FEBS Letters 587 (2013) 2965–2971

FEBS Letters

journal homepage: www.FEBSLetters.org

Influenza NS1 interacts with p53 and alters its binding to p53-responsive genes, in a promoter-dependent manner

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ARTICLE INFO

Article history:
Received 16 May 2013
Revised 12 July 2013
Accepted 5 August 2013
Available online 13 August 2013

Edited by Varda Rotter

Keywords:
p53
Transcription factor
Orthomyxoviridae
Influenza virus
Non-structural protein NS1

ABSTRACT

The interplay between influenza A viruses (IAV) and p53 has only been reported in a limited number of studies, mainly focusing on the antiviral role of p53. We investigated the impact of IAV infection on p53 stability and transcriptional activity. Our results indicate that IAV-induced stabilization of p53 only partially correlates with modulation of p53 transcriptional activity measured during infection. Moreover, we show that the viral non-structural protein 1 (NS1) is able to inhibit p53 transcriptional activity, in a promoter-dependent manner. Based on these data, we propose that NS1 may contribute to p53-mediated cell fate decision during IAV infection.

Structured summary of protein interactions:
p53 physically interacts with **NS1** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

Influenza A viruses (IAV) belong to the *Orthomyxoviridae* family of enveloped viruses that contain a segmented genome of single-stranded negative sense RNA. This family comprises some of the few RNA viruses that undergo replication and transcription within the host cell nucleus using the nuclear machinery to support their propagation [1]. Several studies have shown that IAV are able to modulate and/or hijack several cellular networks and signalling pathways [2,3]. The viral non-structural protein 1 (NS1) is involved in multiple aspects of IAV/host interactions that together inhibit a broad range of cellular functions, notably those related to the antiviral response, and also interferes with host mRNA export and splicing [4]. Indeed, NS1 is believed to be important for regulation of the host cell cycle and was recently shown to be involved in the IAV-induced G0/G1 cell cycle arrest [5]. Moreover, NS1 seems to play an ambivalent role in the regulation of apoptosis during IAV infection, based on contradictory results from studies reporting either its pro- or anti-apoptotic functions [6–8].

In response to stress, the transcription factor p53 rapidly accumulates in the nucleus where it regulates gene expression to maintain genomic and cellular integrity [9]. The numerous genes regulated by p53 are involved in several biological processes, including cell cycle arrest (e.g. P21/CDKN1A), apoptosis (e.g. BAX) or senescence [9]. The interplay between influenza viruses and p53 has only been reported in a limited number of studies which mainly highlight the role of p53 as an antiviral protein [10,11]. Moreover, we recently showed that p53 isoforms are involved in the regulation of these p53-dependent antiviral properties [12].

Most of the studies dedicated to IAV and p53 have described an increase in p53 protein levels during infection [11,13–15]. However, it is still not clear whether this accumulation is correlated with the activation of p53 and consecutive transactivation of its target genes. Wang et al. have suggested a possible interaction between NS1 and p53 leading to the inhibition of p53-mediated transcriptional activity [16]. Interestingly, results from our previous study based on transcriptional profiling of IAV-infected human cells, showed a massive downregulation of the p53 pathway, mostly its downstream part, in response to IAV infection [11].

Based on these observations, we further investigated the impact of IAV infection on p53 stability and transcriptional activity in human lung epithelial cells, with a systematic comparative focus

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.08.006

on NS1. Our results indicate an IAV-induced increase in p53 stability that is only partially correlated with the modulation of p53 transcriptional activity during IAV infection. Moreover, they show that NS1 is able to inhibit p53 transcriptional activity by altering its binding to target genes, in a promoter-dependent manner.

2. Materials and methods

2.1. Cell line, virus and infection

Human lung epithelial A549 cells (ATCC CCL-185, wild type p53) and H1299 (ATCC CRL-5803, p53 null) were grown at 37 °C in DMEM supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin sulphate. Influenza virus A/Moscow/10/99 (H3N2) was propagated in MDCK cells (ATCC CCL-34) in EMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin sulphate and 1 mg/mL trypsin. The virus was then titrated to determine the 50% tissue culture infective dose (TCID50) in MDCK cells, as previously described [17]. Sub-confluent A549 cells were then infected with the influenza virus at multiplicities of infection (MOI) of 0.1 or 2 for 1 h in a minimal volume of DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulphate and 0.5 μg/mL trypsin (infection medium) at 37 °C. Fresh infection medium was then added to the cells before their incubation at 37 °C for different lengths of time.

2.2. Transfection and transactivation assay

Transient transfections were performed using TransIT-LT1 reagent (Mirus), according to the manufacturer's instructions. For the transactivation assay, A549 cells were transfected with 1 μg firefly luciferase reporter vectors. Transfection efficiency was normalised using 100 ng of Renilla luciferase plasmid. After 24 h, cells were infected with influenza virus A/Moscow/10/99 at a MOI of 0.1 or 2 before being harvested at different time points for further analysis. Alternatively, cells were co-transfected with a pCI-empty or pCI-NS1-H3N2 expression plasmid and then harvested at 24 h post-transfection. Luciferase activity was measured in whole cell extracts using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. Three independent experiments were performed in triplicate. The different reporter vectors used were pG13-luc, with a firefly luciferase gene under the control of thirteen copies of the p53-binding consensus sequence (5′- CCAGGCAAGTCCAGGCAGG- 3′ [18]), and p21-luc, Mdm2-luc and Bax-luc, with the firefly luciferase gene under the control of the complete (p21) or partial (Mdm2 and Bax) promoter sequence of the corresponding genes [18–20].

2.3. p53 stability assay

For the p53 half-life experiments, previously transfected or infected cells were treated with 50 μM cycloheximide (CHX). Total protein lysates were harvested at different time-points during a 1 h period and were analysed by western blot [12] to determine relative protein levels (RPL) by densitometry. RPLs between Mock and NS1-transfected or infected cells were subjected to statistical analysis (Student's t test, statistical significance $P < 0.05$).

2.4. Chromatin immunoprecipitation assay (ChIP)

ChIP experiments were performed as previously described [21,22]. Briefly, 2×10^6 H1299 cells were seeded onto a 15 cm plate and co-transfected with 4 μg of pSV-p53 and 4 μg of pCI-empty or pCI-NS1-H3N2 expression plasmid. After 24 h, cells were fixed with 1% formaldehyde for 10 min at room temperature. The cells were then scraped and washed with ice-cold PBS, and then sonicated with salmon sperm DNA and protease inhibitors. Immunoprecipitation was carried out using an anti-p53 monoclonal antibody (DO-1) and Dynabeads (Invitrogen). Mouse IgGs were used as negative controls. A mouse anti-NS1 monoclonal antibody (Santa-Cruz ref sc-130568) was used to control a possible binding of NS1 to promoter regions. The amount of total input DNA per ChIP was adjusted to 25 μg. After immunoprecipitation and DNA purification, samples were analysed by real-time quantitative PCR (RT-qPCR), using specific primers and probes for p21 and Bax. In parallel, the same quantitative analysis was performed on Input DNA. The results were expressed as a percentage of total input DNA. The specific primers and probes used for p21 and Bax were the same as those described by Kaeser and Iggo [21].

3. Results

3.1. IAV infection increases p53 stability

To investigate the impact of influenza infection on p53 stability, we mock-infected or infected A549 cells with influenza virus A/Moscow/10/99 (H3N2) at an MOI of 2 or 0.1. At 4, 24 or 48 h post infection (hpi), we then studied p53 stability by monitoring its levels over a 1 h period post treatment with 50 μM cycloheximide (CHX) (Fig. 1A). In all conditions tested, the mock-infected cells displayed a loss of around 80% of the RPL of p53 in 1 h, thereby reflecting a p53 half-life of approximately 15–20 min. In the H3N2-infected cells, however, the RPL of p53 consistently remained higher than in mock-infected cells, the difference of which was particularly marked at the later time-points. For example, at 24 and 48 hpi, the 1 h stability of p53 was almost complete (p53 RPL of 0.91, P-value <0.001) or complete, respectively (Fig. 1A). These first observations suggested a marked increase in p53 stability during IAV infection, even from 4 hpi (Fig. 1A).

3.2. IAV NS1 expression contributes towards p53 stability

To further investigate the potential involvement of NS1 in this virally-induced stabilization of p53, we transfected A549 cells with either an empty plasmid (pCI-empty) or a plasmid expressing NS1 from the H3N2 strain (pCI-NS1-H3N2). We used the same method as above to evaluate the endogenous p53 stability (Fig. 1B) and western blot to assess the expression of NS1, p53 and its targets (Fig. 1C) 36 h post-transfection. The CHX assay revealed a slight increase in p53 stability in the NS1-expressing cells. For example, at 30 min, the RPL of p53 measured in the NS1-expressing cells was more than two times higher than levels measured in pCI-empty transfected cells (p53 RPL of 0.44 *versus* 0.21, P-value <0.05) (Fig. 1B). Interestingly, stabilization of p53 in the NS1-expressing cells did not reach the same level as that measured in the infected cells, even with higher concentrations or longer kinetics of the transient expression of NS1 (data not shown). Not only does this finding support the contribution of NS1 towards the stabilization of p53, it also suggests that NS1 may not be the only determinant.

3.3. NS1 interacts with p53

To investigate the potential interaction between p53 and NS1, we transfected H1299 cells (p53 null) with an empty plasmid (pCI-empty) or one expressing NS1 (pCI-NS1-H3N2), along with a plasmid expressing p53 (pSV-p53). After 48 h, we analysed the cell lysates using a co-immunoprecipitation assay (co-IP) with an anti-p53 polyclonal antibody (CMI), or control IgG (Fig. 1D). Western blot displayed a band corresponding to NS1 only with the

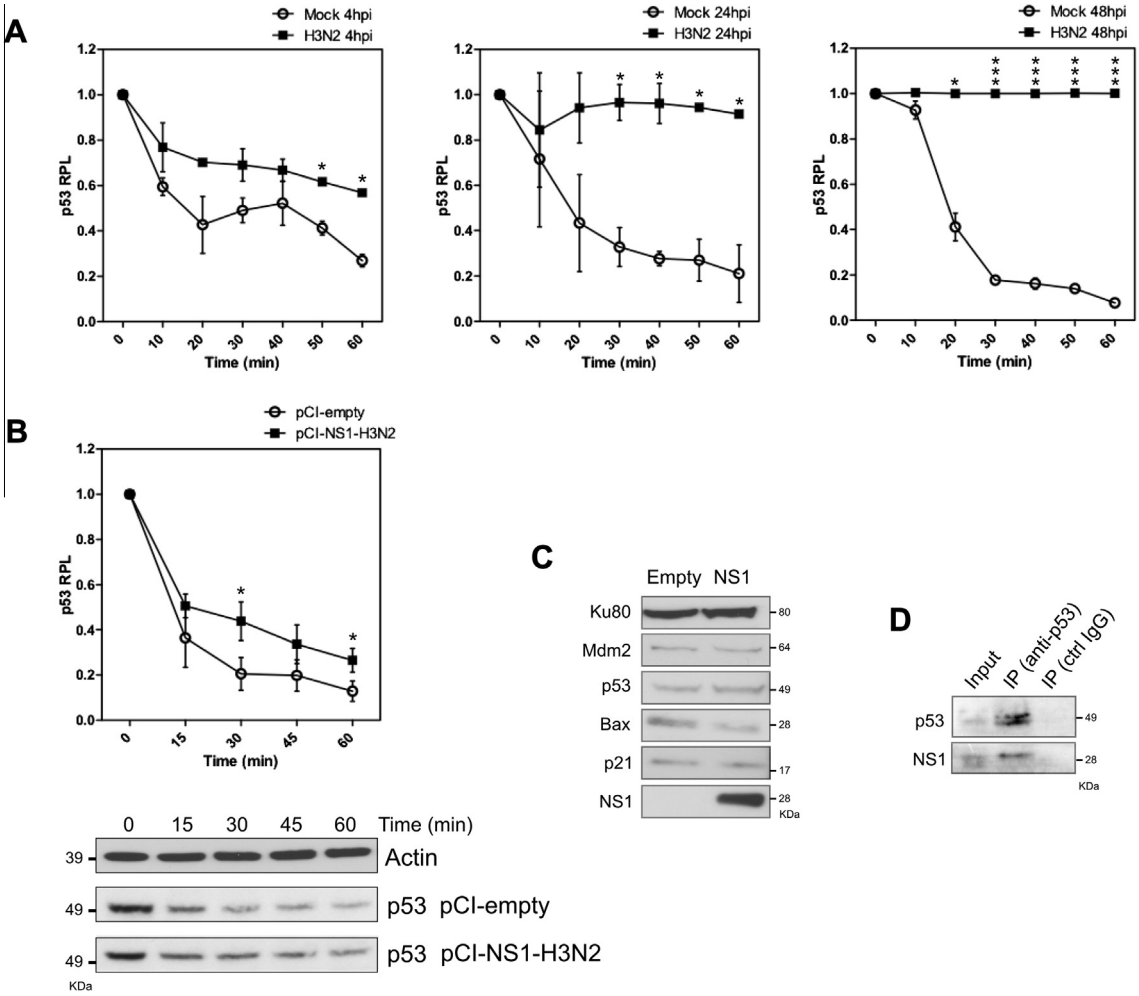

Fig. 1. NS1 is involved in the IAV-induced stabilization of p53. (A) Stability assay in IAV-infected cells. A549 cells were mock-infected or infected with influenza virus A/Moscow/10/99 (H3N2) with an MOI of 2 or 0.1 and analysed at different time-points. Stability was assessed by monitoring relative protein levels (RPL) of p53 during a 1 h time period, post treatment with 50 μM cycloheximide (CHX). Mean values ± standard deviation from three independent experiments are presented. * and *** for *P*-value <0.05 and 0.001, respectively. (B) Stability assay in presence of NS1. A549 cells were transfected with either an empty plasmid (pCI-empty) or a plasmid expressing NS1 from the H3N2 strain (pCI-NS1-H3N2), and p53 stability was evaluated 36 h post-transfection. Mean values ± standard deviation from three independent experiments are presented. **P*-value <0.05. (C) The efficient expression of NS1, p53 and its targets 36 h post-transfection were analysed by western blot. (D) Analysis of the interaction between NS1 and p53 by co-immunoprecipitation (co-IP). H1299 cells were transfected with either an empty plasmid (pCI-empty) or a plasmid expressing NS1 (pCI-NS1-H3N2), and a plasmid expressing p53 (pSV-p53). After 48 h, cells were scraped into ice-cold PBS and analysed using a co-immunoprecipitation assay with an anti-p53 polyclonal antibody (CMI), or a control IgG.

anti-p53 antibody and not with the control IgG thus indicating that the p53 could immunoprecipitate NS1 (Fig. 1D). These results suggest that NS1 associates with p53 and are in line with previous data obtained by Wang et al., based on the additional use of a GFP-tag or Flag [16].

3.4. IAV infection modulates p53 transcriptional activity

To evaluate the impact of IAV infection on p53 transcriptional activity we firstly transfected A549 cells with pG13-luc, which expresses luciferase under the control of thirteen repeats of p53-binding consensus sequences. We then either mock-infected or infected the A549 cells with A/Moscow/10/99 (H3N2) at a MOI of 2 or 0.1 before their analysis at 4 and 10 hpi or 24 and 48 hpi, respectively (Fig. 2A). Western blot analysis allowed a confirmation at the protein level of the infection and the expression of p53 and products of p53 target genes (Fig. 2B). The mock-infected cells displayed a change in luciferase activity and thus p53 transcriptional activity, most likely due to serum starvation of the cells in the infection protocol. Consequently, to avoid any artefactual misinterpretation, we only used luciferase activities obtained in infected samples compared to their mock-infected counterparts, treated with the same protocol and harvested at the same time points. At an MOI of 2, we observed a significant increase in relative luciferase units (RLU) in the infected cells compared to the mock-infected at 4 hpi ($P < 0.05$), but no significant change at 10 hpi (Fig. 2A). Similarly, at an MOI of 0.1 at 24 hpi, the RLU were two times higher in the infected cells compared to the mock infected ($P < 0.001$). In contrast, at 48 hpi luciferase activity had significantly decreased ($P < 0.001$) (Fig. 2A). These first results suggest a biphasic modulation of p53 transcriptional activity during IAV infection, with an increase in activity at the early time points, followed by a marked decrease during the later stages. To confirm our results at the level of p53 target genes, we performed similar experiments, using p21, Bax or Mdm2 luciferase reporter constructs (p21-luc, Bax-luc, Mdm2-luc, Fig. 2A). At 48 hpi we observed a significant decrease in luciferase activity with p21-luc and Mdm2-luc ($P < 0.005$ and $P < 0.001$, respectively), similar to the levels measured with the pG13-luc construct. The increase in p53 transcriptional activity observed at 4 hpi was consistent with the significant increase in luciferase activity

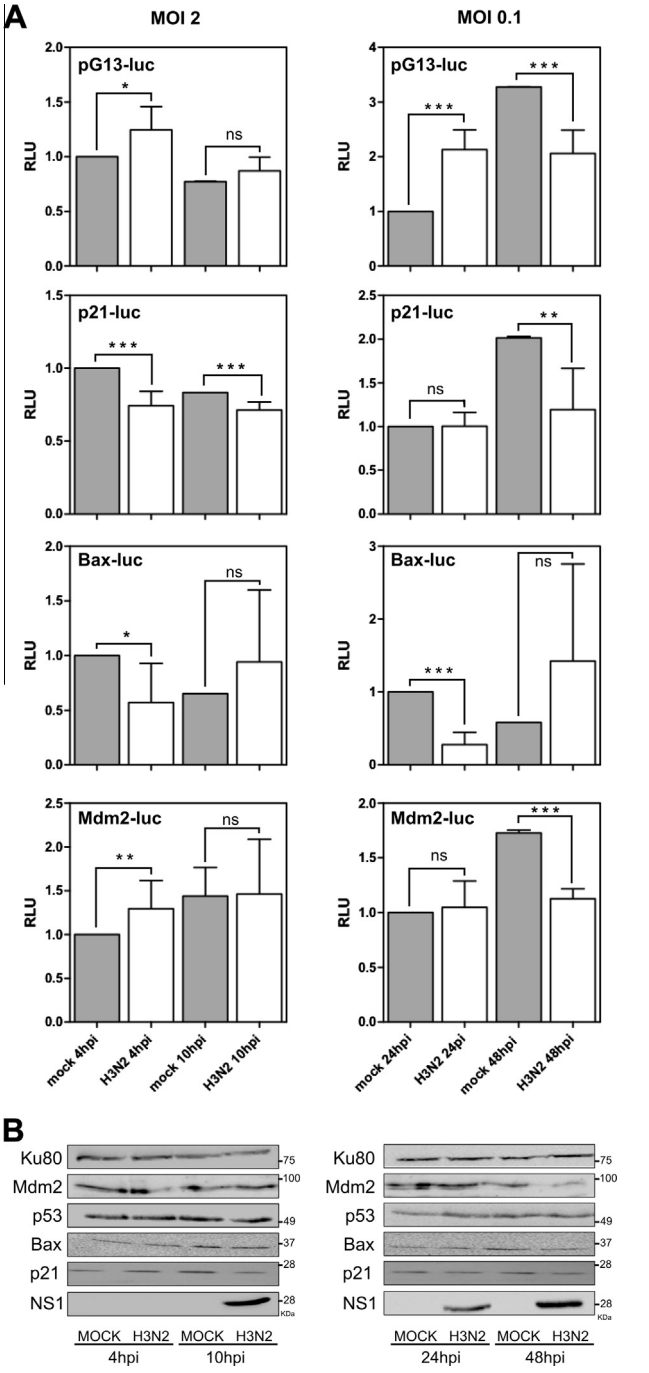

Fig. 2. IAV infection modulates p53 transcriptional activity. (A) A549 cells were transfected with pG13-luc, p21-luc, Bax-luc or Mdm2-luc reporter constructs and then mock-infected or infected with A/Moscow/10/99 (H3N2) at an MOI of 2 or 0.1 for 4 and 10 h post infection (hpi) or 24 and 48 hpi, respectively. Relative luciferase units (RLU) were monitored to evaluate the p53 transcriptional activity. (B) Western blot analysis was used to monitor infection levels and the expression of p53 and products of its target genes. Mean values ± standard deviation from three independent experiments are presented. (ns, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.005$; and $^{***}P < 0.001$).

with Mdm2-luc ($P < 0.005$). In contrast, we measured a significant decrease in luciferase activity for p21-luc and Bax-luc at the same time point ($P < 0.001$ and $P < 0.05$, respectively) (Fig. 2A). In conclusion, we observed distinct activity profiles for three p53 target genes that differed from the global results obtained with pG13-luc, suggesting that influenza virus regulates p53 transcriptional activity in a promoter-dependent manner. A comparative similar approach in a p53-deficient model was not technically possible, due to the marked difference of viral replication kinetics in presence *versus* absence of p53 [10,12,11]. These results also highlight that there is no correlation between p53 stability and its transcriptional activity, as previously suggested by our transcriptomic study of IAV-infected cells [11].

3.5. NS1 inhibits p53 transcriptional activity

To assess the role of NS1 in this IAV regulation of p53 transcriptional activity, we transfected A549 cells with the pG13-luc reporter plasmid together with increasing concentrations of the NS1 expressing plasmid (pCI-NS1-H3N2) (Fig. 3A, left panel). This titration showed that 1 μg of pCI-NS1-H3N2 was required to decrease more than 80% of the RLU, and thus illustrated the considerable impact of NS1 on endogenous p53 transcriptional activity. To further explore the role of NS1, we then co-transfected 1 μg of pCI-NS1-H3N2 and 1 μg of pG13-luc in H1299 cells (p53 null) together with increasing concentrations of the p53 expressing plasmid (pSV-p53) (Fig. 3A, right panel). As expected, in the absence of NS1 (empty), we observed a significant increase in luciferase activity in correlation with increasing concentrations of p53, with RLU fold changes of more than 60 and 192, for 0.1 and 1 μg of pSV-p53, respectively. In the presence of NS1, this increase in luciferase activity was reduced to RLU fold changes of 6.5 and 29, and corresponded to 9 and 6 times lower RLU for 0.1 and 1 μg of pSV-p53, respectively (Fig. 3A, right panel). These results confirm that NS1 inhibits p53 transcriptional activity and that this inhibition is in turn titrated by increasing concentrations of p53.

Using a similar approach to that used in infected cells, we transfected A549 cells with p21, Bax or Mdm2 luciferase reporter plasmid constructs together with 1 μg of empty or NS1-expressing plasmid (Fig. 3B). we observed a highly significant decrease in luciferase activity in the presence of NS1 for p21-luc and Mdm2-luc ($P < 0.001$). In contrast, we did not observe any significant change with Bax-luc (Fig. 3B).

In a second step, we performed a p53 titration in H1299 cells transfected with each specific promoter reporter construct together with 1 μg of empty or NS1-expressing plasmid (Fig. 3C). The inhibitory effect of NS1, as observed in all cases by reduced luciferase activity compared to that obtained with p53 alone, was titrated by increasing concentrations of p53. The titration profile obtained with Mdm2-luc was similar to the results obtained with the pG13-luc, with the same extent of NS1-dependent inhibition and similar titration by p53 (Fig. 3C). In contrast, the activity profiles obtained for p21-luc and Bax-luc were distinct, notably in terms of levels of the NS1-dependent inhibition. Interestingly, p53 titration results obtained in H1299 revealed the NS1 inhibitory effect on Bax-luc (Fig. 3C), whereas it was not visible in the context of p53 wt cells (A549, Fig. 3B), suggesting that the p53/NS1 ratio could be an important factor.

Altogether, our results confirm that IAV NS1 expression strongly inhibits p53 transcriptional activity, in a similar way to that observed in IAV infected cells (Fig. 2) Moreover, the use of specific promoter reporter plasmids and the results from the titration assays suggest that the impact of NS1 differs according to the promoter studied.

3.6. NS1 partially blocks the binding of p53 to its target genes, in a promoter-dependent manner

To further investigate the impact of NS1 on p53 DNA-binding and uncover possible differences between promoters, we chose to focus our analysis on p21 and Bax promoters, which presented distinct activity profiles in the previous experiments (Figs. 2 and 3). ChIPs were performed in H1299 cells, transfected with

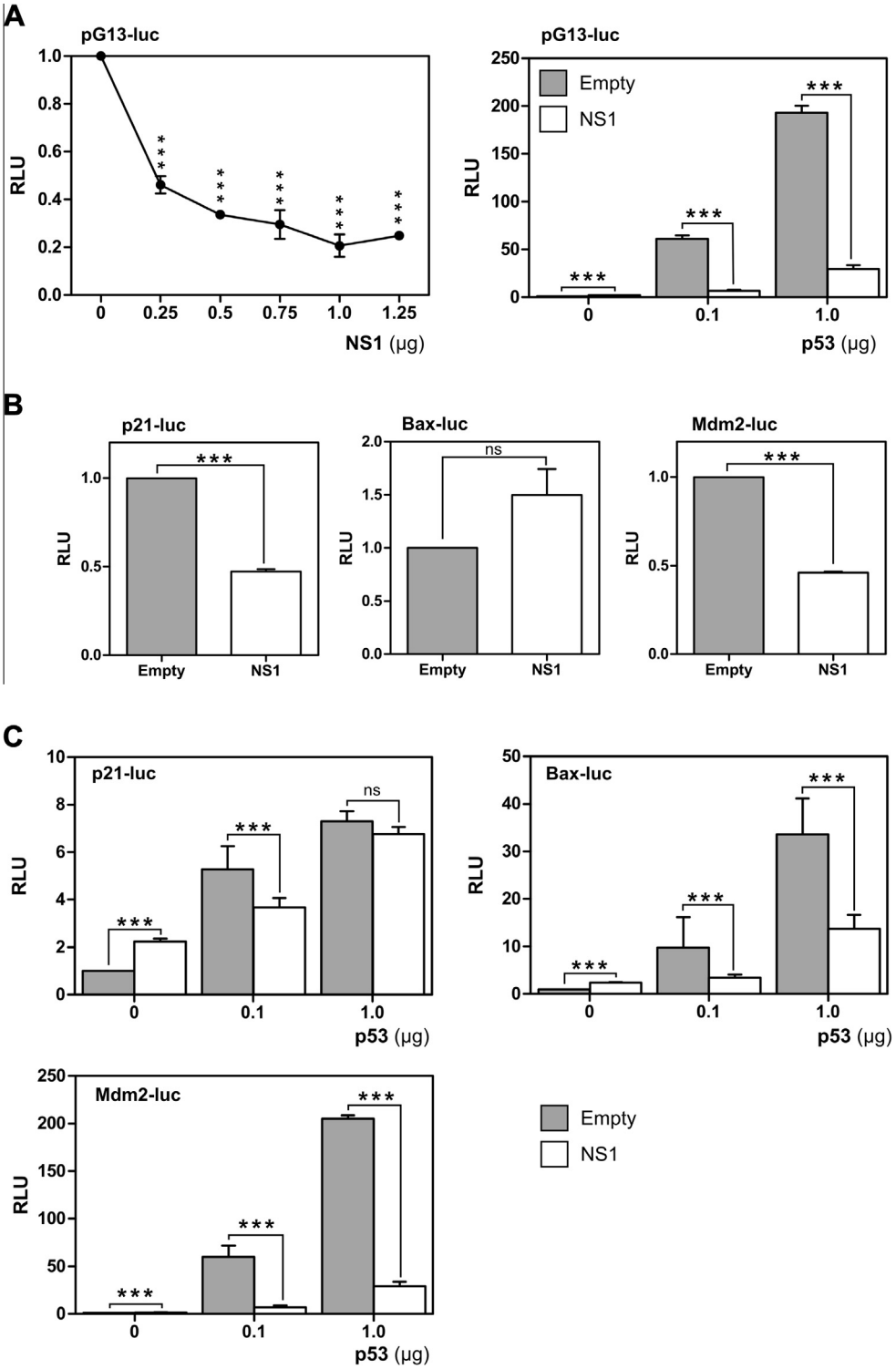

Fig. 3. NS1 inhibits p53 transcriptional activity. (A) A549 cells were transfected with the pG13-luc reporter plasmid together with increasing concentrations of NS1 expressing plasmid, (pCI-NS1-H3N2, left panel). H1299 cells (p53 null) were then co-transfected with 1 μg of pCI-empty or pCI-NS1-H3N2 and increasing concentrations of p53 expressing plasmid (pSV-p53, right panel). (B) A549 cells were transfected with p21, Bax or Mdm2 luciferase reporter plasmid constructs together with 1 μg of pCI-empty plasmid or pCI-NS1-H3N2 C. H1299 cells were co-transfected with 1 μg of pCI-NS1-H3N2 and 1 μg of each specific promoter reporter construct together with increasing concentrations of pSV-p53. In A. B. and C. Cells were harvested at 36 hpi and RLU were measured. Mean values ± standard deviation from three independent experiments are presented. (ns, $P > 0.05$ and ***,<0.001).

pSV-p53 together with either an empty plasmid (pCI-empty) or the NS1-expressing plasmid (pCI-NS1-H3N2), with an antibody against p53 (DO-1) and specific primers and probes for p21 and Bax [21]. The plotted figures were based on the quantitative results by real-time quantitative PCR, and the results were expressed as a percentage of total Input DNA. As expected, p21 and Bax promoters were specifically immunoprecipitated by the DO-1 antibody (IP p53, Fig. 4). The percentage of total input DNA obtained for p21 and Bax was in accordance with previously published results [21]. As a control, we also used an anti-NS1 antibody, with no

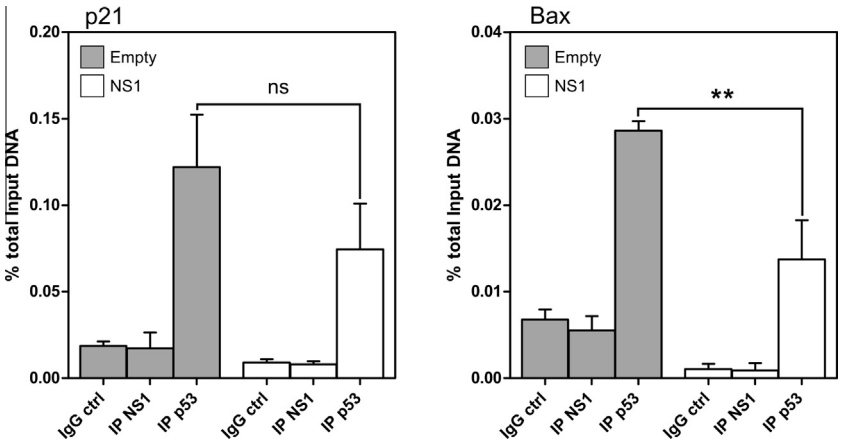

Fig. 4. NS1 partially blocks the binding of p53 at the level of p53-responsive genes, in a promoter-dependent manner. H1299 cells were co-transfected with 4 μg of pSV-p53 and 4 μg of pCI-empty or pCI-NS1-H3N2 expression plasmid. After 24 h, cells were fixed with 1% formaldehyde for 10 min at room temperature. The cells were scraped and washed with ice-cold PBS, and then sonicated with salmon sperm DNA and protease inhibitors. Immunoprecipitation was carried out using Dynabeads (Invitrogen) and different antibodies produced in mouse: an anti-p53 monoclonal antibody (DO-1), or an anti-NS1 monoclonal antibody (Santa-Cruz ref sc-130568). Mouse IgGs were used as negative controls. The amount of total input DNA per chip was adjusted to 25 μg. After immunoprecipitation and DNA purification, samples were analysed by RT-qPCR with specific primers and probes targeting p21 and Bax. Mean values ± standard deviation from two independent experiments are presented. (ns, $P > 0.05$; $^{**}P < 0.005$).

measurable binding compared to background (IP NS1 *versus* IgG ctrl, Fig. 4). When comparing the two experimental conditions, we observed that the level of binding was lower in the presence of NS1, with a significant difference with the Bax promoter (Fig. 4, $P < 0.005$ for ChIP-Bax). This result indicated that NS1 can decrease the binding of p53 to its target genes, which is in agreement with the results obtained in the transcriptional activity assays both in transfection and infection contexts.

4. Discussion

In this study, we have shown that IAV induces an increase in the stabilization of p53 throughout the course of infection in vitro (Fig. 1). These results are in agreement with previous studies reporting increased p53 protein levels in IAV-infected cells, notably during the later stages of infection [11,13,15]. Interestingly, Wang et al. have recently shown that this stabilization was associated with a compromised Mdm2-mediated ubiquitination of p53 [23]. At 24hpi, p53 protein levels were clearly higher in IAV-infected cells than in non-infected cells (Fig. 2B). In contrast, no marked difference of p53 protein abundance was observed at 48hpi (Fig. 2B), maybe due other levels of regulation of p53, not assessed in stability assay (Fig. 1). Our results suggest that NS1 is also involved in this stabilization but to a limited extent. Our co-IP experiments indicated that NS1 interacts with p53, in a transient expression context, confirming previously published reports of an interaction displayed between GFP-p53 and Flag-NS1 [16]. We hypothesise that both compromised ubiquitination of p53 and NS1 interaction may contribute towards IAV-induced stabilization of p53.

In one of the first studies dedicated to p53 and influenza viruses, Turpin et al. showed that p53 activity was increased during influenza infection [13]. On the other hand, NS1 expression has been shown to inhibit p53-mediated transcriptional activity [16]. Our results indicate that p53 transcriptional activity is modulated during IAV infection, with a significant increase at 24 hpi, which would correspond to the experimental conditions used by Turpin et al. [13]. In contrast, this activity is significantly inhibited during the later stages of infection, in correlation with elevated NS1 protein levels (Fig. 2). These results corroborate those we obtained in our transcriptional profiling study, indicating a strong down-regulation of p53 target genes during the later stages of IAV infection [11]. We demonstrated and validated that NS1 strongly inhibits p53 transcriptional activity in a promoter dependent manner, and that this phenomenon is titrated by p53 (Fig. 3). These results suggest that the ratio of p53/NS1 is an important factor and that NS1 may inhibit p53, via a direct interaction. Another viral factor, yet to be determined, seems to counterbalance the NS1-mediated inhibitory effect on p53 transcriptional activity, as suggested by our results obtained in the infected cells.

Our comparative evaluation of p53-mediated transcriptional activity, using specific reporter plasmids for three p53 target genes, has revealed marked differences between promoters, in both infection and transient expression contexts (Figs. 2 and 3). This observation refines our initial results obtained with an artificial promoter composed of repeated p53-binding consensus sequences. However, we cannot exclude the possible role of other transcriptional factors, with response elements in the complete (p21) or partial (Mdm2 and Bax) promoters used in luciferase assays. ChIP experiments demonstrated that NS1 inhibits the binding of p53 to different extents for p21 and Bax (Fig. 4). Combined with our results obtained with the transactivation assays in infected or NS1-transfected cells, this observation confirms that NS1 can inhibit p53 transcriptional activity, in a promoter-dependent manner. These results may contribute to better explain apparently contradictory reports in the literature of the relative role of NS1 in apoptosis [6,7,24] or recent results concerning NS1-induced cell cycle arrest [5].

In conclusion, we have shown that IAV infection modulates p53 transcriptional activity and that NS1 contributes to the inhibitory part of this modulation, possibly via its direct interaction with p53. NS1 alters the binding of p53 to the promoter of its target genes and may contribute to "drive" the p53-mediated cell fate decision, in both a stage-of-infection and relative concentration – dependent manner. In this context, further studies are required to understand the role of NS1 in apoptosis and cell cycle regulation. Moreover, the impact of NS1 on other p53 biological functions needs to also be explored.

Acknowledgements

The authors would like to thank Katarzyna Jacob for her technical help during this study. The work of JCB was supported by Cancer Research UK and Breast Cancer Campaign charities. The work of OT and MRC was funded by the Université Lyon 1, Lyon, France.

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