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Respiratory syncytial virus induces NRF2 degradation through a promyelocytic leukemia protein - ring finger protein 4 dependent pathway

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Abstract

Respiratory syncytial virus (RSV) is the most important cause of viral acute respiratory tract infections and hospitalizations in children, for which no vaccine or specific treatment available. RSV causes airway mucosa inflammation and cellular oxidative damage by triggering production of reactive oxygen species and by inhibiting at the same time expression of antioxidant enzymes, via degradation of the transcription factor NF-E2-related factor 2 (NRF2). RSV infection induces NRF2 deacetylation, ubiquitination, and degradation through a proteasome-dependent pathway. Although degradation via KEAP1 is the most common mechanism, silencing KEAP1 expression did not rescue NRF2 levels during RSV infection. We found that RSV-induced NRF2 degradation occurs in an SUMO-specific E3 ubiquitin ligase - RING finger protein 4 (RNF4)-dependent manner. NRF2 is progressively SUMOylated in RSV infection and either blocking SUMOylation or silencing RNF4 expression rescued NRF2 nuclear levels and transcriptional activity. RNF4 associates with promyelocytic leukemia - nuclear bodies (PML-NBs). RSV infection induces the expression of PML and PML-NBs formation in an interferon (INF)-dependent manner and also induces NRF2 – PMN-NBs association. Inhibition of PML-NB formation by blocking IFN pathway or silencing PML expression resulted in a significant reduction of RSV-associated NRF2 degradation and increased antioxidant enzyme expression, identifying the RNF4-PML pathway as a key regulator of antioxidant defenses in the course of viral infection.

Graphical Abstract

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DECLARATION OF INTEREST

Conflicts of interest: none

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Keywords

Respiratory syncytial virus; antioxidant enzymes; NRF2; SUMOylation; ubiquitination; PML nuclear bodies; KEAP1; RNF4

INTRODUCTION

Respiratory syncytial virus (RSV) is the most important cause of severe lower respiratory tract infections (LRTIs) in infants and in young children [1, 2]. RSV is also a primary cause of severe respiratory morbidity and mortality in senior adults [3], being responsible for 64 million clinical infections and 160,000 deaths annually worldwide [4]. Additionally, RSV is also associated with both the development and severity of asthma [5]. No vaccine and specific treatment available against RSV [4]. In previous studies, we found that RSV infection induces the rapid generation of reactive oxygen species (ROS) and oxidative stress, both *in vitro* and *in vivo*, associated with cellular damage, lung inflammation and clinical disease [6–8]. Antioxidant treatment significantly ameliorates RSV-induced oxidative stress, clinical disease and pulmonary inflammation in a mouse model of infection, indicating a causal relationship between increased ROS production and lung disease [9].

Unbalanced ROS production in the course of RSV infection is due to a progressive reduction in nuclear and cellular level of the transcription factor NF-E2-related factor 2 (NRF2), the primary regulator of antioxidant enzyme (AOE) gene transcription, resulting in decreased expression of AOE in airway epithelial cells (AECs)[10], as well as in RSV-infected mice and in children with LRTI [8]. RSV induces NRF2 deacetylation, ubiquitination, and degradation via the proteasome pathway both *in vitro* and *in vivo*, using a mouse model of infection. Histone deacetylase (HDAC) and proteasome inhibitors block NRF2 degradation and increase NRF2 binding to endogenous promoter ARE sites, resulting in increased AOE expression [11].

NRF2 is normally bound in the cytosol to an inhibitor called KEAP1 (Kelch-like-ECH associated protein 1). This association renders NRF2 inactive by shuttling it toward degradation by the ubiquitin-proteasome pathway [12]. Although KEAP1 is the major

regulatory protein ubiquitinating NRF2 through the Cullin3/RING box1/E3 ubiquitin ligase complex, other KEAP1-independent pathways regulating NRF2 degradation have been identified [13–15]. Our study shows for the first time that RSV-induced NRF2 degradation occurs via the SUMOylation-dependent ubiquitin ligase RING finger protein 4 (RNF4), which is associated with promyelocytic leukemia protein- nuclear bodies (PML-NBs). RSV infection induces the expression of PML and PML-NBs formation in an interferon (IFN)-dependent manner. Inhibition of PML expression and PML-NB formation results in a significant reduction of RSV-associated NRF2 degradation and in increased antioxidant gene expression, identifying the RNF4-PML pathway as a key regulator of antioxidant defenses in the course of RSV infection.

MATERIALS AND METHODS

RSV Preparation

The RSV Long strain was grown in Hep-2 cells and purified by centrifugation on discontinuous sucrose gradients as described elsewhere [16]. The virus titer of the purified RSV pools was 8–9 log₁₀ plaque forming units (PFU)/mL using a methylcellulose plaque assay. No contaminating cytokines were found in these sucrose-purified viral preparations [17]. LPS, assayed using the Limulus hemocyanin agglutination assay, was not detected. Virus pools were aliquoted, quick-frozen on dry ice/alcohol and stored at –80 °C until used.

Cell Culture and Infection of Epithelial Cells with RSV

A549 cells, a human alveolar type II like epithelial cell line (American Type Culture Collection, Manassas, VA) and small alveolar epithelial cells (SAECs)(Lonza Inc., San Diego, CA), normal human AECs derived from terminal bronchioli, were grown according to the manufacturer's instructions. RSV infection in A549 cells were done in F12K medium containing 2% FBS. When SAECs were used for RSV infection, they were changed to basal medium, not supplemented with growth factors, 6 h before and throughout the length of the experiment. At 90 to 95% confluence, cell monolayers were infected with RSV at multiplicity of infection (MOI) of 3. An equivalent amount of 30% sucrose solution was added to uninfected A549 and SAECs, as a control.

For Anacardic acid (10 µM) (172050, EMD Millipore, MA) and Cerdulatinib (5 µM) (PRT062070, Selleckchem, TX) experiments, cells were pretreated with the compounds for 1 h and then infected in their presence for the duration of the experiment. Equal amounts of diluent were added to cells uninfected and infected as control. Total number of cells and cell viability, following various treatments, were measured by trypan blue exclusion. There was no significant change in cell viability with all compounds tested. There was no effect of Anacardic acid on viral replication, while there was a trend in increased viral replication in cells treated with Cerdulatinib.

RNA Interference

KEAP1 (M-012453-00-0005), RNF4 (M-006557-03-0005), PML (M-006547-01-0005) and SUMO2/3 (M-016450-01-0005, M-019730-01-0005) expression was silenced using 25 to 50

nM of specific human siRNA sequences (siGENOME siRNA SMARTpool, Dharmacon). The sequences of siRNA are shown in following table.

siRNA SMARTpool	Sequence
KEAP1	CGAAUGAUCACAGCAAUGA, GGGCGUGGCGUCCUCAAU, CAGCAGAACUGUACCUGUU, GGACAAACCGCCUAAUUC
RNF4	CCAGGGACAGAGACGUAAU, AGAUAACCACAAACGGUA, GCAAUAAUUCUAGACAAG, GAAUGGACGUCUCAUCGUU
PML	GGGGAAAGAUGCAGCUGUA, GCAAAGAGUCGGCCGACUU, GCGCUGGUGCAGAGGAUGA, CCGAUGGCUUCGACGAGUU
SUMO2	GCGUCUUGUUGUUAAAUA, UUGCAUACCUUGUUAUUAU, GUACGUAGCUGUACAUGU, GGCAAUCUGUCUUAAGUA
SUMO3	GCAAGCUGAUGAAGGCCUA, GACAGAGAAUGACCACAUC, CAGAUCAGAUUCAGGUUCG, CAGUUAUGUUGUCGUGUAU
Non-Targeting	UAGCGACUAAACACAUCAA, UAAGGCUAUGAAGAGAUAC, AUGUAUUGGCCUGUAUUAUAG, AUGAACGUGAAUUGCUCAA

The siRNAs were transfected by electroporation system from Amaxa Biosystem, Gaithersburg, MD, which uses cell-type by using Amaxa Basic Nucleofector Kit (Cat # VPI-1005) for SAE cells and Amaxa Cell Line Nucleofector Kit T (Cat # VCA-1002) for A549 cells according to manufacturer instructions to allow high transfection efficiency. Non-Targeting sequences (siGENOME pool D-001206-13-05), Dharmacon) were used as negative control. At 48 h post-transfection, cells were infected with RSV and harvested at the indicated time points to prepare either nuclear extracts or to extract total RNA.

Western Blot

Nuclear extracts of uninfected and infected cells were prepared using hypotonic/nonionic detergent lysis, according to Schaffner protocol [18]. To prevent contamination with cytoplasmic proteins, isolated nuclei were purified by centrifugation through 1.7 M sucrose buffer B for 30 min, at 12,000 rpm, before nuclear protein extraction, as previously described [18, 19]. Protease inhibitor cocktail (EMD Millipore, MA), N-Ethylmaleimide (Sigma, MO), 3-Indoleacetic acid (Sigma, MO) were included in all buffers to preserve protein integrity and post translational modifications. Equal amount of proteins (20 µg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Nonspecific binding was blocked by immersing the membrane in Tris-buffered saline-Tween (TBST) blocking solution containing 5% skim milk. After a short wash in TBST, the membranes were incubated with the primary antibody overnight at 4 °C, followed by the appropriate secondary antibody diluted in TBST for 1 h at room temperature. Proteins were detected using enhanced-chemiluminescence assay. Densitometric analysis of band intensities was performed using UVP VisionWorksLS Image Acquisition and Analysis Software 8.0 RC 1.2 (UVP, Upland, CA). The primary antibodies used for Western blots were anti-NRF2 (ab62352, Abcam, MA), anti-KEAP1 (2729 Cell Signaling Technology, MA) anti-PML (ab179466, Abcam, MA) and anti-lamin B (GWB5CD4D4, GenWay Biotech, CA).

Immunoprecipitation

Immunoprecipitation was done by using Dynabeads Protein G Immunoprecipitation Kit (10007D, Thermo) according to the manufacturer's protocol. Briefly, 300 µg of nuclear

proteins from RSV-infected A549 cells with or without Anacardic acid or Cerdulatinib treatments were immunoprecipitated using 8 µg of anti-NRF2 antibody that were conjugated to protein G magnetic Dynabeads. Complexes were eluted in elution buffer provided in kit and mixed with 2x SDS PAGE buffer and subjected to Western blot analysis using anti-ubiquitin (SC-8017, Santa Cruz Biotechnology, Inc., CA) or anti-SUMO2/3 (ab81371, Abcam, MA) and anti-PML (ab179466 Abcam, MA) antibodies.

Confocal Microscopy

A549 cells ($\sim 2 \times 10^5$ cells per well) were seeded on coverslips in 12 well plates in F12K medium containing 10% FBS and incubated overnight at 37 °C. The cells were then uninfected or infected with RSV (MOI 3) for 16 h in the presence or absence of Anacardic acid (10 µM) or Cerdulatinib (10 µM) in F12K medium containing 2% FBS. The cells were fixed with 4% paraformaldehyde solution for 20 min at room temperature and then washed twice with PBS. The cells were blocked and permeabilized with 0.1% Triton X-100 and 3% BSA in PBS for 30 min at room temperature and washed. The cells were then incubated with primary antibodies – rabbit anti NRF2 (1:100 dilution) (abcam – ab62352) and mouse anti-PML (1:100 dilution) (abcam – ab179466) in 3% BSA in PBS for 1h at room temperature. The cells were washed three times with PBS and incubated with secondary antibodies – anti-rabbit Alexa Flour 488 IgG (H+L) (1:100 dilution) (molecular probes - A-11008) and anti-mouse Alexa Flour 594 IgG (H+L) (1:100 dilution) (molecular probes - A-11005) for 1 h at room temperature. The cells were washed three times with PBS, and the coverslips were mounted onto glass slides using a drop of ProLong Gold Antifade reagent with DAPI (Invitrogen), dried and kept overnight at 4 °C. Imaging was performed on a Zeiss LSM 510 Meta confocal laser scanning microscope at excitation/emission wavelengths of 488/505–550 nm (for green fluorescence), 561/570–620 nm (for red fluorescence), and 405/425–475 nm (for DAPI, colored purple). Pictures of the fluorescence patterns were merged to visualize colocalization. Image analysis done by ImageJ software version 1.51k downloaded at <https://imagej.nih.gov/ij> and for colocalization studies by Image J downloaded at <http://wwwfacilities.uhnresearch.ca/wcif/download.php>. PML and NRF2 colocalization were confirmed by Intensity Correlation Quotient (ICQ) analysis in by using Image J software. PDM values were calculated and ICQ were analysed for several fields. The ICQ is based on the non-parametric sign-test analysis of the PDM values and is equal to the ratio of the number of positive PDM values to the total number of pixel values. The ICQ values are distributed between 0.0 and +0.5 indicating both PML and NRF2 are co-localized together during RSV infection.

Real-Time PCR (RT-PCR)

Total RNA was extracted using ToTALLY RNA kit from Ambion (Cat # AM1910, Austin, TX). RNA samples were quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies) and quality was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Synthesis of cDNA was performed with 1 µg of total RNA in a 20 µl reaction using the reagents in the Taqman Reverse Transcription Reagents Kit from ABI (Applied Biosystems #N8080234), according to manufacturer's instructions. Q-PCR amplification was done using 1 µl of cDNA in a total volume of 25 µl using the Faststart Universal SYBR green Master Mix (Roche Applied Science #04913850001). The final

concentration of the primers was 200nM. 18S RNA was used as housekeeping gene for normalization. PCR assays were run in the ABI Prism 7500 Sequence Detection System.

Triplicate CT values were analyzed in Microsoft Excel using the comparative CT ($\Delta\Delta CT$) method as described by the manufacturer (Applied Biosystems). The amount of target (2- $\Delta\Delta CT$) was obtained by normalizing to endogenous reference (18S) sample. All the Primers

were ordered as PrimeTime qPCR primers from Integrated DNA Technologies (Coralville, IA) and the sequences are shown in following table.

Gene	Primer Sequence
Catalase	5'-GCCTTGGAGTATTTGGTAATGTC-3' 5'-ACCGAGAGAGAATTCCTGAGA-3'
SOD1	5'-TTAATGCTTCCCCACACCTT-3' 5'-CCTCGGAACCAGGACCT-3'
NRF2	5'-TGGCTTCTGGACTTGGAAAC-3' 5'-GACGGT ATGCAACAGGACAT -3'
PML (Mouse)	5'-CTGCTATCGTCCAACCTCGTG-3' 5'-AAAAGATCCTCCTGCCCAAC-3'

In vivo studies

10–12 weeks old female BALB/c mice were purchased from Envigo (Houston, TX) and were housed in pathogen-free conditions in the animal research facility of the University Texas Medical Branch (UTMB), Galveston, Texas, in accordance with the National Institutes of Health and UTMB institutional guidelines for animal care. Experiments were performed with a minimum of four to six animals/group. Under light anesthesia, mice were inoculated intranasally with 5×10^6 PFU of sucrose-purified RSV (Long Strain) in a final volume of 50 μ l diluted in phosphate buffered saline (PBS). Control animals (mock infected, defined as sham) received PBS only. Lungs samples from all groups were harvested at different times post-infection (p.i.) to prepare either total RNA, to quantify PML mRNA by RT-PCR, or nuclear proteins, as previously described [8]. Immunoprecipitation with anti-NRF2 antibody and Western blot using anti-SUMO2/3 antibody were performed as described above.

Statistical Analysis

All results are expressed as mean $\pm$ SEM. Data were analyzed using the GraphPad Prism 5 software. Results were compared among treatment groups by either one-way ANOVA analysis followed by Tukey's post-hoc test or two-way ANOVA analysis followed by Tukey's post-test. Significance was accepted at $p < 0.05$. To streamline figures, all significant results were reported as $p < 0.05$, although in many instances, it was well below that threshold.

RESULTS

Viral-mediated NRF2 degradation is KEAP1-independent

Although the role of KEAP1-Cullin 3 in NRF2 degradation through the proteasome pathway is well established, the mechanism of NRF2 degradation in RSV infection has not been fully investigated. Our recent observation that NRF2 degradation in response to infection occurs in A549 cells, a lung carcinoma-derived cell line which harbors a KEAP1 mutation that hampers its binding to NRF2, raised the question whether KEAP1 would be indeed responsible for the observed reduction in NRF2 cellular levels following RSV infection. Thus, to assess the putative role of KEAP1 in viral-mediated NRF2 degradation we used two distinct approaches, i.e., an siRNA strategy to silence KEAP1 expression in normal SAE cells, or embryonic fibroblasts derived from KEAP1 knock-out mice. For the first approach, SAE cells were transfected with KEAP1-specific or with non-target siRNAs, infected with RSV for 18 h, and harvested to prepare either nuclear extracts for WB analysis or total RNA. KEAP1 levels (by WB) were significantly reduced following transfection with specific siRNA, both in uninfected (Ctrl) and RSV-infected SAE cells (Figure 1A, **left and middle panels**). In uninfected SAE cells (Ctrl), treatment with KEAP1-specific siRNA resulted in significantly higher (4-fold) basal levels of nuclear NRF2, compared to non-target siRNA transfected cells. Infection with RSV caused a significant reduction in nuclear levels of NRF2, however the % reduction was similar in cells transfected with KEAP1-specific or with non-target siRNA, (65% vs 61%)(Figure 1A, **left and right panels**). A similar result was obtained in MEFs lacking KEAP1 expression, in which there was increased basal NRF2 levels and a similar or greater percentage of RSV-induced NRF2 degradation, compared to intact cells (Figure 1B). There was a minor decrease in NRF2 gene expression following RSV infection, which was not statistically different between treatment groups (Supplementary Material, Figure S1). In addition, mRNA levels of the NRF2 target genes catalase and SOD1 were significantly reduced in RSV-infected cells transfected with KEAP1 siRNA, to an extent similar to non-target transfected, infected cells (Figure 1C). Collectively, these results suggest that NRF2 degradation during RSV infection occurs through a KEAP1-independent mechanism.

In addition to KEAP1, other cellular pathways have been identified in controlling NRF2 degradation [13–15]. Among them, it has been shown that NRF2 may undergo SUMOylation in the nucleus and become a substrate of the ring finger protein (RNF)4, a small ubiquitin-like modifier (SUMO)-specific E3 ubiquitin ligase, targeting NRF2 for degradation [13]. To test the possible role of RNF4 in RSV-mediated NRF2 degradation, A549 cells (Figure 2A to C), and SAE cells (Figure 2D to F) were transfected with either RNF4-specific or non-target siRNAs, infected with RSV for 18 h, and harvested to prepare either nuclear extracts or total RNA. SiRNA treatment resulted in significant reduction of RNF4 mRNA levels in both A549 (Figure 2A) and SAE (Figure 2D) cells. Western blots analysis demonstrated a significant reduction in NRF2 nuclear levels (55 %) in RSV infected cells compared to control cells transfected with non-target siRNA. NRF2 nuclear levels in RNF4 siRNA transfected, uninfected cells were slightly higher (0.2 fold) compared to non-target transfected cells. Following infection with RSV, nuclear levels of NRF2 was restored to that of uninfected cells in RNF4 siRNA transfected cells (Figure 2B and E). There was a

minor decrease in NRF2 gene expression following RSV infection, which was not statistically different between treatment groups (Supplementary Material, Figure S2). In addition to these findings, mRNA levels of the NRF2 target genes catalase and SOD1 from RSV-infected A549 and SAE cells transfected with RNF4 siRNA were restored to the levels comparable to control cells (Figure 2CB and F). These results demonstrate that RNF4 plays a central role in the process of viral-induced NRF2 degradation.

As RNF4 ubiquitinate target proteins in a SUMO-dependent manner [13], we then investigated whether RSV infection was indeed associated with NRF2 SUMOylation. Nuclear proteins extracted from A549 cells uninfected and infected for various lengths of time were immunoprecipitated with anti-NRF2 antibody and subjected to WB using anti-SUMO1 or SUMO2/3 antibody. While we did not detect significant changes in SUMO1-NRF2 levels, RSV infection was associated with a significant increase in SUMO2/3-NRF2 starting as early as 3h p.i. (Figure 3A). To determine the role of SUMOylation in NRF2 degradation during RSV infection, we used a chemical inhibitor and a silencing approach by siRNA. A549 and SAE cells were infected with RSV for 18h in the presence or absence of the SUMO inhibitor Anacardic acid and harvested to prepare nuclear proteins or total RNA. Anacardic acid treatment rescued nuclear levels of NRF2 following RSV infection in both A549 and SAE cells (Figure 3B and D), and it was also able to restore expression of the NRF2 target genes catalase and SOD1 to the levels of uninfected control cells (Figure 3C and F). There was a minor decrease in NRF2 gene expression following RSV infection, which was not statistically different between treatment groups (Supplementary Material, Figure S3). A549 cells were transfected with either SUMO2/3 specific or with non-target siRNAs, infected with RSV for 18 h, and harvested to prepare nuclear extracts. Immunoprecipitation with anti-NRF2 antibody and Western blot analysis with anti-SUMO2/3 antibody showed there was more than two fold increase in SUMOylation of NRF2 during RSV infection in non-target treated cells, and that NRF2 nuclear levels were restored close to control levels in cells transfected with SUMO2/3-specific siRNA (Figure 3F). There was minor decrease in NRF2 gene expression in RSV infection and blocking SUMOylation restored to that of uninfected control cells (Supplementary Material, Figure S3). Collectively, these results indicate an important role of SUMOylation in RSV-mediated NRF2 degradation in airway epithelial cells.

NRF2 associates with PML following RSV infection

PML protein is a member of the TRIM family and a major component of PML-NBs. PML and NBs are involved in a wide variety of cellular processes, through facilitation of posttranslational modifications of partner proteins (notably SUMOylation), resulting in partner sequestration, activation, or degradation [20]. PML is primarily upregulated in infection by type I and type II interferons [21, 22]. Upon ROS exposure, NBs recruit RNF4, an event which is associated with ubiquitination of a variety of nuclear proteins which are then targeted for proteasome degradation [23]. To determine whether RSV infection could modulate PML expression, nuclear proteins from A549 cells infected with RSV for various lengths of time were analyzed by WB using anti-PML antibody. RSV infection was associated with a significant time-dependent increase in the expression of different PML isoforms, which are generated by alternative splicing [24](Figure 4A). In cell infected for 15

and 24 h, increased PML levels were associated with increased PML-NB formation, as shown by immunofluorescence and confocal microscopy analysis (Figure 4B).

To investigate whether PML and NRF2 interacted with each other, nuclear protein from A549 cells infected with RSV for 6 and 15 h were immunoprecipitated with anti-NRF2 antibody and subjected to WB using anti PML antibody. There was a significant association of both higher and lower molecular weight PML isoforms with NRF2 in the course of RSV infection, starting at 6 h p.i. (Figure 4C). Using immunofluorescence and confocal microscopy analysis with PDM value calculation, we also detected a significant increase in NRF2 and PML co-localization between 6 and 15 h p.i. (Figure 4D, showing 15 h p.i.).

Inhibition of PML-NB formation rescues NRF2 degradation and transcriptional activity

To investigate the role of PML and PML-NBs in NRF2 degradation, we used two different approaches, the first one based on inhibition of IFN signaling, which is required for viral-induced PML expression, and the second one based on direct inhibition of PML expression by siRNA. For the first approach, A549 cells were infected with RSV in the presence or absence of 5 μ M Cerdulatinib, an IFN receptor signaling inhibitor [25] and PML-NB formation was analyzed by immunofluorescence and confocal microscopy. Treatment with Cerdulatinib significantly reduced (>4 fold) RSV-mediated PML-NBs formation (Figure 5A), inhibited viral-induced nuclear PML expression and almost completely restored levels of NRF2 to those of control uninfected cells (Figure 5B). To further determine the role of PML in NRF2 degradation by direct inhibition of PML expression, A549 cells were transfected with either PML-specific or with non-target siRNAs, infected with RSV for 18 h, and harvested to prepare nuclear extracts. Western blot analysis showed that RSV infection induced NRF2 degradation in cells transfected with non-target siRNAs, while NRF2 nuclear levels were restored close to control levels in cells transfected with PML-specific siRNA (Figure 5C). There was a minor decrease in NRF2 gene expression following RSV infection, which was not statistically different between treatment groups (Supplementary Material, Figure S4 and 5). Restoration of NRF2 nuclear levels in cells treated with Cerdulatinib (Figure 5D) or transfected with PML siRNA (Figure 5E) was associated with rescue of the NRF2 target genes catalase and SOD1 in RSV-infected A549 cells.

Finally, to determine whether inhibition of PML-NB formation by blocking the interferon receptor signaling affected NRF2 SUMOylation and ubiquitination levels, nuclear protein prepared from A549 cells treated with Cerdulatinib were immunoprecipitated with anti-NRF2 antibody and subjected to Western blot using anti SUMO2/3 and ubiquitin antibodies. RSV infection induced a significant increase in both SUMOylation and ubiquitination of NRF2, which was significantly reduced by treatment with the interferon signaling inhibitor (Figure 5F). Collectively, these results indicate that PML-NBs are involved in RSV-mediated NRF2 degradation and disruption of PML-NBs rescues NRF2 nuclear levels and NRF2 dependent gene expression.

NRF2 is SUMOylated *in vivo*

To determine whether NRF2 is SUMOylated during RSV infection *in vivo*, we performed confirmatory experiments in our mouse model of RSV infection. Nuclear proteins isolated

from lungs of mice either PBS-inoculated or infected with RSV for 24h were subjected immunoprecipitation with anti-NRF2 antibody and analyzed by Westernblot with anti-SUMO2/3 antibody. Similar to our findings in A549 cells, RSV infection was associated with a significant increase in SUMOylation of NRF2, along with reduced NRF2 nuclear levels (Figure 6).

As PML seems to be central for NRF2 degradation, we determined whether PML expression was upregulated during RSV infection *in vivo* as well. Mice either PBS-inoculated or infected with RSV were sacrificed at day 1, 2 and 5 p.i. to isolate lung total RNA and PML mRNA levels were assessed by RT-PCR. Mice infected with RSV showed a very significant upregulation of PML gene expression on day 1 p.i. [which coincides with peak of type I IFN in the mouse model of RSV infection [26]] and it was sustained up to day 5 p.i., compared to PBS-inoculated mice (Figure 7). Together these findings support a role of the PML-RNF4 axis in NRF2 degradation *in vivo*, similar to what we have shown *in vitro*.

DISCUSSION

Respiratory syncytial virus (RSV), a single-stranded, negative-sense RNA virus of the *Pneumoviridae* family [according to the new classification of the order of *Mononegavirales* [27]], is the single most important cause of acute lower respiratory-tract infections (bronchiolitis and pneumonia) in children [1]. Each year in the US, 75,000–125,000 hospitalizations related to RSV occur among children aged <1 year, and RSV infection results in approximately 1.5 million outpatient visits among children aged <5 years [1, 28], with an annual economic burden estimated to be greater than \$500 million for hospitalizations, and a considerable additional amount for outpatient care [29]. No effective treatment or vaccine yet exists for RSV and immunity is incomplete, resulting in repeated attacks of acute respiratory tract illness through adulthood [2]. The clinical spectrum of RSV-induced disease in children ranges from a relatively mild “cold” to acute lower respiratory tract infections, which may be so severe to require mechanical ventilation (intensive care). Although our understanding of the mechanisms that determine the severity of lower respiratory tract infections caused by the virus is still incomplete, several recent studies have directly or indirectly indicated an important role of ROS produced by epithelial and inflammatory cells, and subsequent oxidative stress, in the pathogenesis of RSV infection, as inhibiting ROS production by administering antioxidants significantly decreases lung injury and improves clinical disease [9]. RSV infection induces decreased expression of AOE in human airway epithelial cells [10], as well as in RSV-infected mice and in children with severe lower respiratory tract infections (LRTI)[8], leading to cellular oxidative stress, both *in vitro* and *in vivo*. AOE gene transcription is regulated through binding of NRF2 to the antioxidant responsive element (ARE) site located in their promoters [30]. While other viruses associated with ROS production have been shown to induce ARE-dependent responses by activating NRF2, among them hepatitis B and C viruses, human cytomegalovirus, Kaposi’s sarcoma-associated herpes virus, and Marburg virus [31–36], we were the first to show that RSV infection is uniquely associated with a progressive reduction in NRF2 nuclear and cellular levels, leading to inhibition of ARE-dependent gene expression.

NRF2 is a basic leucine zipper transcription factor that is normally bound in the cytosol to an inhibitor called KEAP1 (Kelch-like-ECH associated protein 1). This association normally renders NRF2 inactive by shuttling it toward degradation by the ubiquitin-proteasome pathway. However, when ROS are generated, KEAP1 undergoes a conformational change and releases NRF2, which can then translocate to the nucleus to bind to antioxidant ARE or MAF recognition elements (MARE) and promote gene transcription [30]. Different from many pro-oxidative stimuli, including several virus infections [31–35], we showed that RSV infection is associated with increased NRF2 ubiquitination and degradation through the proteasomal pathway, and decreased acetylation [11], a post-translational modification that promotes DNA binding of NRF2, enhancing gene transcription [37], and it also regulates NRF2 cellular distribution, as deacetylating conditions results in relocalization of NRF2 to the cytoplasmic compartment [38].

Although KEAP1 is the major regulatory protein ubiquitinating NRF2 through the Cullin3/RING box1/E3 ubiquitin ligase complex, other KEAP1-independent pathways regulating NRF2 degradation have been identified [13–15]. One of them involves RNF4, in which nuclear NRF2 is a target for SUMOylation, and becomes substrate of RNF4, a SUMO-specific E3 ubiquitin ligase, targeting NRF2 for degradation [13]. Our data clearly show that RSV induces NRF2 SUMOylation and that NRF2 degradation occurs in a KEAP1-independent manner, as it occurs in A549 cells, which carry a KEAP1 mutation [39], in MEFs derived from KEAP1 $-/-$ mice and in small airway epithelial (SAE) cells lacking NRF2 expression by gene silencing. Inhibition of RNF4 expression in SAE cells by siRNA treatment significantly rescued RSV-induced NRF2 cellular levels indicating that NRF2 is targeted for degradation through the RNF4 pathway.

PML-NBs are membrane-free subnuclear compartments whose formation is initiated and controlled by PML protein, a member of the tripartite motif (TRIM) family, which is upregulated in viral infections by type I and type II interferons [21, 22]. PML is present in cells as several different isoforms, which differ mainly in either the coiled-coil region or the C-terminal end of the protein [40]. At the molecular level, PML proteins can multimerize to form the NBs outer shell, thus creating a partition within the nucleus. PML-NBs recruit multiple PML partner proteins that concentrate inside the bodies, providing an explanation for the involvement of PML in a variety of biological processes, including tumor suppression, apoptosis, cell cycle regulation, and antiviral activities [20]. Specifically, PML-NBs have been proposed to fine-tune and optimize protein–protein interactions and post-translational modifications, contributing in particular to protein SUMOylation [20]. Upon prolonged ROS exposure, NBs recruit RNF4, an event which is associated with ubiquitination of a variety of nuclear proteins which are then targeted for proteasome degradation [23]. Our results indicate that RSV infection induces PML expression and PML-NB formation, in an IFN-dependent manner. In the absence of IFN-dependent signaling, there is no induction of PML protein in response to RSV infection and there is significant reduction of NRF2 degradation, resulting in enhanced antioxidant gene expression.

Although IFN production in response to viral infections is important to induced a cellular antiviral state, RSV is known to be poorly responsive to IFN activity [41], whereas evidence of overexpression of IFN genes in lung and blood of infected children has been shown to be

associated with immune dysregulation, neutrophil inflammation and overall disease severity persisting even after the acute period of infection [42–44]. Our results identify an important cross-talk between IFN and NRF2 pathway in the course of RSV infection, with increasing IFN production/signaling leading to decreased NRF2 activation, which we found it occurs through a novel nuclear pathway. These findings provide initial experimental evidence that modulating the IFN pathway and/or PML-NB formation could represent a possible therapeutic intervention for RSV-induced oxidative lung damage and inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AEC	Airway epithelial cell
AOE	Antioxidant enzyme
ARE	Antioxidant response element
CBP	CREB-binding protein
HDAC	Histone deacetylase
IFN	Interferon
IP	Immunoprecipitation
KEAP1	Kelch like-ECH-associated protein 1
MEF	Mouse embryonic fibroblasts
NRF2	Nuclear factor erythroid 2-related factor 2
PML-NB	Promyelocytic leukemia protein - Nuclear bodies
RNF4	Ring Finger Protein 4
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SAEC	Small airway epithelial cell
SOD1	Superoxide dismutase 1
SUMO	Small ubiquitin-like modifier

Ub Ubiquitin

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Highlights

- RSV infection in lung cells induces
 - NRF2 degradation in a KEAP1-independent manner.
 - SUMOylation of NRF2, with the nuclear ubiquitin ligase RNF4 mediating NRF2 degradation.
 - PML expression via IFN, and blocking PML-NB formation rescues NRF2 degradation.

Figure 1.

(A) Nuclear protein prepared from SAE cells transfected with nontarget siRNA or KEAP1 siRNA, uninfected or infected with RSV for 18 h, were subjected to Western blot analysis with anti-NRF2 antibody. Membranes were stripped and reprobed with anti-KEAP1 and anti-Lamin B antibodies for loading control. The blots are representative of three independent experiments. Densitometric analysis of NRF2 band intensity is shown after normalization to Lamin B. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. (B) Nuclear protein prepared from wild type (WT) or KEAP1 knockout mouse embryonic fibroblasts, uninfected or infected with RSV for 24 h, were subjected to Western blot analysis with anti-NRF2 antibody. Membranes were stripped and reprobed with anti-Lamin B antibody for loading control. The blots are representative of three independent experiments. Densitometric analysis of NRF2 band intensity is shown after normalization to Lamin B. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. (C) SAE cells transfected with nontarget siRNA or KEAP1 siRNA, uninfected or infected with RSV for 18 h, were harvested to prepare total RNA. KEAP1 (left panel), catalase (middle panel) and SOD1 (right panel) gene expression were quantified by RT-PCR. Data are representative of three independent experiments. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM.

Figure 2.

A549 cells (A to C) and SAE cells (D to F) transfected with non-target siRNA or RNF4 siRNA, uninfected or infected with RSV for 18 h, were harvested to prepare total RNA or nuclear proteins. RNF4 (A and D), catalase and SOD1 (C and F) gene expression were quantified by RT-PCR, while NRF2 levels were analyzed by Western blot analysis (B and E). Membranes were stripped and reprobed with anti-Lamin B antibody for loading control. Densitometric analysis of NRF2 band intensity is shown after normalization to Lamin B. Data are representative of three independent experiments. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to nontarget transfected, infected cells.

Figure 3.

(A) Nuclear proteins of A549 cells infected with RSV for 3, 6 and 15 h were immunoprecipitated using anti-NRF2 antibody and subjected to Western blot using anti-SUMO2/3 antibody. Membranes were stripped and reprobed with anti-NRF2 antibody to determine the level of immunoprecipitated NRF2. Lower panel shows NRF2 Western blot of input proteins and Lamin B as loading control. The blots are representative of three independent experiments. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to uninfected control cells. Nuclear extracts from A549 cells (B) or SAE cells (D) uninfected or infected with RSV for 18h in the presence or absence of 10 μ M Anacardic acid were subjected to Western blot analysis using anti-NRF2 antibody. Membranes were stripped and reprobed with anti-Lamin B antibody for loading control. The blots are representative of three independent experiments. Densitometric analysis of NRF2 band intensity is shown after normalization to Lamin B. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to untreated, RSV infected cells. (C) A549 cells or (E) SAE cells uninfected or infected with RSV for 18 h in the presence or absence of 10 μ M Anacardic acid were harvested to prepare total RNA. Catalase and SOD1 gene expression were quantified by RT-PCR. Data are representative of three independent experiments. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to untreated, RSV infected cells. (F) Nuclear protein prepared from A549 cells transfected with nontarget siRNA or SUMO2/3 siRNA, uninfected or infected with RSV for 18 h, were immunoprecipitated using anti-NRF2 antibody and immune complexes were analyzed by Western blots using anti-SUMO2/3 and anti-ubiquitin antibodies. Membranes were stripped and reprobed with anti-NRF2 antibody to determine the level of immunoprecipitated NRF2.

Lower panel shows NRF2 Western blot of input proteins and Lamin B as internal control. Blots are representative of three independent experiments. Densitometric analysis of SUMOylated NRF2 and input NRF2 band intensities are shown. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * P < 0.05 relative to untreated RSV infected cells.

Figure 4.

(A) A549 cells were infected with RSV for 6, 15 and 24 h and nuclear protein were subjected to Western blot using anti-PML antibody. Membranes were stripped and reprobed with Lamin B for loading control. Densitometric analysis of PML band intensity is shown after normalization to Lamin B. The blots are representative of three independent experiments. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * P < 0.05 relative to uninfected control cells. (B) Immunofluorescence and confocal microscopy analysis of uninfected or RSV infected A549 cells (6–24h p.i.). Nuclei (DAPI), PML (red) and merged images are shown. Scale bar, 10 μ m. Image analysis was done by ImageJ software. The plotted graph represents total cell fluorescence (CTCF) signal intensity from 50 cells of six different fields. Data are shown as mean $\pm$ SEM. * P < 0.05 relative to uninfected control cells. (C) A549 cells were infected with RSV for 6 h and 15 h and nuclear protein were immunoprecipitated using anti-NRF2 antibody and subjected to Western blot using anti-PML antibodies. Two panels of different exposure times are shown for better visibility of higher and lower molecular weight PML bands. Membranes were stripped and reprobed with anti-NRF2 antibody to determine the

level of immunoprecipitated NRF2. Lower panel shows NRF2 Western blot of input proteins and Lamin B as loading control. The blots are representative of three independent experiments. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to uninfected control cells. **(D)** Immunofluorescence and confocal microscopy analysis of uninfected or RSV infected A549 cells (15h p.i.). Nuclei (DAPI), NRF2 (green), PML (red) and merged images are shown. Scale bar, 10 μ m. Enlarged image of cells showing co-localization of NRF2 and PML, as well as PDM image of the same cells, are shown.

Figure 5.

(A) Immunofluorescence and confocal microscopy analysis of A549 cells uninfected or infected with RSV for 18 h in the presence or absence of 5 μ M Cerdulatinib. Nuclei (DAPI), PML (red) and merged images are shown. Scale bar, 10 μ m. Image analysis was done by ImageJ software. The plotted graph represents total cell fluorescence (CTCF) signal intensity from 50 cells of six different fields. Data are shown as mean $\pm$ SEM. * P < 0.05 relative to untreated, RSV infected cells. (B) Nuclear protein prepared from A549 cells uninfected or infected with RSV for 18 h in the presence or absence of 5 μ M Cerdulatinib were subjected to Western blot analysis using anti-PML and anti-NRF2 antibody. Membranes were stripped and reprobed with anti-Lamin B antibody for loading control. The blots are representative of three independent experiments. Densitometric analysis of PML and NRF2 band intensities were shown after normalization to Lamin B. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * P < 0.05 relative to untreated, RSV infected cells. (C) A549 cells transfected with

nontarget siRNA or PML siRNA, uninfected or infected with RSV for 18 h, were harvested to prepare nuclear protein and subjected to Western blot analysis with anti-NRF2 and -PML antibody. Membranes were stripped and reprobed with anti-Lamin B antibody for loading control. The blots are representative of three independent experiments. Densitometric analysis of PML and NRF2 band intensities are shown after normalization to Lamin B. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to PML siRNA transfected cells to nontarget siRNA transfected cells. (D) A549 cells uninfected or infected with RSV for 18h in the presence or absence of 5 μ M Cerdulatinib were harvested to prepare total RNA. Catalase (left panel) and SOD1 (right panel) gene expression were quantified by real-time PCR. Data are representative of three independent experiments. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to untreated, RSV infected cells. (E) A549 cells transfected with nontarget siRNA or siRNAs for PML, uninfected or infected with RSV for 18 h, were harvested to prepare total RNA. Catalase (left panel) and SOD1 (right panel) gene expression were quantified by real-time PCR. Data are representative of three independent experiments. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to PML siRNA transfected cells to nontarget transfected cells. (F) Nuclear protein prepared from A549 cells uninfected or infected with RSV for 12 h in the presence or absence of 5 μ M Cerdulatinib were immunoprecipitated using anti-NRF2 antibody and immune complexes were analyzed by Western blots using anti-SUMO2/3 and anti-ubiquitin antibodies. Membranes were stripped and reprobed with anti-NRF2 antibody to determine the level of immunoprecipitated NRF2. Lower panel shows NRF2 Western blot of input proteins and Lamin B as internal control. Blots are representative of three independent experiments. Densitometric analysis of SUMOylated NRF2 and ubiquitinated NRF2 band intensities are shown. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to untreated RSV infected cells.

Figure 6.

Nuclear protein isolated from lungs of mice that were either PBS inoculated (P1–P4) or infected with RSV (R1–R4) for 24h were immunoprecipitated using anti-NRF2 antibody and subjected to Western blot using anti-SUMO2/3 antibody. Membrane was stripped and reprobed with anti-NRF2 antibody. Lower panel shows NRF2 Western blot for input of the IP. Lamin B was used as loading control. The blots are representative of three independent experiments. Densitometric analysis of SUMOylated NRF2 and input NRF2 is shown after normalization to Lamin B. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to PBS inoculated mice.

Figure 7.

Total RNA isolated from lungs of mice that were either PBS-inoculated or infected with RSV for 1, 2 or 5 days. PML gene expression was quantified by RT-PCR. Data are representative of three independent experiments. The groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data are shown as mean $\pm$ SEM. * $P < 0.05$ relative to PBS inoculated mice.