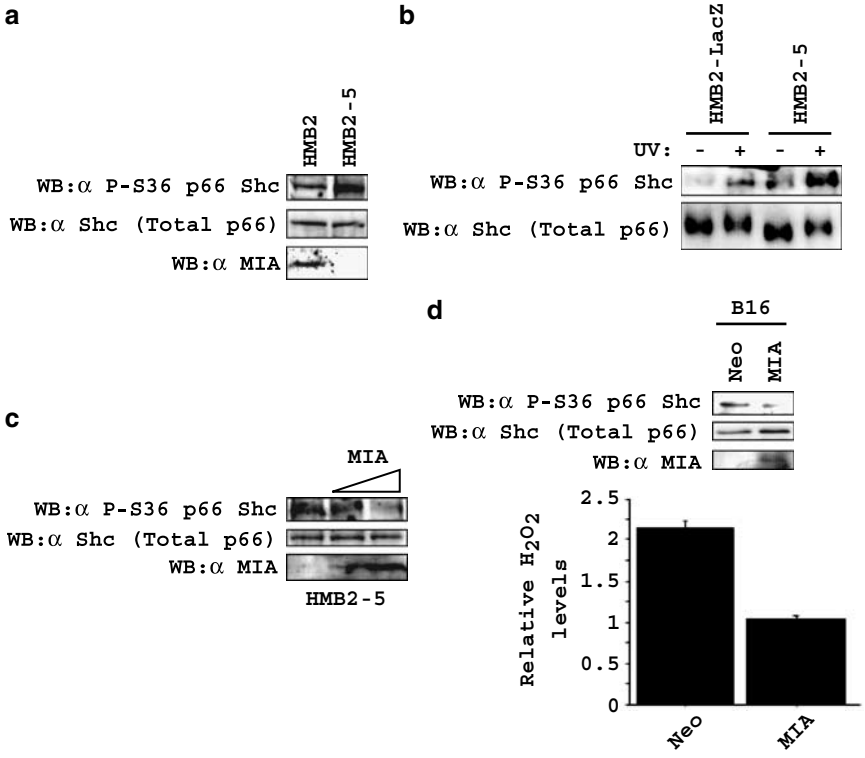

Figure 7 Endogenous MIA inhibits phosphorylation of p66Shc on Serine 36 in melanoma cells. (**a**) Basal phosphorylation on S36 of endogenous p66shc in the MIA-expressing HMB2 metastatic human melanoma cell line, and its MIA-deficient derivative HMB2-5. (**b**) UV radiation-induced phosphorylation in the MIA-expressing HMB2-LacZ and the MIA-deficient HMB2-5 human melanoma cell lines. Cells were challenged with UV irradiation (UVC 254 nM at 60 J/m^2 for 10 min). (**c**) Effect of MIA re-constitution on basal phosphorylation of S36 of endogenous p66shc in the MIA-deficient HMB2-5 cell line. (**d**) Effect of MIA expression on basal phosphorylation of S36 of endogenous p66shc, and basal H_2O_2 levels, in the B16 murine melanoma cell lines. MIA: MIA-expressing B16 cell line; Neo: control neomycin B16 cell line

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