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Research Article

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Involvement of the nucleolar protein Nucleophosmin (NPM1) in Human Respiratory Syncytial Virus replication

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Abstract

Human respiratory syncytial virus (hRSV) accounts for most lower respiratory tract infections during infancy and childhood. We previously demonstrated that the hRSV Matrix (M) protein interacts with the nucleolar protein Nucleophosmin 1 (NPM1) in HEK 293T cells. In this study we confirmed this interaction by using an in vitro pull-down assay. Knockdown of NPM1 with shRNA against NPM1 mRNA as well as the use of an NPM1 oligomerization inhibitor, affected hRSV proteins expression. These results indicate that the association of NPM1 with M is important for hRSV replication and is a possible target for antiviral strategies.

Key words: Human respiratory syncytial virus, hRSV, Matrix, Nucleophosmin 1, NPM1, B23.1, virus-host cell interaction

The Human Respiratory Syncytial Virus (hRSV) belongs to the order *Mononegavirales*, family *Pneumovirinae*, genus *Orthopneumovirus* [1]. It is an enveloped virus with a negative-sense single-stranded non-segmented RNA genome, and infects the respiratory tract with various manifestations, such as bronchiolitis and pneumonia. hRSV is the primary pathogen responsible for hospitalizations by acute lower respiratory tract infection, one of the leading causes of death in children under five years of age worldwide. Despite this, there is no approved vaccine or effective drug against hRSV. Its genome of about 15 Kb encodes 11 proteins. The nucleoprotein (N) associates with the viral RNA, phosphoprotein (P) interacts with N and with the RNA-dependent RNA polymerase (L) to form the nucleocapsid, three envelope proteins (F, G, SH), a matrix protein (M), a nucleocapsid-associated transcription factor M2-1, another protein involved in genome replication M2-2, the second product of the M2 gene, and two nonstructural proteins (NS1, NS2) [5].

M aligns itself with the inner surface of the viral envelope, interacting with F and G tails and with the nucleocapsid. It is related to the maturation of viral filaments, the organization of virion components in the plasma membrane and the trafficking of viral proteins to the budding site, being essential for the assembly of particles in the replication of hRSV [5, 17]. M localizes in the nucleus of infected cells early in infection through the action of the conventional nuclear transporter importin β1 [10]. It has been observed that chromatin association of M is critical to host transcriptional inhibition, and recombinant hRSV with M impaired for chromatin association shows decreased replication in cell culture, and reduced disease in BALB/c mice [19]. Later in infection, M traffics to the cytoplasm through the action of the host export protein CRM-1, where it inhibits viral transcriptase activity through its RNA binding activity and facilitates virus assembly by coordinating the activity of other hRSV components [9, 27]. Inhibition of M nuclear export impaired the final stages of infection and reduced viral infectivity [16]. The expression of M protein alone in lung cell cultures is related to a lower rate of cell proliferation compared to control cells. Analysis showed that M expression can induce cell cycle arrest in G1 and G2/M phases [2].

Nucleophosmin 1 (NPM1/B23.1) is an abundant phosphoprotein localized mainly in the nucleoli that undergo different phosphorylation events during the cell cycle. It is present in all cells and is a highly conserved protein [23]. NPM1 is biochemically defined as a histone chaperone, being necessary for the correct assembly and disassembly of nucleosomes [8]. NPM1 is found mainly in the granular region of the nucleolus, which is correlated with favoring ribosomal DNA transcription, potentially through its chaperone activity and, the processing of ribosomal RNA precursors [21]. It plays a vital role in the biosynthesis of ribosomes through its properties that ensure the proper transport of its components and preventing protein aggregation during their assembly. Moreover, NPM1 facilitates the transport of other proteins across the nuclear membrane, due to its ability to move between the nucleolus, nucleoplasm, and cytoplasm [4]. Recent studies have also highlighted that NPM1 is involved in additional nonclassical roles, including cell cycle regulation, DNA repair, cellular stress responses, and viral replication [3, 13]. Finally, it is becoming more and more apparent that different viruses targeting nucleolus proteins can induce significant alterations of NPM1 behavior. These alterations can participate in specific processes that are crucial for the outcome of virus infection, like viral DNA or RNA replication, virus assembly, and control of intracellular trafficking [12].

In a previous study we have shown that the NPM1 protein is co-immunoprecipitated with the hRSV Matrix protein, indicating their interaction in HEK 293T cells [24]. In this report, we have hypothesized that M

could recruit NPM1 to assist in the hRSV replication. To this end, we first confirmed this interaction by an in vitro pull-down assay. Moreover, we demonstrated that the expression of viral phosphoprotein P, essential for viral replication, is significantly inhibited in HEK 293 cells with the knockdown of NPM1. Furthermore, using an inhibitor of NPM1 oligomerization (NSC348884), we observed a decreased expression of all viral proteins. Therefore, the association of NPM1 with hRSV M protein may represent an essential mechanism for hRSV replication.

NPM1 was cloned in the pGEX-4T-3 vector and M was cloned in the pET28a vector, and were individually expressed, purified, and used for in vitro interaction tests. These plasmids were used to transform electro-competent bacteria *Escherichia coli* BL21 by electroporation. Collected colonies were seeded in 2 ml of LB as a pre-inoculum, which was then transferred to 200 ml of LB at 37°C and the culture was monitored until reaching an O.D. of 0.6. IPTG was added to a final concentration of 0.33 mM, followed by overnight incubation at 28°C. The bacteria were centrifuged, and the protein was extracted and purified from the pellet.

For the GST-fused NPM1 protein, GST-NPM1, from the pGEX-4T-3 vector, the purification system was based on the binding of the Glutathione S-Transferase (GST) protein to glutathione anchored to Sepharose Beads (GE Healthcare Glutathione Sepharose 4B Beads), which in the text we refer to as GST and GS-beads, respectively. The bacterial pellet was resuspended in 10 ml of lysis buffer [50 mM Tris pH 7.8; 2 mM DTT; 1 mM EDTA; 4 mM benzamidine; 0.2% Triton X-100 and one pill Roche® antiprotease cocktail (Serine and Cysteine proteases)], added 10 mg/ml lysozyme, and left at -20°C for 2 hours. After thawing, 2 µl, 2.5ku, of Novagen® benzonase was added and left under gentle agitation until obtaining a liquid consistency. This solution was centrifuged for 20 minutes at 3,000 x g. The supernatant was incubated with 1 ml of GS-beads in PBS pH 7.0 for 3-4 hours at room temperature or overnight at 4°C with agitation. Then, the beads were washed three times with lysis buffer, three times with PBS and stored at 4°C. For the 6His-tagged M protein (6xHis), His-M, from the pET28a vector, the bacterial pellet was resuspended in 10 ml of lysis buffer [20mM Tris pH 8.0; 500 mM NaCl; 10 mM Imidazole; 0.2% Triton X-100 and 1 Roche® antiprotease cocktail pill], added 10 mg/ml lysozyme, and left at -20°C for 2 hours. The same thawing and centrifugation protocol was performed until the supernatant was incubated with 1 ml of Nickel His-60 Ni Superflow™ Resin Clontech Laboratories, Inc. beads, and left under gentle agitation for 2 hours. The beads were washed twice with imidazole diluted in 20 mM Tris and 500 mM NaCl pH 8.0 buffer at three different concentrations: 10 mM, 50 mM, and 100 mM. After such washes, the beads were stored at 4°C.

The GST-NPM1 protein expressed and purified with GS-beads, was used complexed with the beads to study the interaction with M. The M protein was purified with nickel beads and eluted with 1 M of imidazole. It is worth mentioning that M protein in solution forms oligomers, precipitating with time. To keep the M protein soluble and available for interaction, it was ultracentrifuged at 100,000 x g for 5 minutes and held at 4°C. After centrifugation, the supernatant was mixed with the GST-NPM1 beads, in PBS containing RNA or BSA, incubated for 1 hour and washed three times with PBS. For negative controls, interaction with P or M2-1 viral proteins was tested. P and M2-1 genes were also cloned in the pGEX-4T-3 vector, and GST-P and GST-M2-1 beads were obtained as described for GST-NPM1 beads. In Fig. 1, it is possible to observe the interaction between the GST-NPM protein and the M protein, not with the negative controls.

Since the interaction between NPM1 and M proteins might be critical, we wondered if the knockdown of NPM1 would affect the replication of hRSV. To this end, HEK-293 cells were transfected with linearized pLKO and pLKO-shNPM1 (Sigma®) using Lipofectamine and PLUS reagents (Thermo Scientific®) to generate NPM1 knockdown cells. After 24 hours, cells were maintained in media containing puromycin (2 µg/mL) for two weeks to select a pool of resistant cells. After selection, HEK-293 cells with stable NPM1 knockdown were maintained with 1 µg/mL puromycin for infection with hRSV. As shown in Figs. 2a and 2b, cells transfected with the shRNA targeting the NPM1 protein mRNA showed significant inhibition of NPM1 ($p < 0.0001$). Knockdown of NPM1 markedly reduced the expression of the viral phosphoprotein P, an essential cofactor of viral RNA polymerase L, up to 24 hours after infection ($p < 0.01$) (Figs. 2c and 2d).

To determine whether the NPM1 oligomerization affects hRSV replication in infected HEp-2 cells, we used the small molecular inhibitor NSC348884 (Sigma®). This drug can impair the formation of NPM1 dimers, by binding to its globular domain of the N-terminal portion (residues 1-110) [25]. NSC348884, solubilized in DMSO (Merck®), was used in HEp-2 cells at concentrations of 2,5µM and 5µM. To measure the effect of the inhibitor on viral replication, Hep-2 cells were seeded in 6-well plates (80%-90% confluence). After 24 hours they were infected with hRSV. 24 hours post-infection the medium was changed to DMEM with 2% FBS medium and antibiotics, plus various concentrations of NSC348884. After 12 hours, cells were collected, and the extracts were analyzed. Fig. 3a shows the expression of NPM1, whose pattern is not modified by the presence of the drug compared to control cells (DMSO), since the drug does not interfere

in the expression of NPM1, but in its oligomerization [25]. After 24 hours of infection and treatment with the drug at concentrations of 2.5 μM and 5 μM, it was observed that the expression of viral protein P was inhibited, and the concentration of 5 μM was significantly more effective in promoting such inhibition ($p < 0.05$) (Fig. 3b and 3c). By observing such a pattern of inhibition of viral phosphoprotein P, a protein essential for the proper replication of hRSV, it was used an anti-RSV antibody, to observe if the inhibition by the drug would affect other viral proteins. In Fig 3d, we can see that the drug, in both concentrations, inhibits the expression of all hRSV viral proteins, suggesting that the budding process was also affected, since F and G proteins were also inhibited.

Although the nucleolus is traditionally the site of rRNA synthesis and ribosome assembly, over the last years it has become clear that the nucleolus plays a role in a wide range of critical cellular processes, including cell cycle control, apoptosis, mRNA maturation and regulation, transcription, DNA replication, and DNA repair [3]. The interaction between viruses and the nucleolus is a pan-virus phenomenon exhibited by DNA and RNA viruses. Many virus-encoded proteins traffic to and from the nucleolus, and several host cell nucleolar proteins are redistributed to other cellular localizations during the virus replication cycle [12, 13, 26]. Various studies have shown that NPM1 is redistributed to other cellular localizations during virus infection, such as the Japanese encephalitis virus [29], adenovirus [30], and Porcine circovirus type 3 [28]. In addition, several other viral proteins interact with NPM1, such as the NDV Matrix protein [7], the HIV-1 Rev protein and the HSV-1 US11 [22].

In the present study, we have confirmed that nucleolar protein NPM1 was identified as an hRSV M-interacting factor by in vitro GST-pull down. The in vitro interaction between these two proteins occurs better in the presence of BSA (Bovine Serum Albumin). However, this interaction also appears in the presence of RNA, with slightly lower efficiency (Fig. 1). This suggests that the domain of interaction between NPM1 and M is outside their domain of interaction with RNA [11, 27], as the RNA present in the lysate could not disrupt the interaction between them.

The critical role of NPM1 in hRSV replication was subsequently confirmed by the fact that NPM1 knockdown affected the regular expression of viral phosphoprotein P, a protein directly involved in viral genome replication (Fig. 2). According to our previous working hypothesis [24], NPM1 could be recruited by the hRSV M protein to assist in the assembly and budding of viral particles. However, we observed here that the inhibition of NPM1 interferes with the replication process itself, inhibiting the expression of P protein in infected cells. The involvement of NPM1 in hRSV replication was also confirmed by the fact that the NPM1 oligomerization inhibitor (NSC348884) treatment markedly reduced the levels of hRSV proteins expression (Fig. 3). Inhibition of NPM1 has been seen to disfavor viral replication of several other viruses [28, 30]. One of them, the Newcastle Disease Virus (NDV), uses M to disrupt the nucleolus and inhibit the action of NPM1 on ribosomal biogenesis and protein synthesis of host cells, and favor its replication [7].

hRSV replication has severe impacts on host mitochondrial function, which support infectious virus production [15]. hRSV replication also is promoted by autophagy-mediated inhibition of apoptosis, inducing cell survival [18]. Since at the beginning of infection hRSV M protein associates with host cell chromatin and interferes with host cell transcription [14], it may influence these processes. Thus, it can be suggested that the interaction between hRSV M and NPM1 has the possible function of interfering in the biogenesis of ribosomes, host gene transcription, cell survival, and cell cycle since NPM1 has been associated with participating in all these tasks [6, 20, 21]. However, further investigations would be necessary to prove this hypothesis.

In summary, the interaction of NPM1 with the hRSV M protein has been confirmed in this study. When its expression is inhibited or an inhibitor of its oligomerization is used, it significantly inhibits viral protein expression in infected cells, demonstrating the contribution of NPM1 to hRSV replication. Taken together, the results presented here provide essential information for further understanding the mechanism of hRSV replication, suggesting NPM1 as a potential target for the therapy of hRSV infection.

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Authors Contributions
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ana Beatriz Ruiz Afonso Barbosa, Andressa Peres de Oliveira, Fernando Riback Silva and Isadora Carolina Betim Pavan. The first draft of the manuscript was written by Ana Beatriz Ruiz Afonso Barbosa and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics declarations
Conflict of interest
The authors have no relevant financial or non-financial interests to disclose.
Ethical approval
This article does not contain any studies with human participants performed by any of the authors.

Figures and legends

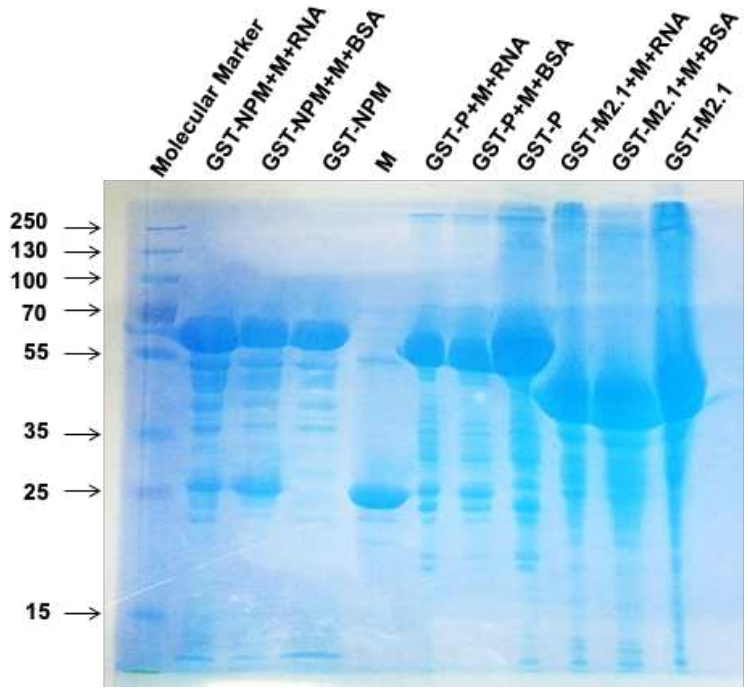

Fig. 1. In vitro interaction between M and NPM1. BL21 bacteria transformed with pGEX-NPM1, pGEX-P, pGEX-M2-1, or pET-M were induced with IPTG, and their extract was pulled down with GS-beads or nickel resin, respectively. Bound His-M protein was eluted with 1 M imidazole and mixed in buffer (PBS) containing RNA (tRNA from bovine liver, Type XI Sigma®, 10ug/ml) or BSA (Sigma®, 10ug/ml). These mixtures were subjected to a new pull down with the GS-beads complexed with GST-NPM1, GST-P or GST-M2-1. Analysis of proteins in 12% polyacrylamide SDS-PAGE stained with Coomassie Blue is shown. M protein has an apparent MW of 28 kDa.

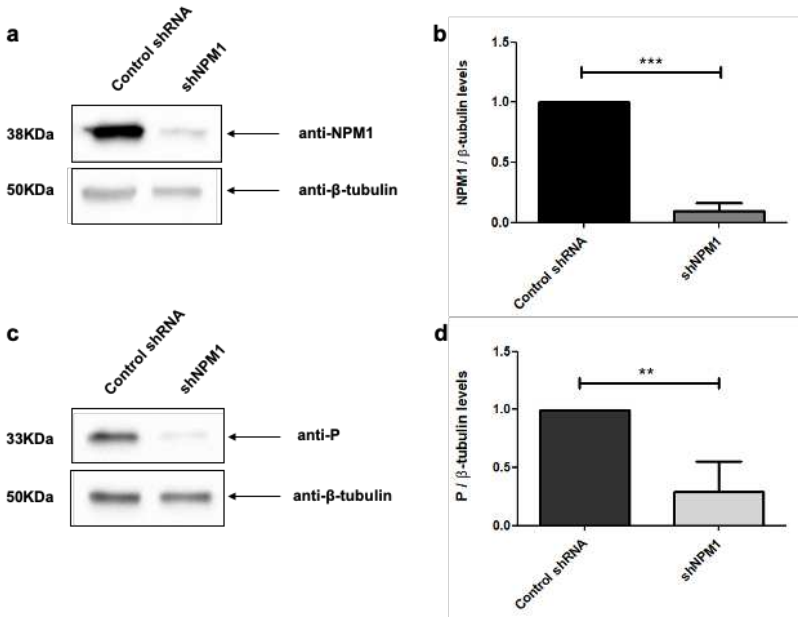

Fig. 2. Effect of NPM1 depletion on viral P protein expression. (a) Effect of NPM1 shRNA on the expression of endogenous NPM1. HEK-293 cells with stable NPM1 knockdown were maintained with 1 µg/mL puromycin. Cell lysates were examined by Western blotting with anti-NPM1 antibody (Sigma-Aldrich® 1:5,000). Endogenous β-tubulin expressions were used as internal control. (b) The relative levels of NPM1 in NPM1 shRNA-treated cells. The density of bands in (a) was quantified by densitometry. The relative levels of NPM1 were calculated from three independent experiments: the band density of NPM1/the band density of β-tubulin, with standard deviations indicated by error bars. (c) Effects of NPM1 shRNA on the expression of viral phosphoprotein P. HEK-293 cells with NPM1 knockdown and control were infected with hRSV at an MOI of 10. At 24 hours post-infection, the cell lysates were examined by Western blotting with anti-P antibody (Abcam® 1:1,000). Endogenous β-tubulin expression was used as an internal control. (d) Relative levels of P protein in NPM1 shRNA-treated cells. The density of bands in (c) was quantified by densitometry. The relative P levels were calculated from three independent experiments: the band density of P/the band density of β-tubulin, with standard deviations indicated by error bars. In (b) and (d) the significance of the difference was performed by the Independent-Samples T-test (***$p < 0.0001$ and **$p < 0,01$, respectively), using the GraphPad Prisma 5.03 software.

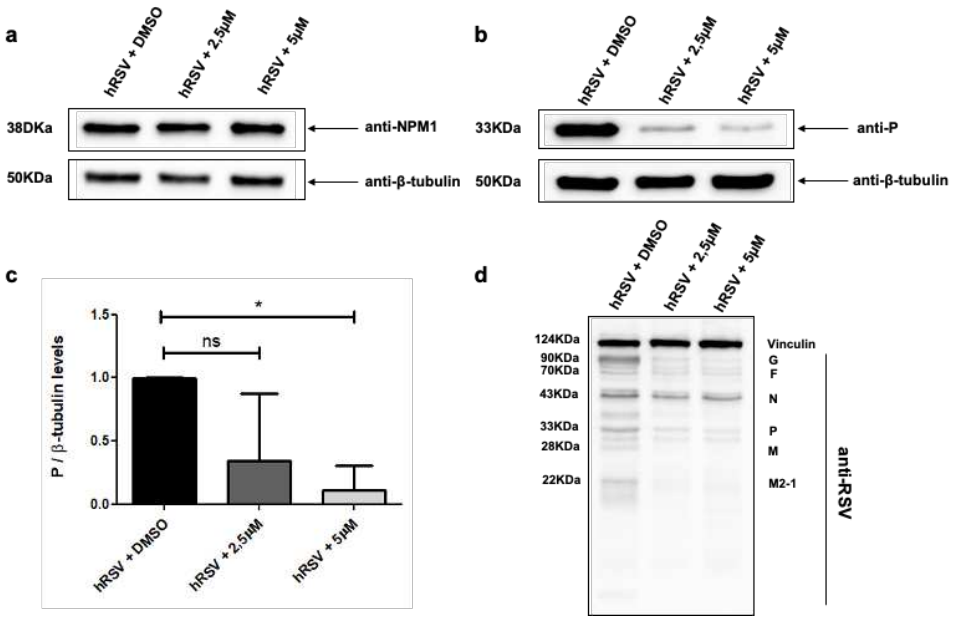

Fig. 3. Effects of NPM1 oligomerization inhibitor, NSC348884, on hRSV protein expression. (a) Effects of NSC348884 on the expression of endogenous NPM1. Lysates of control and treated cells with the indicated NSC348884 concentrations were examined by Western blotting with anti-NPM1 antibody. Endogenous β-tubulin expression was used as an internal control. (b) Effects of NSC348884 on the expression of viral phosphoprotein P. HEp-2 cells were infected with hRSV at an MOI of 10. At 24 hours post-infection the indicated concentrations of NSC348884 were added. After 12 hours, the cell lysates were examined by Western blotting with anti-P antibody (Abcam® 1:1,000). Endogenous β-tubulin expression was used as an internal control. (c) Relative P protein levels in cells treated with the drug. The density of bands in (b) was quantified by densitometry. The relative levels of P were calculated from three independent experiments: the band density of P/the band density of β-tubulin, with standard deviations is indicated by error bars. (d) Effect of NSC348884 on the global expression of viral proteins. HEp-2 cells were infected and treated as in (b). Cell lysates were examined by Western blotting with anti-RSV antibody (Abcam® 1:1,000). Endogenous vinculin expression is presented as an internal control. In (c) the significance of the difference was performed by the One-way ANOVA (Tukey's test) (*$p < 0,05$), using the GraphPad Prisma 5.03 software.