Supplementary

8-oxoguanine DNA glycosylase1 recognizes oxidatively-generated epitranscriptomic marks on nascent mRNAs to promote RSV replication

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Author contributions: K.W., W.H., and I.B. designed, performed, analyzed cell culture and animal experiments. L.P., X.Z. helped K.W to perform binding assays and immunoprecipitation. K.W., I.B., wrote the manuscript with help of A.C, AR.B. MG.G., R.B., assisted in production, purification and characterization of RSV M2-2 protein. T.B. generated OGG1 knock out hSAECs using CRISPR/Cas9 genome editing. R.G., A.C provided purified characterized suspensions of RSV virions and advised RSV infection of animals. A.B. R.Z. and S.V designed, performed, and analyzed protein-protein interaction assays. AR.B and X.B. advised RNA CLIP, and RNA-IP experiments. All authors discussed results and approved the content of the manuscript.

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Supplementary Figure 1

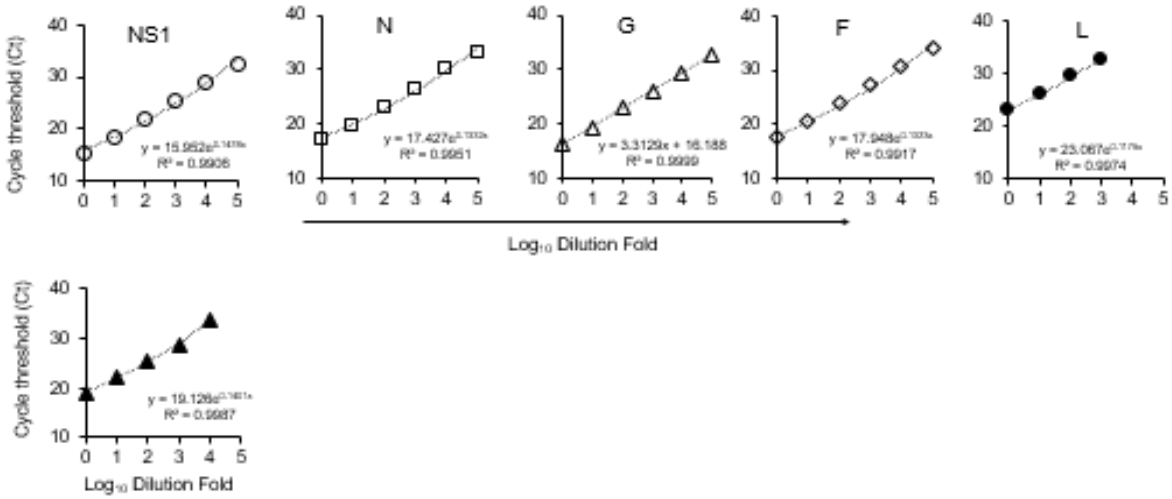


Fig S1 Revers transcription-coupled quantitative real-time polymerase chain reaction (RT-qPCR) amplification plots of target sequences for primer validation. RSV-infected (MOI = 0.1) hSAECs were harvested at 24h. Total RNAs were isolated using RNeasy Mini kit (Qiagen) and RNA samples (one µg) converted into cDNA by using oligodT or random primers to assess RSV mRNA and genomic RNA levels, respectively. For both mRNA and genomic RNAs ten-fold dilutions of the cDNAs were used to determine Ct values.

Upper panels: mRNA levels coding for NS1, non-structural protein; N, nucleoprotein; G, attachment glycoprotein G; F, fusion protein; L, viral RNA-dependent RNA polymerase. PCR primers for mRNAs are shown in Table 1.

Lower panel: Genomic RNA. Primers were designed to amplify sequences of inter-genome -- upstream from the attachment glycoprotein G and 3'-end of G gene. Genome coordinates 3' 4629 base to 4940 base. PCR primers are shown in Table 1.

Strength of the relationship between dilution and CT-values were estimated by a Pearson correlation coefficient. Each correlation coefficients (R2) and regression equation is shown.

Supplementary Figure 2

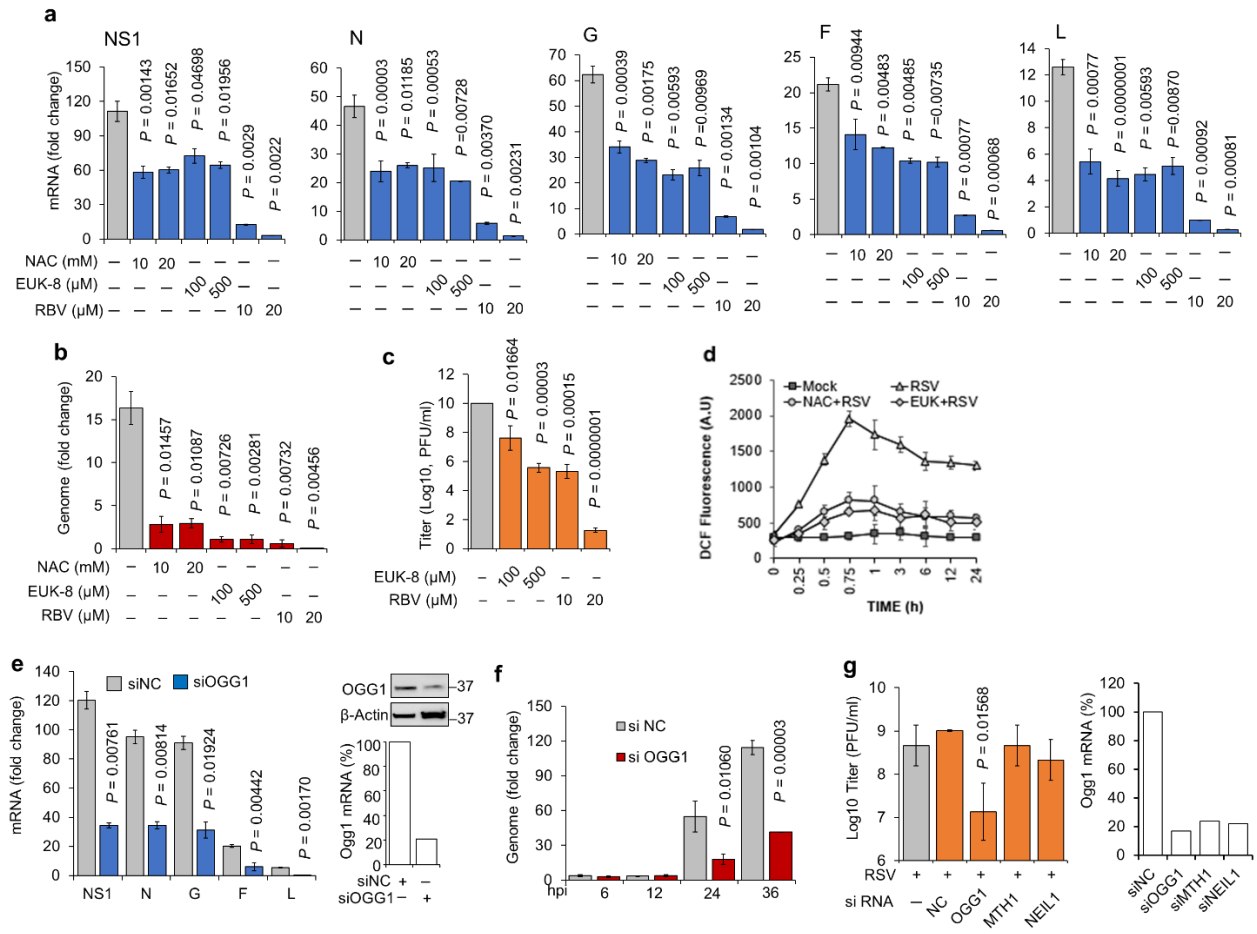


Fig. S2. Decrease in ROS levels by antioxidants and OGG1 deficiency lower RSV replication

(**a**) Decrease in viral mRNA levels in cells treated with antioxidants (AO). A549 cells were pretreated for 3h with NAC (10 or 20 mM) or EUK-8 (100 or 500 μM) and then were infected with RSV (MOI = 0.1). In controls, ribavirin was added at 10 or 20 μM concentration. At 2h cells were washed and growth media was added containing the corresponding AOs. At 2h and 24h post-exposure, cells were harvested and total RNAs were isolated using RNeasy Mini kit (Materials and Methods). One μg RNAs were converted into cDNA by using oligodT to assess RSV mRNA levels (see legend to supplementary Fig. 1). mRNA coding for NS1, non-structural protein 1; N, nucleoprotein, G, attachment glycoprotein G; F, fusion protein; L, viral RNA-dependent RNA polymerase was determined by qRT-PCR. Statistical significance was determined by Student's *t*-test (n = 3).

(**b**) Decreases in levels of ROS by antioxidants lowers genome replication. A549 cells were treated and infected as in legend to a. In controls, ribavirin was added at 10 or 20 μM concentration. Total RNAs were isolated as in legend to a and one μg RNAs were converted into cDNA by random hexamers. Primer pair was designed to specifically amplify genomic (antigenome) RNA, --sequences of inter-genome (upstream from attachment glycoprotein G) and part of G mRNA coding sequences (genome coordinates 4629 base to 4940 base). Statistical significance was determined by Student's *t*-test (n = 3).

(**c**) EUK-8 decreases yield viral progeny similar to ribavirin. A549 was pre-treated with EUK-8 (100, 500 μM) or RBV (10, 20 μM) as in legend to a, and infected with RSV (MOI = 0.01). At 48h post-infection, cells were snap-frozen (-80 ^{o}C), virus suspension was clarified by centrifugation and RSV yield in supernatant fluids were determined by plague assays (Materials and Methods). Statistical significance was determined by Student's *t*-test (n = 3).

(**d**) Kinetic changes in RSV induced ROS levels ±antioxidants. Parallel cultures of hSAECs were mock- or pretreated for 3h with NAC (20 mM) or EUK-8 (500 μM) and then were infected with RSV (MOI = 1). At the indicated time points after RSV addition 10 μM CM-H2DCF-DA added for 15 min, cells were washed, and lysed. Cell lysates were clarified by centrifugation and DCF fluorescence in supernatant fluids were determined by using a Synergy H1 Hybrid Multi-Mode Reader (BioTek) with excitation / emission at 485 nm / 535 nm. Results are expressed as change in fluorescence units.

(**e**) Silencing of OGG1 expression lower mRNA levels of RSV. OGG1 was depleted by a cocktail of silencing RNAs in A549 cells, then infected with RSV (MOI = 0.1). Total RNAs were isolated at 24 post-infection and cDNAs were prepared using oligodT primers. Changes in mRNA levels of NS1, N, G, F and L were determined by qRT-PCR. Changes are expressed as fold, compared to 2 h post RSV addition. Right panel, extent of OGG1 depletion as shown by qRT-PCR and Western blotting. NS1, non-structural protein 1; N, nucleoprotein, G, major attachment glycoprotein G); F, fusion protein; L, viral RNA-dependent RNA polymerase. *P* values are calculated using Student's t-test (n=3).

(**f**) Silencing OGG1 expression decreased viral genome level. A549 cells were OGG1 depleted using a cocktail of silencing RNAs, then infected with RSV (MOI = 0.1). Total RNAs were isolated at 2, 6, 12, 24, and 36h post-infection. One ug RNAs per samples were converted into cDNAs using random primers. Changes in levels of genomic vRNA was determined by qRT-PCR, using primer pair that amplifies sequences of inter-genome (upstream from the major attachment glycoprotein G gene) and 3'-end of G gene (genome coordinates 3' 4629 base to 4940 base). Statistical significance was determined by Student's *t* - test (n = 3).

(**g**) OGG1 depletion, but not other glycosylases, inhibits production of RSV progeny. OGG1, MTH1, NEIL1 was depleted by corresponding cocktail of silencing RNAs in A549 cells, then infected with RSV (MOI = 0.1). At 48h post-infection, cells were snap-frozen (-80 ^{o}C), thawed and virus suspension was clarified by centrifugation. RSV yield in supernatant fluids was determined by plague assays (Materials and Methods). Right panel, extent of gene silencing as shown by qRT-PCR. Statistical significance was determined by Student's *t*-test (n = 3).

Supplementary Figure 3

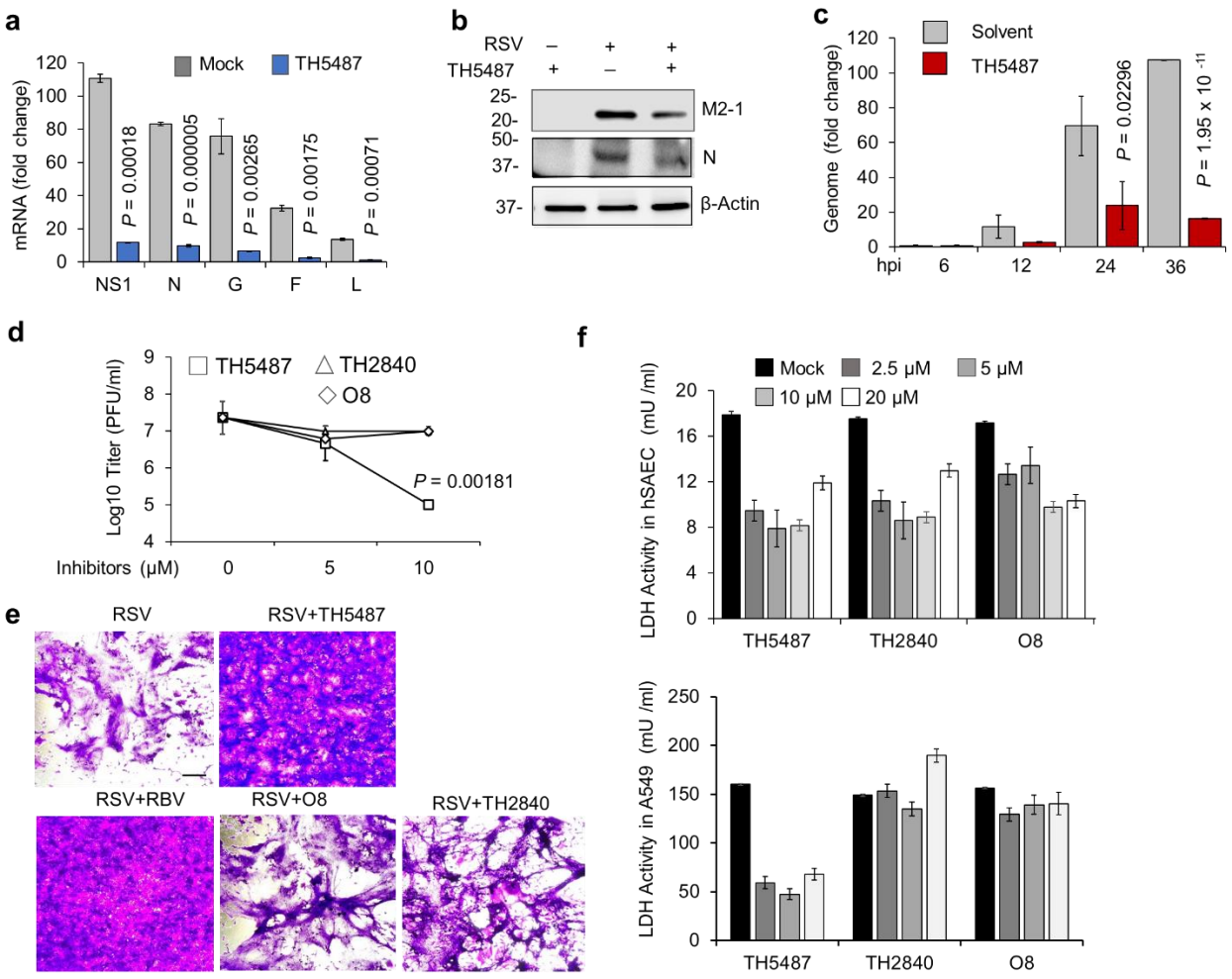


Fig. S3 Inhibition of OGG1' binding to viral RNAs via 8-oxo(r)Gua decreases output of RSV infection.

(**a**) OGG1 inhibitor TH5487 decreases viral mRNA level. A549 cells were infected with RSV (MOI = 0.1). Inhibitors were added at -2h (prior to), after RSV absorption (+2h) and every 8 h thereafter. Total RNAs were isolated at 24 post-infection and cDNAs were prepared using oligodT primers. Changes in mRNA levels were determined by qRT-PCR and changes are expressed as percentage of RNA yield compared to mock-treated control. mRNA coding for NS1, non-structural protein 1; N, nucleoprotein, G, attachment glycoprotein G; F, fusion protein; L, viral RNA-dependent RNA polymerase. *P* values are calculated using Student's t-test (n=3). (**b**) Inhibitor of OGG1' substrate binding decreases levels of M2-1 and N proteins. A549 cells were RSV-infected (MOI = 1) and treated with 10 μM TH5487 as in legend to a. At 24h post-infection cells were lysed and M2-1 and N protein levels were determined by Western blot

analysis. Representative images of Western blots from mock and TH5487-treated cells are shown. Images are representative of two biological replicates.

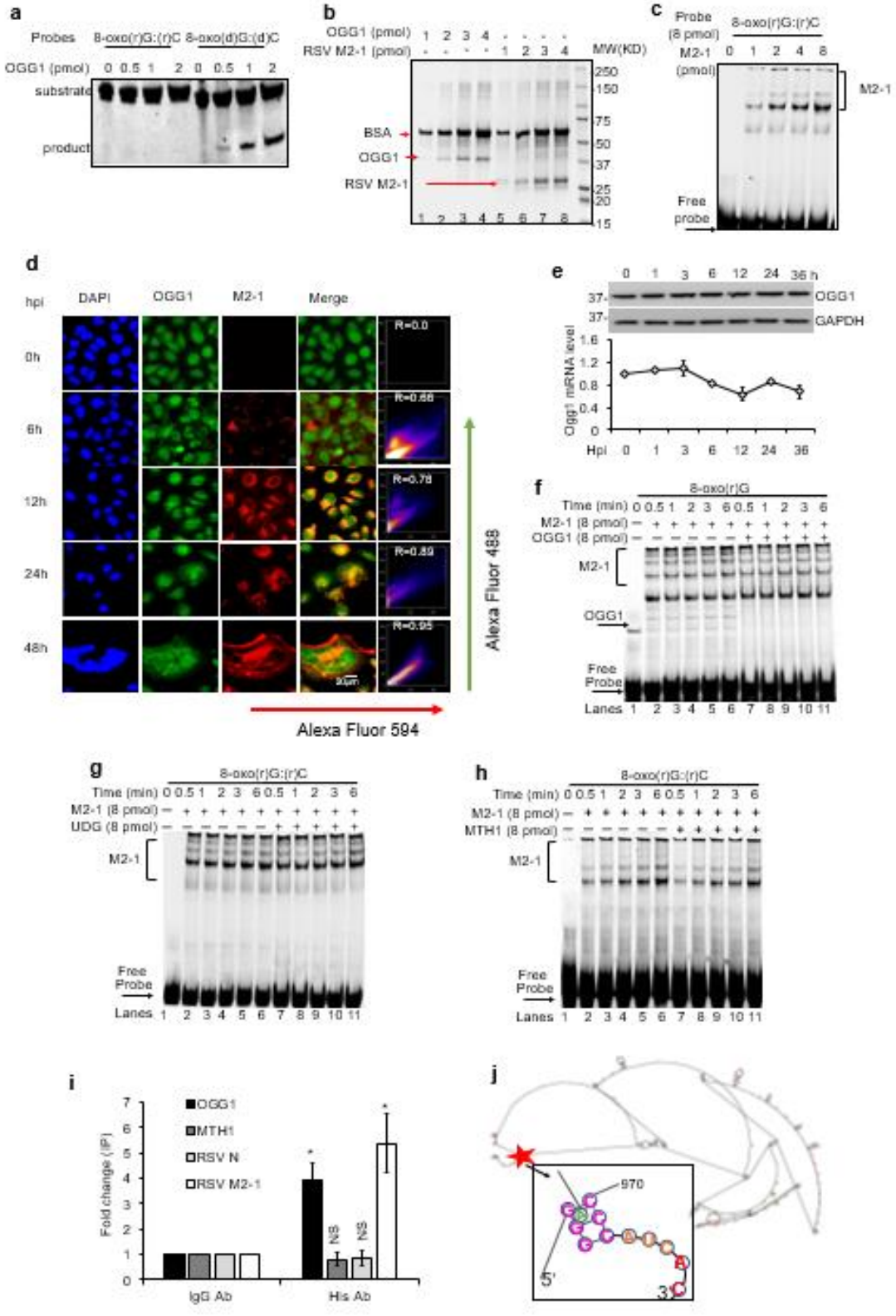
(**c**) Inhibition of OGG1 decreases viral genome level. A549 cells were RSV-infected (MOI = 0.1) and treated with TH5487 (10 μM) as in legend to a. Total RNAs were isolated at 2, 6, 12, 24, and 36h post-infection. One μg RNAs per samples were converted into cDNAs using random primers. Changes in levels of genomic vRNA were determined by qRT-PCR using primer pair that amplifies sequences of inter-genome (upstream from the attachment glycoprotein G) and 3'-end of G gene (genome coordinates 3' 4629 base to 4940 base). Statistical significance was determined by Student's *t*-test (n = 3).

(**d**) Inhibition of OGG1 binding to 8-oxo(r)Gua but not OGG1' glycosylase activity decreased RSV yield. A549 cells were RSV-infected (MOI = 0.1) and treated with 5 or 10 μM TH5487 as in legend to a. In controls, similar concentrations of TH2840 (an inactive analog of TH5487), and O8 (inhibits OGG1 glycosylase activity) was used. At 48h post-infection virus titers were determined by plague assays (Materials and Methods). Statistical significance was determined by Student's *t*-test (n = 3)

(**e**) Inhibition of OGG1' substrate binding but not its glycosylase activity decreases RSV-induced cytopathology. A549 cells were RSV-infected and treated as in legend to a. At 48h post-infection cells were formalin fixed and stained with crystal violet. Representative images were photographed by using an OLYMPUS Microscope System BX53P, with a built-in digital CCD color camera (DP73WDR). Magnification: 20X; scale bar: 100 μm.

(**f**) Toxicity of OGG1 inhibitors as determined by release of lactose dehydrogenase (LDH). hSAEC and A549 cells were treated with increasing concentrations (2.5, 5, 10, 20 μM) of OGG1 inhibitors (TH5487, O8) or TH2840 (an inactive analog of TH5487) at time 0 and 8h intervals thereafter. Levels of LDH in supernatant fluids of cells was determined at 48h time point as described to Materials and Methods. hSAECs (upper panel), A549 cells (lower panel).

Supplementary Figure 4



(**a**) **OGG1 lacks enzymatic activity on dsRNA**. OGG1 was mixed with 8-oxoGua containing 8-oxo(r)Gua:(r)C (dsRNA) or 8-oxo(d)Gua:(d)C (DNA) probes and cleavage assays were performed. Assays were terminated by adding loading buffer (formamide, NaOH). The cleaved product was separated from the intact probe on a 15% polyacrylamide gel containing 8 M urea in Tris-borate-EDTA buffer (pH 8.4). Bands were visualized by using Amersham™ Imager 680. Lanes 1-4, 8-oxo(r)Gua:(r)C (RNA probe): /5'-Cy5/-rCrArArArArUrGrGrArGrGrUrUrArArUrArU /8oxorG/rGrGrArArArUrGrArUrGrGrArA-3'). Lanes 5-8: 8-oxo(d)Gua:(d)C (DNA probe): /5'-Cy5/-dAdGdAdGdAdAdGdAdAdGdAdAdGdAdAdGdAdA/8oxodG/dAdGdAdTdGd GdGdTdT dAdTdTdCdGdAdAdCdTdAdGdC-3').

(**b c**) **Quality control of recombinant M2-1 protein**. His-tagged M2-1 was expressed locally from sequence-optimized RSV M2-1 gene cloned into pET28a and purified as in Materials and Methods. The purity of His-M2-1 recombinant protein was confirmed by spectrophotometry (OD 260nm/280nm ratio (Materials and Methods) and PAGE electrophoresis (lanes: 5,6,7,8). In parallel, increasing amounts of OGG1 (ProSpec) show a single band (b). Concentration dependent binding of M2-1 to viral RNA probe (c). Single stranded 3'-Cy5-tagged RNA (8-oxo(r)Gua) probe (8 pmol) was incubated with increasing amounts of M2-1 (Materials and Methods). M2-1 binding was determined by EMSA. Bands were visualized by using Amersham™ Imager 680. Lane 1: probe only; lanes 2, 3, 4 and 5: 1, 2, 4, 8 pmol M2-1, respectively.

(**d**) **Co-localization of OGG1 and M2-1 *in vivo***. A549 infected with RSV (MOI = 1) was fixed at 0, 6, 12, 24 and 48 h), then IF assays were performed using OGG1 and M2-1 Abs. Randomly selected fields were photographed using an OLYMPUS Microscope System BX53P, with a built-in digital CCD color camera (DP73WDR). Magnification: 40X; scale bar: 20 μm. Most-right column: Pearson' fluorophores-moment correlation coefficient (Alexa 594 and Alexa 488) was performed using Image J v1.51 software (Materials and Methods).

(**e**) **There are no changes in overall expression of OGG1 at RNA and protein level after RSV infection**. A549 cells were RSV infected (MOI = 1) for various length of time (1, 3, 6, 12, 24 and 36 h), then mRNAs and proteins level of OGG1 were determined using RT-PCR and Western blotting, respectively.

(**f**) **OGG1 has no effect on M2-1 binding to single stranded viral RNA**. For each assay, 8 pmol 5'-Cy5-labeled probe was incubated with or without OGG1 on ice then 8 pmol M2-1 was added for increasing length of time (0.5, 1,2,3,6 min) in binding buffer (Materials and Methods) then EMSA was performed. M2-1 binding was visualized by using Amersham™ Imager 680.

(**g h**) **MTH1 or UDG has no effect on M2-1 binding to double stranded viral RNA**. MTH1 or UDG was incubated with 8 pmol 3'-Cy5-labeled dsRNA probe on ice then 8 pmol M2-1 was added for increasing length of time (0.5, 1,2,3,6 min) in binding buffer then EMSA was performed (Materials and Methods). M2-1 binding was visualized by using Amersham™ Imager 680.

(**i**) **OGG1 and M2-1, but not MTH1 or N protein interacts with viral mRNA**. mRNAs isolated form RSV-infected (MOI = 1, for 12h) cells were mixed with His-tagged OGG1, M2-1, MTH1 or N protein in binding buffer and IP-ed using Ab to His. mRNA level in IPs were determined by qRT-PCR (n = 3).

(**j**) **A secondary structure of G-mRNA**. mRNA sequence of the attachment glycoprotein G was submitted to "*RNAstructure*" version 6.2 (http://rna.urmc.rochester.edu/RNAstructure.html) software (Zhou and Routh 2020) to predict base pairing probabilities. Inset: 5-end G mRNA of secondary loop structure is enlarged and potential engagement of OGG1 with 8-oxo(r)Gua in 5-UTR is shown.

Zhou, Y. & Routh, A. Mapping RNA-capsid interactions and RNA secondary structure within virus particles using next-generation sequencing. *Nucleic Acids Res* **48**, e12, doi:10.1093/nar/gkz1124 (2020).