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Acetylation is indispensable for p53 antiviral activity

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Tumor suppressor p53 is known to be a direct transcriptional target of type I interferons (IFNs), contributing to virus-induced apoptosis, and in turn activating itself the interferon pathway. Acetylation, among many other post-translational modifications of p53, is thought to exert a crucial role regulating p53 activity. Here, we examined the contribution of this modification on the antiviral activity mediated by p53. Our results show that virus infection induces p53 acetylation at lysine 379, and that this modification is absolutely required for p53-dependent transcriptional transactivation of both, pro-apoptotic and IFN-stimulated genes induced by virus infection, and for p53-mediated control of virus replication. Thus, our study identifies p53 acetylation as an indispensable event that enables the p53-mediated antiviral response.

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Introduction

Tumor suppressor protein p53 has the ability to trigger proliferation arrest or cell death through the transcriptional activation of its target genes to prevent cell transformation and to limit virus replication.[1-9] As part of this later function, p53 plays an important role in the activation of the interferon pathway, a system that is crucial in the antiviral response of the cell.[10]

p53 activity is tightly regulated in the cell by a complex network of interacting partners and post-translational modifications.[11-13] When the cell is confronted with different sources of stress, such as DNA damage, hypoxia or oncogene activation, p53 expression raises and the protein is activated by multiple covalent modifications such as phosphorylation and acetylation. Similarly, virus infection has been shown to cause transcriptional expression of p53 through an interferon-dependent mechanism, and activation by phosphorylation at serine residue 18.[7] However, no acetylation of p53 in response to virus infection has been reported so far.

The acetylation of p53 has different functional consequences and among them, it destabilizes its interaction with the negative regulator Mdm2, enhancing p53's DNA binding activity on specific promoters.[14-16] In the context of virus infection, acetylation of p53 might be also very relevant to limit virus replication since different viral proteins have been shown to target this modification.[17-22] However, the importance of p53 acetylation on the antiviral activity of this tumor suppressor has not been explored yet.

Here we identify virus infection as a novel inducer of p53 acetylation and evaluate the role of this modification on the control of vesicular stomatitis virus (VSV) replication. Loss of p53 acetylation abolishes its ability to transactivate target genes in response to VSV infection, and to control virus replication. These results underscore the importance of p53 acetylation in its antiviral activity.

Results and Discussion

Virus infection induces p53 acetylation at lysine residue 379. We were interested in addressing whether virus infection triggered p53 acetylation. To this end, we infected mouse embryo fibroblasts (MEFs) with VSV or HSV-1 at a MOI of 5 PFU/cell, and analyzed the acetylation of p53 at different times after infection by immunoblot analysis using an antibody against p53 acetylated at the lysine residue 379 (the mouse equivalent of human lysine 382). As shown in **Figure 1A**, p53 acetylated at lysine 379 was detected as early as two hours after infection with HSV-1 or VSV.

SIRT1 is a class III histone deacetylase within the sirtuin family of related proteins that can have oncogenic or tumor-suppressors effects, depending on the status of p53.[23,24] SIRT1 targets a plethora of substrates for deacetylation, including the p53 protein with specificity for this lysine residue.[25,26] Therefore, we examined the upregulation and activation of p53 in MEFs derived from genetically modified mice that lack expression of SIRT1 (SIRT1-KO), normal wild type animals (WT), and mice

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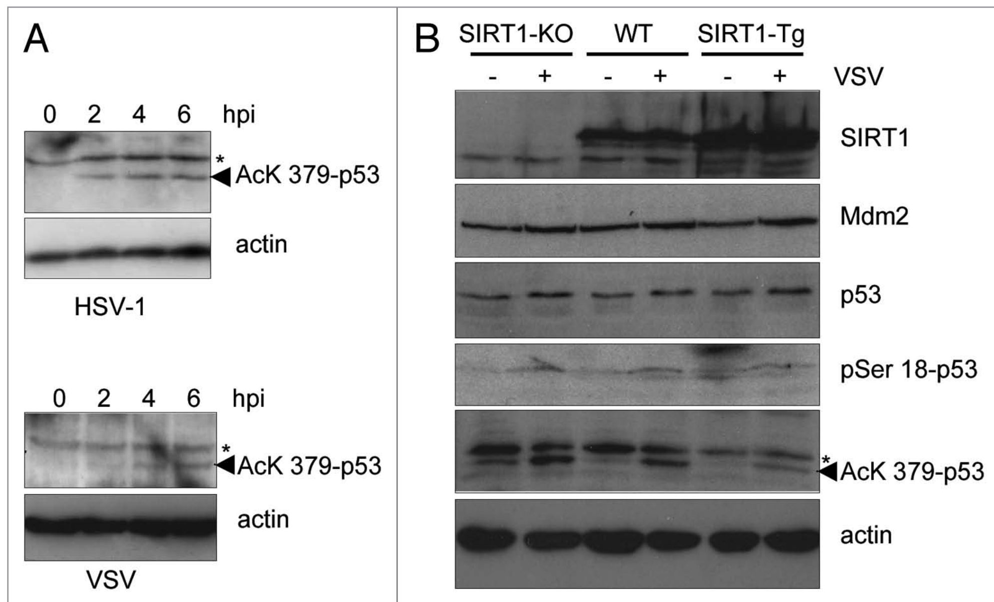

Figure 1. MEFs infection with HSV-1 or VSV induces p53-acetylation. (A) WT MEFs were infected with HSV-1 (upper part) or VSV (lower part) at MOI of 5 PFU/ml and at the indicated times, protein gel blotting using antibodies to acetyl-p53 K379 was performed. (B) MEFs derived from SIRT1$^{-/-}$, WT or SIRT1-tg mice were infected with VSV at MOI of 5 PFU/ml and four hours after infection, immunoblot analysis with the antibodies indicated was performed. Asterisk indicates a non-specific band.

engineered to harbor extra copies of the SIRT1 gene under the control of its own regulatory genomic sequences (SIRT1-Tg),[27] after infection with VSV. As expected, VSV infection induced an increase in the amount of total p53 as well as phosphorylation of p53 at serine-18 (**Fig. 1B**). This upregulation and phosphorylation of p53, as well as an increase in the levels of the p53-downstream target Mdm2, were unaffected by the different amounts of SIRT1 expressed in the cells (**Fig. 1B**). However, and in agreement with the results shown above, VSV infection induced the acetylation of p53 at lysine-379, and this modification was clearly reduced with increasing SIRT1 gene dosage (**Fig. 1B**), as expected for the specific deacetylase activity of this protein. These results demonstrate that p53 is acetylated in response to virus infection at lysine residue 379, independently of serine 18 phosphorylation and of Mdm2 upregulation.

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Phosphorylation of p53 at serine 18 by virus infection is caused by changes in the chromatin structure in virus-infected cells.[7] We speculate that p53 acetylation may be a consequence of p53 phosphorylation and/or of the increase in PML levels that occur in response to virus infection. In this sense, phosphorylation at serine 15 increases the interaction between p53 and CBP, leading to an increase in p53 acetylation.[28] Furthermore, following viral infection cells induce type I IFN, which in turn upregulates the expression of numerous antiviral proteins including PML,[29-31] and this upregulation induces the acetylation of p53 at lysine 382.[32]

Transcriptional transactivation of p53-activated genes in response to virus infection requires p53 acetylation. Activation of p53 in response to virus infection induces transcriptional activation of some of its classical target genes such as Mdm2 or Puma,[7] and of some IFN-inducible genes such as interferon regulatory factor 9 or 5 (IRF9, IRF5).[10] To elucidate the influence of p53 acetylation on p53-mediated transcriptional transactivation induced in response to virus infection, we generated a derivative of the p53-null human lung carcinoma line H1299 expressing wild-type p53 (p53-WT) or a p53 mutant in which the eight known acetylated sites are all substituted with arginine (p53-8KR), that has been previously described in reference 16. Then, the cells were infected with a recombinant VSV expressing GFP (rVSV-GFP) and at different times after infection, GFP expressing cells were isolated by fluorescence-activated cell (FACs) sorting to measure the transactivation of p53-response genes by real time quantitative PCR analysis (RT-QPCR). As shown in **Figure 2A**, expression of wild-type p53 led to strong induction of both, pro-apoptotic Bax and Puma genes, as expected. In addition, VSV infection induced the transactivation of IFN-inducible genes, such as regulated upon activation in normal T cell expressed and secreted (RANTES) and IRF7. However, this transactivation was clearly reduced in those cells transfected with empty vector or the acetylation-defective p53-8KR mutant (**Fig. 2A**). Similar results were obtained after RT-QPCR of FACs-sorted p53-null MEFs transiently transfected with pcDNA, p53-WT or p53-8KR. VSV infection induced the transactivation of IFN-inducible genes in a p53-dependent manner and loss of acetylation completely abolished this p53-dependent transcriptional transactivation (**Fig. 2B**). Our findings demonstrate that acetylation is required for p53-dependent transcription of pro-apoptotic genes in response to virus infection, in agreement with previous reports that demonstrate that loss of acetylation abrogates p53-mediated activation of proapoptotic targets such as Bax, Puma and Pig3 in response to genotoxic stress.[16] In addition we also demonstrate that p53 acetylation is required for

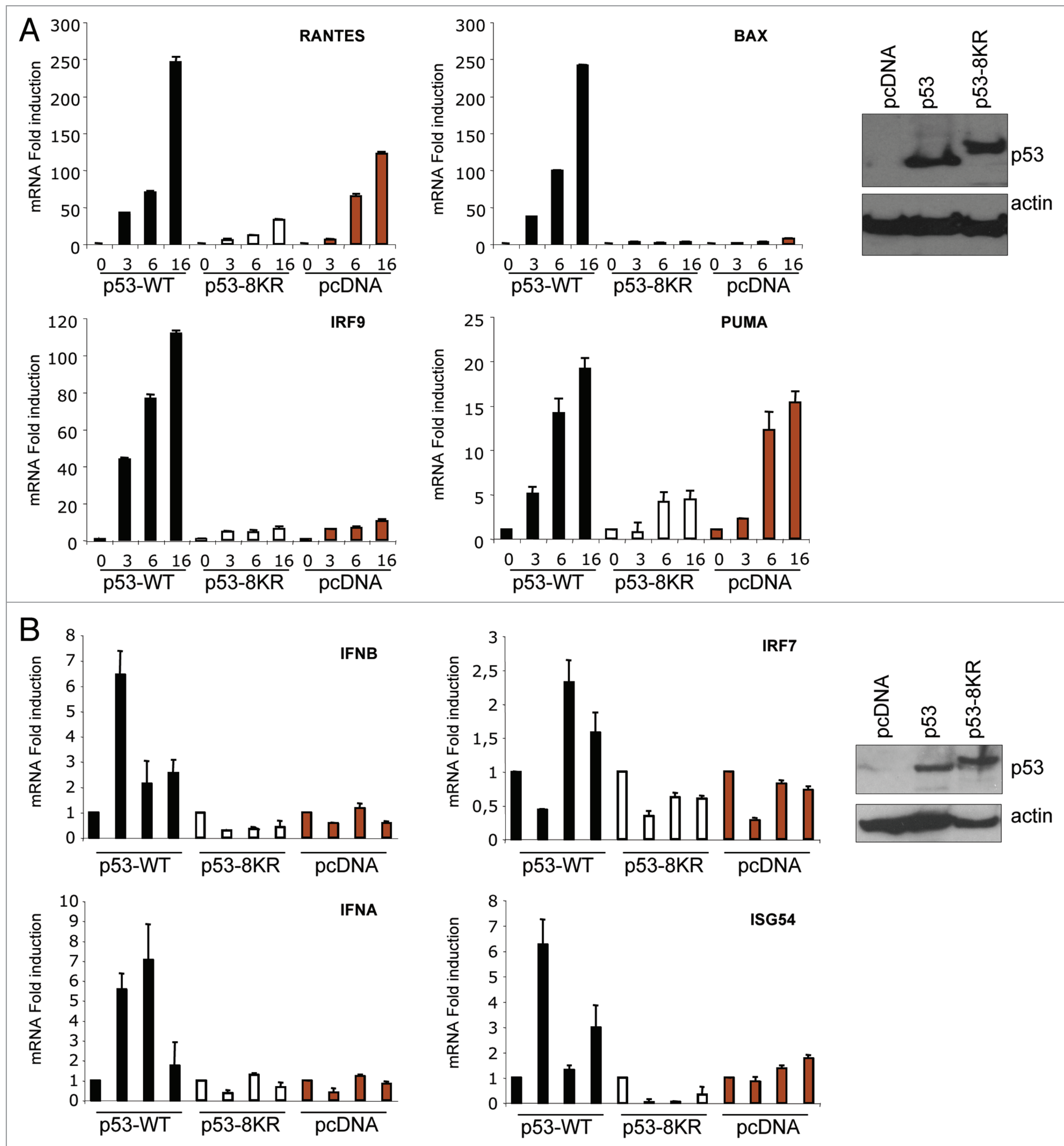

Figure 2. Transcriptional transactivation of p53-activated genes in response to virus infection requires p53-acetylation. (A) H1299 cells stably transfected with pcDNA, p53-WT or p53-8KR were infected with rVSV-GFP for the indicated times. Then, GFP expressing cells were selected by FACs sorting and Q-RTPCR analysis of the infected cells was performed. (B) MEFs derived from $p53^{-/-}$ mice transiently co-transfected with GFP and pcDNA, p53-WT or p53-8KR at a ratio 1:10 were infected with VSV at MOI of 5 PFU/ml for the indicated times. Then, GFP expressing cells were selected by FACs and Q-RTPCR analysis was performed. The right parts represent the expression levels of p53 in each experiment.

p53-mediated transcriptional activation of genes implicated in innate immunity.

Acetylation is essential for p53-mediated antiviral activity. The importance of p53 acetylation for p53 antiviral activity is underscored by the fact that proteins from different viruses can inhibit p53 acetylation.[17-22] To examine the role of p53 acetylation on p53-mediated antiviral activity, p53-deficient MEFs transiently transfected as described above were infected with VSV at a MOI of 5 PFU/cell and at 24 or 48 h after infection, we determined virus titers in supernatants. As expected, the expression

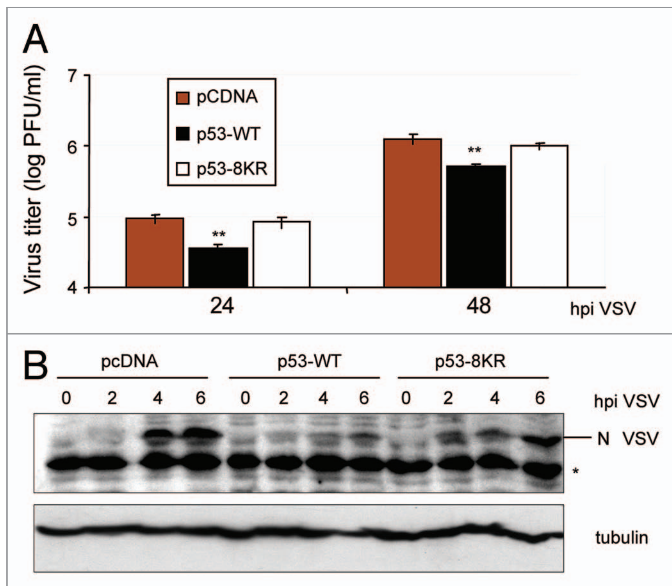

Figure 3. Acetylation is essential for p53-mediated antiviral activity. (A) MEFs derived from p53$^{-/-}$ mice were transiently transfected with pcDNA, p53-WT or p53-8KR and then infected in triplicate with VSV at MOI of 5 PFU/ml and quantification of the virus yield at the indicated time was assessed. The same results were obtained in at least three different experiments Data represents means ± SE for one experiment. *$p < 0.05$ compared with pcDNA transfected cells, Student's test. (B) p53$^{-/-}$ MEFs were transiently transfected with pcDNA, p53-WT or 53-8KR and then infected with VSV at MOI of 5 PFU/ml and at the indicated times, protein gel blotting using antibodies against the M protein from VSV was performed. Asterisk indicates a non-specific band.

of wild type p53 induced a statistically significant decrease in the viral titer in comparison with the one obtained in pcDNA-transfected cells. Importantly, no reduction in the viral titer was observed in cells transfected with the 8KR mutant (**Fig. 3A**), indicating that p53 acetylation is required for the protective role of p53 against VSV infection. This notion was further corroborated by direct inspection of viral protein synthesis after infection of p53-deficient MEFs transfected with pcDNA, p53-WT or p53-8KR with VSV at a MOI of 5 PFU/ml. Viral protein synthesis was clearly reduced in those cells expressing p53-WT in comparison with the VSV protein level detected in the pcDNA or p53-8KR transfected cells (**Fig. 3B**).

In summary, our data demonstrate that p53 is acetylated in response to virus infection and that acetylation is an indispensable event for the transcriptional activation of p53-dependent genes in response to virus infection and the subsequent control of virus replication. These results might represent the basis to explain the observed inhibition of p53 acetylation by viral proteins as a mechanism of viral escape from p53-mediated antiviral activity.

Methods

Cell culture, virus and transfections. SIRT1-tg and SIRT1$^{-/-}$ mice have been previously described in reference 27 and 33. MEFs were isolated and cultured as described previously in reference 34. All MEFs were used before spontaneous immortalization. BHK-21, H1299 and BSC-40 cells were maintained in DMEM supplemented with 10% fetal calf serum (Gibco), 5 mmol/L L-glutamine (Invitrogen) and penicillin-streptomycin (Invitrogen). Infections were performed using Herpes simplex virus type 1 (HSV-1), vesicular stomatitis virus (VSV) of Indiana strain or recombinant VSV expressing GFP (rVSV-GFP) kindly provided by Dr. Adolfo Garcia-Sastre (Mount Sinai School of Medicine), and VSV yields were measured by plaque assays in BSC-40 cells. To obtain the p53 or Flag-p53-8KR expressing cell lines, H1299 cells were transfected with pcDNA-p53-WT or pCIN4-Flag-HA-p53-8KR and selected for 2 weeks on 1 mg/ml G418 (Invitrogen), until p53-expressing clones were obtained. p53-null MEFs were transiently co-transfected with GFP and pcDNA, p53-WT or p53-8KR at a 1:10 ratio of plasmids. Transfection of H1299 was done using FuGene (Roche) and MEFs were transfected with lipofectamine 2000, respectively, following the manufacturer's instructions.

Protein gel blot analysis and antibodies. Cells were washed in PBS, scraped in SDS-gel loading buffer and boiled for 5 min. Proteins of total extracts were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the corresponding antibodies. The following antibodies were used: anti-mouse p53 (CM5, Novocastra), anti-human p53 (Santa Cruz Biotechnology), anti-VSV, anti-Ser18-p53 (Cell Signaling), anti-K379-p53 (Cell Signaling), anti-SIRT1 (Abcam, AB12193), anti-Mdm2 (Santa Cruz Biotechnology) and anti-actin (MP Biomedicals).

Quantitative reverse transcription-PCR (qRT-PCR) analysis. For qRT-PCR analysis, RNA was isolated from cells using TRIzol (Invitrogen). Quantitative RT-PCR was performed using 100 ng of sample RNA and SYBR green (Roche) in an ABI PRISM 7900HT instrument following manufacturer's instructions.

Cell sorting and flow cytometry. For H1299 sorting, cells were infected with rVSV-GFP at a multiplicity of infection (MOI) of 1 PFU/ml. Twelve hours after infection, cells were trypsinized and resuspended in DMEM supplemented with 10% FBS at 2 x 10^6 cells/ml. For MEFs sorting, p53-null MEFs co-transfected at a 10:1 ratio of plasmids were infected with VSV at a MOI of 5 PFU/ml for the indicated times. GFP positive cells were sorted in sterile conditions using a MOFLO cell sorter (Beckman Coulter).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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