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

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49 **Conflict of interest**

50 The authors declare that no conflict of interest exists

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Abstract

Respiratory syncytial virus (RSV) infection induces an oxidizing environment linked to increased viral load, expression of pro-inflammatory genes, and excessive lung inflammation. The mechanisms of how reactive oxygen species (ROS) promotes viral gene expression have remained largely elusive. Here we show that nascent (n)RNAs of RSV acquire 8-oxo-7,8-dihydroguanine (8-oxo(r)Gua) -a covalently modified guanine base in their 5'-UTR peritranscriptionally, while paired with the 3'-terminus of viral gene(s). 8-oxo(r)Gua is bound by 8-oxoguanine DNA glycosylase1 (OGG1), a complex that physically interacts with and recruits the anti-terminator protein M2-1 to increase viral gene transcription. Knockdown of OGG1 (but not other DNA glycosylases) or inhibition of its binding, significantly decreased RSV mRNA, protein levels and yield of progeny in cultured cells and airways. Collectively, these data suggest that Gua oxidation in vRNA, serves as an epitranscriptomic mark that repurposes OGG1 to increase lytic viral replication. Pharmacological inhibition of OGG1 binding to the epitranscriptomic mark could have clinical utility to decrease manifestations of RSV infection.

Introduction

Respiratory syncytial virus (RSV) is one of the most frequent causes of severe lower respiratory tract infections¹. Worldwide, RSV infection is responsible for upper/lower clinical manifestations in nearly all children under age two, resulting in over 3 million hospitalizations and nearly 200,000 in-hospital deaths². In the U.S., more than 100,000 children are hospitalized yearly due to severe consequences of RSV infection, making it the most frequent disease among hospitalized infants³. Re-infections with RSV may occur throughout life and are mostly limited to the upper airways. However, in immunocompromised adults and the elderly, RSV infection spreads into the lower airways, producing severe bronchiolitis and pneumonia, respiratory morbidity and mortality^{4,5}. RSV infections produce clinical impact similar to that seen with influenza, rhino-, and coronaviruses^{2,6}. No effective vaccine or drug is currently available, and patient's treatment is limited to supportive medical care⁷.

RSV infects airway epithelial cells by binding cellular receptors, producing envelope fusion with the plasma membrane, delivering nucleocapsids into the cytoplasm^{8,9}. Once internalized, the negative-sense viral genome encodes for ten mRNAs regulated by 3' and 5' noncoding sequences, transcribed by the RNA-dependent RNA polymerase complex [RdRp; large polymerase (L), phosphoprotein (P)], that are translated into eleven proteins¹⁰. RdRp initiates transcription and replication of nascent (n)RNA transcripts using the same genomic template including vRNA capping (via the conserved region V of the L protein), and polyadenylation¹⁰⁻¹². RdRp recognizes the gene start (GS) and end (GE) of each gene on the genome, guiding mRNA synthesis^{13,14}, which is modulated by the anti-terminator matrix protein 2 (M2-1) also called the transcription elongation factor¹⁵. The primary cellular sites of RSV transcription and replication are cytoplasmic inclusion bodies (IBs)¹⁶.

Efficacy of vRNA transcription and replication are also dependent on cellular factors¹⁷. For example, host cytoskeletal, membrane, heat-shock proteins or those involved in protein trafficking have all been associated with RSV replication¹⁸⁻²⁰. Additionally, cellular enzymes, including methyl- and acetyl-transferases, deposit/induce covalent base modifications to vRNAs (epitranscriptomic marks; e.g., *N*6-methyladenosine (m6A), 5-methylcytidine (m5C), or *N*4-acetylcytidine)²¹⁻²⁴. Other types of covalent modifications include 5 - ribosyl uracil (pseudo-uridine) and inosine also generated enzymatically; however, there are no "reader" or "eraser" enzymes identified to date²⁴. Epitranscriptomic marks facilitate various steps in viral replication, viral mRNA stability, translation, and/or shield viral RNAs from recognition by host RNA-specific innate receptors^{21,22,25}. Similar epitranscriptomic modifications control viral latency and lytic replication of DNA viruses²⁶.

104 RNAs are 10-25-times more susceptible to oxidation than DNA *in vivo* due to their
105 single-stranded nature, their lack of or partial association with protecting proteins, and their
106 close proximity to sites of ROS generators²⁷⁻²⁹. In an oxidizing cellular milieu, ROS primarily
107 attack guanine (Gua) heterocycles due to their having the lowest oxidation potential among
108 nucleic acid bases³⁰. Gua oxidation results in 8-oxo-7,8-dihydroriboguanine (8-oxo(r)Gua) or 8-
109 oxo-7,8-dihydrodesoxyguanine (8-oxo(d)Gua) in RNA and DNA respectively³¹. The mutagenic
110 8-oxo(d)Gua lesions are removed from DNA by 8-oxoguanine DNA glycosylase³², however, in
111 RNA it may affect codon-anticodon pairing during translation, or when in excess, induce
112 apoptosis through cellular RNA surveillance mechanisms^{28,33}. In vRNA, oxidation at Gua is
113 unexplored; however, the addition of antioxidants or inhibition of ROS production significantly
114 lowers RSV as well as human metapneumo-virus (HMPV) replication³⁴⁻³⁷, raising the potential
115 for 8-oxo(r)Gua playing roles in vRNAs.

116 The aim of the present study was to examine the role(s) of oxidative base modification(s)
117 particularly to Gua in the RSV transcriptome and understand its consequences. Our results
118 show that RSV infection-induced ROS co-transcriptionally generated 8-oxo(r)Gua in the 5'-
119 UTRs of nascent vRNAs while paired with the 3'-end of genes. 8-oxoguanine DNA
120 glycosylase1 (OGG1), an oxidized Gua base specific DNA repair protein^{32,38}, physically
121 interacted with 8-oxo(r)Gua in vRNAs and recruited the RSV-encoded transcription elongation
122 factor M2-1 to promote mRNA synthesis. Knockdown of OGG1 (but not other repair proteins) or
123 inhibition of OGG1 binding to the oxidatively modified Gua(s) decreased viral mRNA, protein
124 levels as well as yield of RSV progeny (100 to 1000-fold) in both airways and cultured cells.
125 These data imply that RSV has adopted cellular biosynthetic machinery via Gua oxidation in
126 vRNA as an epitranscriptomic marker to recruit the cellular reader, OGG1 for efficient virus
127 replication. Our findings not only provide a new mechanistic understanding the effects of ROS
128 on vRNA transcription, but point to the potential utility of OGG1 inhibitors in drug development
129 for clinical purposes.

Results

Oxidatively modified guanine base lesions in viral mRNAs

Given the profound effect of oxidative stress on RSV replication, pathogenesis, as well as vulnerability of Gua(s) to ROS^{28,36}, we examined oxidative modification to vRNA by assessing Gua base modification(s). The immortalized human small airway epithelial cell cultures (hSAEC –a cell type target of RSV infection involved in disease pathogenesis^{39,40}) were infected with RSV and harvested to isolate RNAs using buffers containing the iron chelator desferioxamine (DFO) to prevent RNA oxidation⁴¹. Purified RNAs were incubated with 8-oxo(r)Gua-specific antibody (Ab)⁴¹, cross-linked and samples were immunoprecipitated (IP-ed, RNA-IP). In parallel, (FLAG)OGG1-expressing hSAECs were infected as above and vRNA associated with proteins was cross-linked and chromatin-IP-ed (CLIP-ed) with Ab to the FLAG epitope tag. RNA-protein crosslinking was performed using formalin, to avoid ultraviolet (UV) irradiation-induced generation of ROS and oxidative modifications to the RNA⁴². In controls, transcriptional processivity and anti-termination factor (M2-1) and nucleoprotein (N) proteins associated vRNAs were CLIP-ed using the corresponding Abs. IP-ed and CLIP-ed RNAs were isolated and converted into cDNA using oligo-dT primers to assess abundance of transcripts for nonstructural protein-1(NS1), N, attachment glycoprotein G (G), fusion protein (F) and L using quantitative real time (qRT)-PCR (primer sequences: **Table 1**; primer validation and amount of RNA used in qRT-PCR were determined in preliminary studies (**Supplementary Fig. 1a,b**). Results show that Abs to 8-oxo(r)Gua or FLAG(OGG1) enriched all RSV mRNAs tested (**Fig. 1a**). The level of IP-ed mRNA was the highest for NS1 (encoded by the 3'-end proximal gene) (**Fig. 1a**, most left panel), and mRNA levels from subsequent genes were decreased, -the most promoter-distal L gene product was the least (**Fig. 1a**, most right panel). These data are in line with those showing that RdRp re-engages with less efficiency the more distant genes from the 3'-end of the genome^{14,43}. In controls, Ab to M2-1 IP-ed viral mRNAs, similar to that of CLIP-ed, IP-ed by Ab to FLAG(OGG1) and Ab to 8-oxoGua, while levels of N protein-Ab IP-ed viral mRNAs were similar to that of IgG (**Fig. 1a**). These results are in agreement with M2-1' RNA binding core domain and its roles in *de novo* mRNA synthesis⁴⁴ and those showing that the N protein binds genome and anti-genome as they have encapsidation signals, which are absent in mRNAs¹⁰. There was no interaction observed between viral mRNA the repair protein MTH1 (a human homolog of *E. coli* Mutator 1), an enzyme that specifically recognizes 8-oxo(r)Gua generated in the cytoplasmic ribonucleotide pool⁴⁵.

To obtain information on the cellular site of vRNA oxidation, cells were infected as above, and dual-color microscopic imaging was performed 24 h post-infection. IBs were

visualized using Ab to N protein (green, Alexa Fluor 488) and then co-stained with Ab to 8-oxo(r)Gua (red, Alexa Fluor 594). These data show that oxidatively modified RNAs at Gua(s) primarily co-localized to IBs, although they are also found in the cytoplasm (**Fig. 1b**). In support of specificity, our analysis showed that oxidatively modified proteins (carbonylated and nitrosylated) were seen at the periphery and/or adjacent to IBs (**Fig 1c,d**). Because IBs are the sites of vRNA synthesis, these results also imply that oxidative modification to Gua in the RSV transcriptome possibly occurs co-transcriptionally, either by direct oxidation or by incorporation of oxidatively modified Gua nucleoside triphosphate (8-oxo(r)GuaNTP). The latter may be due to the lack of an innate capacity of RdRp to differentiate between GNTp and 8-oxo(r)GuaNTP, similar to mammalian RNA polymerases⁴⁶.

OGG1 converts oxidatively modified Gua into enhanced RNA expression

Based on binding of 8-oxo(r)Gua-Ab to and association of OGG1 with vRNAs (**Fig. 1a, b, c**), we investigated whether the oxidative Gua modification plays a role in RSV RNA transcription. To test our hypothesis, 8-oxo(r)Gua in vRNA was decreased by normalizing RSV-induced ROS through treatment with AOs, EUK-8 (100 or 500 μ M) or N-acetyl-L-cysteine [NAC, a GSH precursor] at 10 or 20 μ M]³⁴. Total RNAs were isolated, IP-ed using Ab to 8-oxoGua and converted into cDNA for qRT-PCR. Results showed that AO treatment significantly lowered ROS levels (**Supplementary Fig. 2d**), and abundance of 8-oxo(r)Gua-containing mRNA coding for the attachment protein G, compared to that isolated from infected mock-treated cells (**Fig. 2a**). Similarly, there were lower mRNA levels of NS1, N, G, F and L (**Fig. 2b**) and genomic vRNA (**Fig. 2c**), as well as viral progeny (**Fig. 2d**). This phenomenon was not restricted to hSAECs, as we found that a decrease in 8-oxo(r)Gua levels correlated well with RSV replication (mRNA, genome and progeny) in established airway epithelial cells, A549, which is a model of infection (**Supplementary Fig. 2a,b,c**). In controls, ribavirin, a guanosine analog, an RdRp inhibitor producing co-transcriptional inhibition⁴⁷ was used, which showed an inhibitory effect irrespective of cell type (**Fig. 2b,c,d; Supplementary Fig. 2a,b,c**).

Results showing that decrease in ROS levels led to a lower abundance of 8-oxo(r)Gua-containing vRNA and physical association of OGG1 with vRNAs (**Fig. 1a,c**) suggested to us that oxidatively modified Gua along with OGG1 has a role in viral replication. To test OGG1 was siRNA-silenced or permanently knocked out by CRISPR-Cas9 genomic editing in hSAECs (hSAEC^{OGG1-/-}), control, silenced or hSAEC^{OGG1-/-} cells were infected with RSV. Total RNAs were isolated, converted into cDNA, and vRNA levels were assessed by qRT-PCR. Results showed significantly lower mRNA (NS1, N, G, F and L) levels in OGG1 silenced hSAECs (**Fig. 2e**) and

hSAEC^{OGG1-/-} cells (**Fig. 2f**). Additionally, there were decreased levels of M2-1 and N proteins in knockout cells compared to those expressing OGG1 as determined by Western immunoblotting (Wb) (**Fig. 2g**, left and right panels). Similar results obtained using OGG1-silenced A549 cells suggested that the observed effects are not restricted to hSAECs (**Supplementary Fig. 3a**). Absence of OGG1 significantly decreased levels of genomic RNAs in hSAECs and A549 cells at 12h, 24h and 36h post-infection (RNA levels at 2h post-exposure served as input, **Fig. 2h,i** and **Supplementary Fig. 3d**). Lack of OGG1 led to a more than 10-fold decrease in viral yield in hSAEC (**Fig. 2j, k**) and A549 cells (**Supplementary Fig. 3b**). Transgenic expression of OGG1 in hSAEC^{OGG1-/-} restored RSV output to a level similar to that of OGG1 expressing cells (**Fig. 2k**). Silencing other DNA glycosylases, endonuclease VIII-like 1 (NEIL1, recognizes and repairs oxidatively modified ring-fragmented purines, pyrimidines, 5-hydroxyuracil⁴⁸), or the 8-oxo(r)Gua-specific MTH1⁴⁵ had no effect on RSV replication (**Supplementary Fig. 3a**). These results suggest that OGG1 bound to 8-oxo(r)Gua in RNA is actively utilized to maximize output of RSV infection.

To examine whether changes in cellular physiology due to OGG1 depletion (selection, puromycin) affected RSV replication, we inhibited OGG1 binding to 8-oxo(r)Gua in vRNAs by using the selective binding-site inhibitor, TH5487⁴⁹. hSAECs were RSV-infected and treated with TH5487 (10 μ M or the equivalent volume of solvent). RNAs isolated were subjected to qRT-PCR analysis. Results show that TH5487 treatment of infected cells significantly decreased mRNA levels coded by NS1, N, G, F, and L genes (**Fig. 3a**). Consistent with these data, we observed significant decreases in the levels of N and M2-1 proteins (**Fig. 3b**, left and right panels) and genomic RNA (24 and 36 hpi; **Fig. 3c**) levels. The inhibitory effects of TH5487 were not cell type specific because it also inhibited RSV output in A549 cells (**Supplementary Fig. 3b,c,f,h**). Inhibition of OGG1's binding to oxidatively modified Gua in vRNA decreased RSV-yield in a concentration dependent (5, 10 μ M) manner in hSAECs (**Fig. 3d**) and in A549 cells (**Supplementary Fig. 3b**). TH2840, an inactive analog of TH5487 or O8, which inhibits only OGG1's enzymatic activity^{49,50}, had no effect on RSV titers (**Fig. 3d, Supplementary Fig. 3b**). In line with this, TH5487 inhibited RSV-induced cytopathology while TH2840 and O8 had no effect (**Supplementary Fig. 3c**). Treatment with TH5487, TH2840 or O8 showed no toxicity in hSAECs or A549 cells (**Supplementary Fig. 3i**, left and right panels).

To validate the data derived from cultured cells, mice were challenged with purified RSV (10⁶ PFU/lung) and mock treated or treated with TH5487 at -2h, +1h and 12h intervals over 96 h (30 mg/kg, intraperitoneal (i.p.)⁴⁹. At 2h, 48h and 96h post-challenge, the lungs were harvested and total RNAs isolated. The RNAs were converted into cDNA by using oligo-dT and random

primers to assess RSV mRNA and genomic RNA levels, respectively. Quantitative RT-PCR results showed that the inhibition of OGG1's substrate binding significantly lowered levels of N, G, and F mRNAs, and genomic RNA compared to mock-treated RSV-infected animals (**Fig. 3f, g**). There was no sign of TH5487 toxicity in infected animals as shown previously⁴⁹. Data from cell culture and animal experiments strongly suggest that OGG1 binding to oxidatively modified Gua in viral RNAs is essential for efficient RSV replication. Future studies will determine the clinical potential of small molecule inhibitor(s) of OGG1.

The 5'-end of nRNAs are hotspots of oxidative Gua modifications and the preferential site of OGG1 binding

To determine the site(s) of oxidative modification of Gua in vRNAs, hybridization-coupled electro-mobility shift assays (EMSA) were performed. We designed synthetic Cy5-labeled 40 nt long DNA probes homologous to the 3'-end (from 4552 to 4592 nt; containing GS) and the 5'-end (from 5433 to 5474 nt, containing GE) of the G genomic RNA (sequences are complementary to the G mRNA) (**Fig. 4a, Table 3**). G:C contents of these probes were 45% and 47%, respectively. The sequence of the third probe is homologous to the region located between GE of small hydrophobic protein (SH) and GS of G gene (4506-4546 nt; intergenic sequences complementary to antigenome). The RNA isolated from RSV-infected cells was incubated with individual probes, denatured at 95-°C for 5 min, and hybridized. Un-hybridized probes and RNAs were digested with mung bean nuclease⁴⁸, then OGG1 was added, cross-linked and mixtures were subjected to EMSA. Results showed that OGG1 extensively bound to the probe-RNA hybrid formed at the 5'-end of viral mRNA, while a weak hybridization signal was observed at the 3'-end of mRNAs (**Fig. 4b**, lane 6). The probe that was complementary to antigenome gave a weak signal, suggesting that the hybridization observed at the 3'-end of mRNA may be considered as background signal (**Fig. 4b**, lane 6). RNase-H completely eliminated EMSA signals (**Fig. 4b**, lane 7, 8 and 9), indicating that OGG1 specifically bound to the 5'-end of the mRNA region that acquired the oxidative modification of Gua, 8-oxo(r)Gua. Accordingly, OGG1 bound to the synthetically made DNA-RNA hybrid containing 8-oxo(r)Gua in the RNA strand in a concentration dependent manner (**Fig. 4c**). The active-site inhibitor, TH5487, prevented OGG1 binding to the DNA-RNA hybrid containing 8-oxo(r)Gua, lending additional support for binding specificity (**Fig. 4d**). These results suggest that oxidative modification of Gua occurs at the 5'-UTR of mRNAs.

Binding of OGG1 to the DNA-RNA hybrid at the 5'-end of mRNA suggests that oxidative modifications of Gua occur in the RNA complementary to GS in the genome. To test this

possibility, we designed Cy5-tagged single stranded (ss) 40 nt RNA probes with and without 8-oxo(r)Gua complementary to the 5'-end of the G protein gene. Based on the documented nature of charge migration⁵¹, the probes contained 8-oxo(r)Gua at the 3'-end of G stretches (5'-GGGG*CAAAU-3') of the 5'-UTR. To mimic a dsRNA structure formed during vRNA synthesis^{10,52}, the sequence of the ssRNA probes is identical to the sequence of nRNA, which allows it to anneal with the complementary sequence GS of genomic G gene (4552 to 4592 nt). Results from EMSAs show that OGG1 binds 8-oxo(r)Gua containing dsRNA and not ssRNA (**Fig. 4e**, lanes 6,7,8), an interaction that could be prevented by TH5487 (**Fig. 4f,g**). OGG1 had no enzymatic activity on dsRNA (**Fig. 4h**, lanes 2,3,4), while it efficiently excised damaged bases from dsDNA³² (**Fig. 4h**, lanes 6,7,8; **Supplementary Fig. 4a**). Therefore, removal by OGG1 of 8-oxo(r)Gua (cleavage of the N-glycosylic bond in RNA) and subsequent degradation of RNA is an unlikely scenario *in cellulo*. These results also imply that OGG1 interacts with 8-oxo(r)Gua formed co-transcriptionally at the transcription active site.

OGG1 physically interacts with the transcriptional elongation factor M2-1

In eukaryotic cells OGG1 binds to 8-oxo(d)Gua, an epigenetic-like mark in gene promoters, and physically interacts with transcription factors, including NF-κB, to facilitate its DNA occupancy⁵³. By analogy, we propose that OGG1 binds to 8-oxo(r)Gua at the 5'-end of RNA and interacts with viral protein(s), potentially aiding efficiency of transcriptional machinery. To test this possibility, FLAG-tagged OGG1, (FLAG)OGG1, expressing hSAECs were infected with RSV, lysed and IP-ed using Ab to FLAG. Complexes that were IP-ed were subjected to Wb analysis using a polyclonal Ab to RSV proteins. The results showed that (FLAG)OGG1 co-IP-ed with M2-1 (~25 kDa subunit of the tetrameric M2-1 is shown after SDS-PAGE; (**Fig. 5a**). To confirm this, M2-1 was identified using a monoclonal Ab to M2-1 (**Fig. 5b**). The lower panel of **Fig. 5a and 5b** shows intrinsic and transgenic expression of OGG1 using Ab to OGG1. We also noted that there was another ~40 kDa OGG1-interacting protein immuno-precipitate (**Fig. 5a**, *right panel); however, its identity remains unknown. To test whether OGG1 directly interacts with M2-1, we purified it to homogeneity⁵⁴ (**Supplementary Fig. 4b,c**), mixed it with OGG1, and performed pull-down assays. Results showed that OGG1 pulled down M2-1 protein, and Ab to M2-1 IP-ed with OGG1 (**Fig. 5c,d**), suggesting a direct interaction between M2-1 and OGG1. There was no interaction between M2-1 and MTH1, which was used as control protein (**Fig. 5e**).

OGG1 typically localizes to the nucleus and mitochondria³², while M2-1 is found in the cytoplasm, primarily in virus-induced IBs^{15,55}. To obtain information on the site of the OGG1-M2-1 interaction *in cellulo*, microscopic imaging (immunohistochemistry; IHC) and *in situ* proximity

ligation assays⁵⁶ were performed using RSV infected hSAECs. Time course studies showed an accumulation of OGG1 in the cytoplasm of RSV-infected cells from 6 hpi, as shown by IHC and Wb analysis (**Fig. 5f, g, Supplementary Fig. 4d**), while its level did not change in the nuclear compartment (**Fig. 5g, Supplementary Fig. 4e**, upper panels), suggesting that *de novo* synthesized OGG1 remained in the cytoplasm. After overlaying IHC images, strong co-localization of OGG1 and M2-1 was observed, especially in IBs. This observation was confirmed by calculating the Pearson fluorophore-moment correlation coefficient⁵⁷. E.g., in contrast to 6 hpi, correlation coefficient (R) at 12, 24 and 42 hpi were $R = 0.93$, $R = 0.93$ and $R = 0.89$, respectively (**Fig. 5f**, most right-hand panels). The positive proximity ligation assays strongly suggest there was a physical interaction between OGG1 and M2-1 within IBs (**Fig. 5h,i**).

OGG1 recruits M2-1 to the transcriptionally active site(s) at the 5'-end of vRNA

The physical interaction observed between OGG1 and M2-1 *in vitro* and *in cellulo* (**Fig. 5a,b,c,d,f**) and the fact that OGG1 bound to 8-oxo(r)Gua at the 5'-end of nRNA (**Fig. 4b, c, d**), suggest that OGG1 may promote interaction of M2-1 with the transcriptionally active site at GS. To model this scenario, we utilized ss and ds RNA probes containing $\pm 8\text{-oxo(r)Gua}$. The presence or absence of 8-oxo(r)Gua in ss or ds RNAs had no effect on M2-1 binding (**Fig. 6a**). M2-1 bound to ssRNA and dsRNA as a tetramer (arrow heads) and also formed higher molecular size complexes (**Fig. 6a**) similar to those described for glutaraldehyde cross-linked forms of RNA-M2-1 complexes⁵⁸. Compared to ssRNA (**Fig. 6a**, lane 1,2) there was a 25-fold decrease in M2-1's association with dsRNA $\pm 8\text{-oxo(r)Gua}$ (**Fig. 6a**, lane 3, 4). To test whether M2-1 binding to dsRNA was specific, we show that Ab to M2-1 super-shifted all M2-1-dsRNA complexes (**Fig. 6b**, lane 3). Ab to OGG1 inhibited its binding to 8-oxo(r)Gua containing dsRNA (**Fig. 6b**, lane 2). IgG was used as control (**Fig. 6b**, lane 1).

To examine if OGG1 promotes binding of M2-1 to RNA, OGG1 was pre-incubated with a dsRNA probe $\pm 8\text{-oxo(r)Gua}$ and M2-1 was added. OGG1 increased M2-1 occupancy on 8-oxo(r)Gua-containing dsRNA in a concentration dependent manner (**Fig. 6c**, lanes 6 to 10 and **Fig. 6c**, right panel), while without 8-oxo(r)Gua in the dsRNA, OGG1 had no effect (**Fig. 6c**, lanes 1 to 5 and **Fig. 6c**). Time course experiments showed that OGG1 accelerated the interactions between 8-oxo(r)Gua-containing dsRNA and M2-1 (**Fig. 6d**, lanes 7 to 11 and **Fig. 6d** right panel). For instance, the level of dsRNA-associated with M2-1 was similar at $t = 1$ min in the presence of OGG1 to that of $t = 6$ min without OGG1 (**Fig. 6d**, lanes 2 and 7, **Supplementary Fig 4g**). Interestingly, there was no M2-1-OGG1-dsRNA complex observed; however, OGG1 formed a separate complex with dsRNA (**Fig. 6d**, arrow). Active site inhibitor

(TH5487) decreased the effect of OGG1 on the interaction between M2-1 and dsRNA in a concentration dependent manner (**Fig. 6e**, right panel). Without 8-oxo(r)Gua OGG1 had no effect (**Fig. 6c**, lanes 1 to 5 and right panel), and in the absence of OGG1, TH5487 had no effect on M2-1 binding (**Fig. 6f**). These data imply that prior OGG1 binding to dsRNA is needed for efficient association of M2-1 with dsRNA. In control experiments, MTH1 had no effect on M2-1 binding to dsRNA (8-oxo(r)Gua:C) (**Supplementary Fig. 4h**). From these results, we speculate that OGG1 binds to dsRNA – a hybrid between nRNA and the 3'-end of viral gene(s) via co-transcriptionally generated 8-oxo(r)Gua, where it interacts with and recruits M2-1 to the transcriptionally active site(s). OGG1 had no effect on the interaction of M2-1 with ssRNA containing 8-oxo(r)Gua (**Supplementary Fig. 4f**).

We next explored the fate of OGG1 once the full-length mRNA disengages from genome. To do so, mRNAs isolated from RSV-infected cells were mixed with His-tagged OGG1 in binding buffer, and IP assays were performed. Levels of mRNA encoding for G protein in IPs was determined using qRT-PCR. Compared to IgG, Ab to His-(OGG1) had enriched viral RNAs coding for G protein in IPs (**Supplementary Fig. 4j**). Heat denaturation of RNA (65°C for 5 min) prevented OGG1 binding. In controls, M2-1 extensively IP-ed with G-mRNA, while N protein lacked interaction with mRNA (**Supplementary Fig. 3j**). Taking that the 5'-end of nRNAs is the preferential site of OGG1 binding (**Figs 4b,c,d**), we performed a series of experiments that examined OGG1 binding to a Cy5-tagged 40 nt base-long ssRNA (identical to 5-end of G-mRNA). Results showed that OGG1 bound to the non-denatured RNA probe only when it contained 8-oxo(r)Gua (**Fig. 6g**, lanes: 5 to 8) similar to dsRNA (**Fig. 6g**, lanes: 9 to 12). Heat denaturation (65°C for 5 min) of RNA abolished OGG1 binding (**Fig. 6g**, lanes 1 to 4) implying that OGG1 binds to the substrate via temperature-sensitive secondary RNA structure(s) at the 5-end of G-mRNA. The predicted secondary structure of the probe is shown in **Fig. 6g** (right panel). A secondary structure of G-mRNA is shown in **Supplementary, Fig. 4i** as predicted by “*RNAstructure*” software⁵⁹. OGG1 lacks enzymatic activity on dsRNAs (**Fig. 4h, Supplementary Fig. 4a**), therefore we speculate that OGG1 remains in complex with full-length viral mRNAs (**Fig. 6h**). The post-transcriptional roles of OGG1 is a matter for future studies.

Discussion

RSV is one of the leading causes of life-threatening respiratory system illnesses globally in infants, preschool children, immunocompromised adults and the elderly; yet, treatment is mostly limited to supportive medical care^{1,4,7}. RSV infection-induced generation of ROS is linked to expression of inflammatory genes and severity of clinical disease; however, the mechanism by which the oxidizing environment modulates virus replication remains unknown. Here we report that vRNAs acquire ROS-generated 8-oxo(r)Gua as an epitranscriptomic mark that is “read” by the repurposed DNA repair protein OGG1. Inhibition of 8-oxo(r)Gua generation through AOs, OGG1 knockdown or inhibition of its binding to epitranscriptomic mark significantly decreased the level of viral mRNAs, proteins, and viral progeny in cultured cells and airways. Reconstitution experiments show that OGG1 binds to the 5'-UTR of nRNAs containing 8-oxo(r)Gua while paired with the 3'-end of viral gene(s) interacts with and recruits the transcriptional elongation factor, M2-1 to transcriptionally active sites. Taken together, these data suggest that ROS generated in RSV-infected cells is utilized to oxidize Gua in vRNA, which serves as an epitranscriptomic mark that repurposes OGG1 to enhance RSV replication. These studies also illustrate how the virus usurps cellular proteins to enhance its replication.

RNAs are highly vulnerable to oxidative modifications, due to their single stranded nature, partial protection by RNA-binding proteins, and unprotected Watson-Crick edges of nucleotides²⁷⁻²⁹. The nucleobase of guanosine is especially vulnerable to modifications as it has the lowest oxidation potential⁶³. Therefore, 8-oxoGua is often generated in RNAs and in nucleotide triphosphate pool by ROS²⁸, in an oxidizing intra-cellular environment, such as in RSV-infected cells³⁶. Therefore, association of lesion-specific OGG1 with or IP by 8-oxoGua Ab of mRNAs of NS1, N, G, F and L genes in RSV-infected cells is not surprising. Importantly, the level of OGG1-associated vRNA was similar to that of RNA that was IP-ed using Ab to M2-1. M2-1 interacts with RNA via its zinc-binding and core domains^{44,60}. OGG1 lacks a zinc-finger motif⁶¹, so its interaction with vRNA likely occurs through its recognition domain, which is specific for 8-oxoGua in DNA and RNA.

Although oxidative modification to Gua can occur at any Gua islet (GGG, GGGG) within genomic RNA and mRNAs, hybridization-coupled EMSA clearly indicated that 8-oxo(r)Gua was formed at the 5'-end of viral nRNAs. There were only insignificant levels of 8-oxo(r)Gua in genomic RNA. These findings are in line with the sequence 5'-GGGGCAAU-3' at the 5'-UTR of NS1, NS2, N, P, M, SH, G, F, and M2, and sequence 5'-GGGACAAAU-3' of L mRNA^{10,13}. The exact position of 8-oxo(r)Gua in these islets is yet to be determined; however, based on the previously characterized nature of charge migration, it is most likely located at the end of G

stretches⁵¹. It is known that during viral replication, the dsRNA hybrid may reach 30 nucleotides or more. For instance, in the case of VSV, mRNA is separated from the genome and then capped after reaching 31 nucleotides⁵². Thus, to reconstitute an *in cellulo* scenario, 8-oxo(r)Gua was synthetically placed at the end of 3'-end of G series in a 40-nucleotide long RNA that is complementary to the 3'-end of genomic RNA (probe), which mimics dsRNA (probe) similar to the polymerization active site at GS⁵². Binding of OGG1 to dsRNA with 8-oxo(r)Gua was similar to DNA containing 8-oxo(d)Gua. Although M2-1 is an RNA binding protein, it showed poor interaction with dsRNA $\pm$ 8-oxo(r)Gua compared to ssRNA $\pm$ 8-oxo(r)Gua. However, OGG1 significantly increased M2-1 binding (over 10-fold) only to dsRNA containing 8-oxo(r)Gua. The exact molecular mechanism by which OGG1 enhances M2-1 binding is yet to be elucidated; however, it is possible that OGG1-induces structural changes in dsRNA that are creating a specific binding interface in the hybrid between the genome and nRNA that facilitates M2-1 interaction with the polymerization complex (**Fig. 6h**). This scenario could be similar to that seen in mammalian cells, where OGG1 binds to the epigenetic mark 8-oxo(d)Gua in gene regulatory regions, thereby serving as a nucleation site and increasing DNA occupancy of transcription factors^{64,65}. During mRNA synthesis, M2-1 interacts with P protein and vRNA in a competitive "on and off" manner, because the RNA recognition epitopes of M2-1 overlap with the site interacting with P protein⁶⁶. Together with the data showing that OGG1 increases RSV transcription, it is conceivable that OGG1 not only interacts physically with M2-1 but also facilitates its stable interaction with vRNA. Indeed, in OGG1 proficient cells, the level of mRNA was approximately 10-times higher, compared to cells lacking OGG1 or treated with the small molecule inhibitor of OGG1. These data also imply that the lack of OGG1 or the OGG1 inhibitor prevents efficient assembly of transcriptional initiation complex and transcriptional elongation in cultured cells and in lungs.

Based on these data, 8-oxo(r)Gua is functionally similar to covalently modified nucleotides, such as m⁶A, and m⁵C, in genomic and mRNAs of RNA viruses (e.g., hMPV, RSV, measles, influenza, flavi-, and corona viruses) with fundamental implications in their life cycles^{21-23,67,68}. In contrast to epitranscriptomic moieties that are deposited and removed by cellular enzymes called "writers" and "erasers", respectively²², 8-oxo(r)Gua is directly generated by the interaction of Gua with one of several reactive species (primarily hydroxyl radical⁶³). Thus ROS is considered to be a covalent modifier ("writer") of Gua by oxidizing --subtracting an electron generating the epitranscriptomic mark 8-oxo(r)Gua of which "reader" is the repurposed base specific DNA repair protein OGG1. As a "reader" OGG1 facilitates M2-1 binding to 5'-UTR, thereby promoting mRNA synthesis. Of note, OGG1 has an innate ability to migrate/diffuse

431 along nucleic acid strands without energy requirement³⁸. Thus, OGG1 may migrate along with
432 M2-1 on vRNA to facilitate transcription elongation. Another possibility is that OGG1 remains
433 engaged with the 8-oxo(r)Gua-containing 5'-UTR of mRNA via temperature sensitive secondary
434 stem-loops. If this is the case OGG1-5'-UTR could have roles in initiation of translation similar to
435 those 5'-UTR-protein complexes of influenza, corona-, and flavi-viruses⁶⁹. The propensity of 8-
436 oxo(r)Gua to pair with adenine may promote formation of specific secondary structures or affect
437 the configuration of the viral RNA which, together with OGG1, allow RSV to exploit the host
438 translation machinery.

439 Microscopic and molecular co-localization studies show that 8-oxo(r)Gua-containing
440 vRNAs primarily localized to IBs, highly dynamic structures in the cytoplasm. Interestingly,
441 carbonylated and nitrosylated proteins are seen at the periphery and/or adjacent to IBs.
442 Although these observations ought to be further investigated, one may propose that repurposed
443 host proteins are protecting viral synthetic machinery and vRNAs from excessive oxidative
444 modifications. The observation that RNAs containing 8-oxo(r)Gua localize within IBs, along with
445 supporting data shown here, implies that oxidative modifications caused by ROS of Gua in the
446 RSV transcriptome occur co-transcriptionally. Another important observation is that OGG1, a
447 nuclear protein, accumulated in the cytoplasm of RSV-infected cells and co-localized with M2-1
448 within IBs, where components of the RNA polymerase complex proteins (P, L) are located.
449 Interestingly, OGG1 level in the nucleus was unchanged suggesting that the newly made OGG1
450 remained in the cytoplasm. Because cytoplasmic and nuclear extracts consistently showed the
451 ~39 kDa OGG1, we exclude the possibilities that new variants were made by alternative splicing
452 or that the mitochondrial OGG1 (42.5 kDa) is involved. The molecular mechanism by which
453 OGG1 is targeted to IBs remains unclear, but we note that site-specific posttranslational
454 modifications (acetylation, phosphorylation, O-GlcNAcylation and others) of OGG1 could be the
455 culprit. Importantly, the Pearson fluorophore-moment correlation coefficient⁵⁷ clearly showed
456 that OGG1 co-localized with M2-1 within IBs and physically interact as shown by *in situ*
457 proximity ligation assays and their co-IP from RSV-infected cells.

458 The fate of OGG1 on viral mRNA remains unclear; however, we note that OGG1 has an
459 innate ability to migrate/diffuse along nucleic acid strands without energy requirement³⁸. Thus,
460 OGG1 may migrate along with M2-1 on RNA to facilitate transcription elongation. Another
461 possibility is that OGG1 remains engaged with the 8-oxo(r)Gua-containing UTR of mRNA via
462 temperature sensitive secondary stem-loops of RNA (**Fig. 6h**). For instance, the 5-UTRs of
463 influenza, corona-, and flavi- viruses, involved in initiation of translation, are reported to form

functional protein-RNA complexes⁶⁹. Thus, it is conceivable that OGG1 associated with secondary elements of RSV mRNAs may be used to hijack the cellular translation machinery.

This work represents the first study on the importance of an oxidatively modified base lesion in a noncoding region of viral mRNA during the RSV life-cycle. Stretches of Gua in RNA are sink of electrons⁵¹, and oxidation of Gua may occur at any site(s) of mRNAs and may lead to errors during protein synthesis as reported in mammalian cells⁷⁰. 8-oxo(r)Gua can also be generated in the genome and anti-genome of RSV, although they are encapsidated, a scenario that occurs in chromatin⁶⁵. Previous studies have considered that mutational errors are made by RdRp during replication due to the combination of a lack of proofreading activity and low fidelity⁷¹. Oxidized Gua in viral genome or antigenome could pair with A and can be responsible for the previously documented variability of RSV strains and their virulence and evolvability⁷², although this needs to be experimentally shown.

In conclusion, the data presented here show that 8-oxo(r)Gua in 5'-UTR of vRNAs functions as an epitranscriptomic mark. Its presence in vRNAs is conveyed into function by the repurposed base specific DNA repair protein OGG1. Because OGG1 has no enzymatic activity (erasing activity) on RNAs, OGG1 is considered a "reader" in the context of epitranscriptomic and translates the oxidatively-generated mark into efficient virus replication. Indeed, the absence of either 8-oxo(r)Gua or OGG1 or inhibition of their interaction by small molecule decreased the output of RSV infection. These novel findings not only widen our understanding of the mechanism of RSV gene expression, but also represent new antiviral targets, which may facilitate the development of novel therapeutics. Because of the electron deficient oxidizing environment in virus-infected cells, covalent modifications of Gua could be a common feature in viral RNAs, making these findings potentially applicable to influenza, rhinovirus, corona-, and metapneumo-viruses.

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Material and Methods

Cell cultures. Hep-2 cells (ATCC number CCL-23) were grown in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS). A549 cells were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) supplemented with 10% (v/v) FBS (HYCLONE), 10 mM glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Telomerase/CDK4 immortalized human small airway epithelial cells (hSAECs)⁴⁰ were maintained in SAEC growth medium (PromoCell, Item #:C-21270), containing Growth Medium Supplement Mix (PromoCell Item #: C-39175), (7.5 mg/mL bovine pituitary extract, 0.5 mg/mL hydrocortisone, 0.5 µg/mL human epidermal growth factor, 0.5 mg/mL epinephrine, 10 mg/mL transferrin, 5 mg/mL insulin, 0.1 µg/mL retinoic acid, 0.5 µg/mL triiodothyronine, 50 mg/mL gentamicin, and 50 mg/mL BSA). Cells were regularly tested for Mycoplasma contamination by the Tissue Culture Core Facility at UTMB (<https://www.utmb.edu/microbiology/core-facilities/tccf>).

CRISPR/Cas9 genome editing. OGG1 deletion from hSAECs was performed using a Clustered Regulatory Interspaced Short Palindrome Repeat (CRISPR)-associate system (Cas) 9 genome editing as previous described⁴⁹. In brief, targeting sequences of OGG1 5'-GATGCGGGCGATGTTGTTGTTGG-3' and 5'-AACAACATCGCCCGCATCACTGG-3' were cloned into pSpCas9 (BB)-2A-Puro expression vector. Following transfection of the vector by Lipofectamine 2000 (Item #: 11668027, ThermoFisher Sci/Invitrogen) into hSAEC, 3 µg/mL of puromycin (Corning, Item #: 61-385-RA) was added, cells were sub-cultured into 24-well plates and clones were established. Clones were further examined for OGG1 expression by qRT-PCR and Western immunoblotting. OGG1 knockout cultures were maintained in SAEC growth medium (PromoCell, Item #: C-21270), containing Growth Medium Supplement Mix (PromoCell, item #: C-39175) in the presence of 2 µg/mL puromycin (Corning, Item #: 61-385-RA).

RSV propagation, purification, infection and titration. The human RSV A2 strain (ATCC VR-1544) was propagated using HEp-2 cells (ATCC CCL-23). For experiments, cell monolayers (80 to 90% confluence) were infected with RSV at a pre-calculated multiplicity of infection (MOI). In brief, OGG1-expressing, -depleted or OGG1 knockout cells or those expressing transgenic OGG1 were infected with RSV (strain: A2). Following a 2h adsorption period, the unbound virus was removed, the cell monolayer was washed, and culture medium containing 2% FBS was added. To assess viral gene expression, cells were infected at MOI = 0.1 and RNAs were extracted at the times indicated in results. For IPs, and cross-linked coupled IPs cells were

540 infected at MOI = 3. For microscopic imaging (IHC and PLA), cells were infected at MOI = 1. To
541 generate RSV stock or determine the effect of inhibitors on yields of progeny, cells were
542 infected at MOI = 0.1. RSV titration: cells were infected, snap frozen then thawed and clarified by
543 centrifugation. Serial (10-fold) dilutions of the supernatant fluids were added to HEp-2 cells
544 grown in 12-well plates (80% confluence), and viral titers were determined by the
545 methylcellulose plaque assay⁷³. To challenge lungs, a purified RSV suspension was used. RSV
546 was purified on discontinuous sucrose gradients (Infant Bronchiolitis and Viral Core. Director Dr
547 Garofalo, Professor, UTMB) as described previously⁷³. Aliquots of sucrose purified (cytokine
548 and lipopolysaccharide free) preparations were stored at -80°C until use.

549 **Animal challenge and treatment** Ten-week-old female and male BALB/c mice (The Jackson
550 Laboratory, Bar Harbor, ME, USA) housed in the animal research facility of the UTMB
551 (Galveston, Texas) were used for these studies. Randomly selected groups of mice (50% ♂ and
552 50% ♀) were challenged via intranasal (i.n) route with RSV (10⁶ PFU per mice) in 60 µl of
553 phosphate-buffered saline solution (PBS; pH: 7.4) under mild isoflurane anesthesia. TH5487
554 (SelleckChem; 30 mg per kg, in a 200 µL volume of solvent (5% DMSO, 10% Tween 80 in
555 saline) was administered via the peritoneal route 1h before and every 8h after RSV challenge.
556 All experiments performed here are in accordance with the NIH Guide for Care and Use of
557 Experimental Animals and approved by the University of Texas Medical Branch (UTMB) Animal
558 Care and Use Committee (approval no. 0807044D).

559 **Assessment of cellular ROS levels.** Intracellular ROS levels were determined by using the
560 fluorogenic probe 5- (and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester
561 (CM-H2DCFDA; Invitrogen, Eugene, OR). In brief, cells were mock- or RSV-infected and loaded
562 with 10 µM CM-H2DCF-DA at 37°C for 10 minutes. Cells were then washed with PBS and lysed
563 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40), then clarified
564 by centrifugation. Changes in DCF fluorescence in supernatant fluids were determined by using
565 a Synergy H1 Hybrid Multi-Mode Reader (BioTek) with excitation / emission at 485 nm / 535
566 nm. Results are expressed as change in fluorescence units (FU).

567 **Gene specific siRNA depletion.** Triplicate cultures of hSAEC or A549 cells were transfected
568 with siRNA targeting the hOGG1 (Dharmacon, L-005147-00-0020), hMTH1 (Dharmacon, L-
569 005218-00-0020), hNEIL1 (Dharmacon, L-008327-00-0020) mRNAs or with universal siRNA
570 negative control (Dharmacon, D-001810-01-20) using Lipofectamine RNA iMAX transfection

reagent (13-778-075, Invitrogen, Carlsbad, CA, USA). The efficiency of gene silencing was determined by qRT-PCR and Western blot analysis. Sequences of primers are listed in **Table 2**.

Transgenic over expression of OGG1. OGG1 deleted hSAECs were transfected with FLAG-tagged OGG1. Sequence fidelity of OGG1 was determined in Molecular Genomic Facility of UTMB (<https://www.utmb.edu/mgf/next-generation-sequencing-core>). One µg plasmid per 35 mm dish (800,000 cell) were transfected using Lipofectamine 3000 as described in manufacturer instruction. Twenty-four h later, cells were mock or infected with RSV (3 MOI per cells) for various lengths of time (0, 12, 24, 36 h). At the time of infection, expression of (FLAG)OGG1 was determined by qRT-PCR using primer sets (**Table 1**) and Western blot analysis (Abcam, ab135940).

M2-1 Expression and Purification. An optimized sequence of RSV M2-1 gene fused with a N-terminal Histidine (His) tag was cloned into the expression vector pET28a and confirmed by sequencing (Creative Biogene Laboratories, Shirley, NY). Protein (His-M2-1) production was performed essentially as described previously^{54,58}. Briefly, the *E. coli* BL21 (DE3) Star (ThermoFisher) was transformed with the expression vector pET28a-His-M2-1 and cultured in LB medium in the presence of 50 µg/mL kanamycin to an absorbance of 0.5–0.7 at 600 nm before inducing His-tagged M2-1 expression with 0.5 mM IPTG, 50 µM ZnSO₄ for 3 hours at 37°C. Harvested cells were flash-frozen in liquid nitrogen and stored at –80°C. For purification of His-M2-1, the frozen cell paste (15 g) with the overexpressed protein was re-suspended at 4°C in buffer A (50 mM Tris-HCl pH 7.4, 300 mM NaCl, 1 mM β-mercaptoethanol) with one complete EDTA-free protease inhibitor tablet. The re-suspended cells were disrupted by three passes through a micro fluidizer (15,000 PSI, Microfluidics, Newton, MA). The cell debris were removed by centrifugation at 18,000 rpm at 4°C for 45 minutes. Clarified supernatant was filtered through a 0.22-µm filter (Millipore, Burlington, MA), loaded onto a 5 mL nickel HisTrap HP (GE Healthcare, Piscataway, NJ) fast-flow column previously equilibrated in buffer A. The column was first washed with buffer A containing 20 mM imidazole, and then with high salt buffer (buffer A with 1M NaCl) to remove loosely-bound nucleic acids from the protein. Elution of His-M2-1 from the His-Trap column was performed by a linear gradient of 50–1000 mM imidazole in buffer A. The fractions containing M2-1 were combined, diluted with buffer A containing 50 mM NaCl, and further purified by cation-exchange Hi-Trap SP column (GE Healthcare, Piscataway, NJ) using a linear gradient of 50-1000 mM NaCl that further removed nucleic acids. The purified His-M2-1 recombinant protein was exchanged into the low salt buffer

(20 mM Tris-HCl pH 7.4, 150 mM NaCl) and its purity was confirmed by spectrophotometry (OD_{260nm/280nm} ratio) and Western blot analysis.

RNA extraction and qRT-PCR. Total RNAs were extracted using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Crude RNAs were DNaseI-treated and loaded onto an RNeasy column and subjected to washes with RW1 and RPE buffers. RNAs were eluted with the RNase-free water included in the kit. In specific experiments, mRNAs were isolated using a magnetic mRNA isolation kit (NEB, S1550S). The RNA concentration was determined spectrophotometrically on an Epoch Take-3™ system (BioTek, Winooski, VT) using Gen5 v2.01 software. The quality of the RNAs were confirmed via the 260/280 nm ratio (varied between 1.9 – 2.0). 1000 ng total RNA was used to generate cDNA with either oligo-dT or random hexamer primers (Takara, Item #: RR037A). qPCR was performed using specific primers for RSV NS1, N, G, F and L mRNA. The level of RSV genome was determined using primer pairs amplifying inter-genome (upstream from the attachment glycoprotein G) and the 3'-end of G gene (genome coordinates 3' c). As internal control, cellular GAPDH was used (sequence of primers are listed in **Table 1**). Changes in mRNA and genomic RNA levels were expressed as fold increases over levels at 2h (after adsorption) in infected cells using the $2^{-\Delta\Delta C_t}$ method.

Co-Immunoprecipitation (Co-IP) and Western blot analysis. Parallel cultures of FLAG-tagged OGG1 expressing cells (1×10^7 hSAEC) were RSV- (MOI = 3) or mock-infected, and 24 h later cells were lysed using cell lysis buffer (Cell Signaling Technology; Item #: 9803; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40) containing 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate and protease inhibitors (1 mM Na₃VO₄, 1 mM NaF, and 20 µg/mL aprotinin, leupeptin, phenylmethanesulfonyl fluoride). Cell lysates were clarified (14,000 g at 4°C for 30 min) and then incubated with Protein G beads (Millipore Sigma, Item #: LSKMAGAG10) coupled to anti-FLAG antibody (Abcam, ab205606) for 3 h at 4°C with rotation. Cell lysates were also incubated with uncoupled beads or antibody to a control IgG (Santa Cruz). Beads were washed 3 times in PBS, and the proteins were eluted by boiling in 1x loading buffer. Eluted proteins were separated by SDS-PAGE electrophoresis, and western blot analysis was performed to detect immunoprecipitated RSV proteins. Proteins were transferred into nitrocellulose membranes, blocked with 5% non-fat dry milk in TBS-T (20 mM Tris base, 500 mM NaCl, and 0.05% Tween-20, pH 7.5), and then incubated for 3 h with mouse anti-RSV Nucleoprotein monoclonal (Abcam, ab94806), mouse anti-RSV M2-1 monoclonal (Abcam, ab94805) and subsequently with horseradish peroxidase-conjugated secondary antibody

(1:4000 dilution; SouthernBiotech, Birmingham, AL, USA). The signals were detected using the ECL Plus chemiluminescent detection system (Bio-Rad, Item #: 1705061). Even loading was confirmed by probing membranes with GAPDH antibody (Item #: 14C10; Cell Signaling Technology).

Microscopic Imaging. hSAECs were plated on collagen pre-treated cover glasses (Roche Applied Sciences) and infected with RSV (MOI = 1) or mock-infected for increasing lengths of time. Cells were fixed with acetone : methanol (1:1) for 20 min at room temperature, dried and stored. At the time of use, cells were rinsed and permeabilized using 0.1% (w/v) Triton-X-100 diluted in phosphate-buffered saline (PBST) for 5 min. Cells were incubated with 1% BSA for 1 h at room temperature and primary antibodies were added to individual slides at a dilution recommended by the manufacturer or determined in preliminary studies (1 to 100; 1 to 300) in PBST for 1 h at 37°C. Abs were specific to OGG1 (Novus, NB100-106), 8-oxo(r)Gua (Millipore Sigma, MAB3560), RSV M2-1 (Abcam, ab94805), anti-nucleoprotein (Abcam, ab94806), anti-Nitro tyrosine (Millipore Sigma, 06-284), and anti-2,4-Dinitrophenylhydrazine (DNP, Abcam, ab178020). After washing cells 3 times in PBST, secondary antibody conjugated to Alexa Fluor 488 (goat anti-rabbit) or Alexa Fluor 594 (goat anti-mouse) was added for 1 h at 37°C. After washing in PBST (3-times), cells were dried and mounted with Vecta shield/DAPI, 4'6-diamidino-2-phenylindole hydrochloride (Vector Laboratories, CA). Over 30 randomly selected fields of view per sample were photographed using a WHN10×/22 eyepiece and a 60× objective (field of view is 1.1 mm, and camera correction is 0.63) on an OLYMPUS Microscope System BX53P.

Proximity ligation assay (PLA). hSAECs plated on collagen-treated coverslip slides were infected with RSV (1 MOI) for 24 h and fixed with 4% paraformaldehyde, and 0.1% (w/v) permeabilized Triton-X-100 diluted in phosphate-buffered saline (PBST) for 5 min. After washing in PBS, Duolink® blocking solution was added for 1 h at room temperature and PLA was performed using the Duolink PLA kit (OLink Bioscience, catalog no. LNK-92101-KI01) per the manufacturer's instructions and as we described previously⁵⁶. In brief, cells were incubated with the Ab to OGG1 (Novus, NB100-106) and RSV M2-1 (Abcam, ab94805) primary antibodies for 1 h at 37°C. Cells were washed twice with 1X wash buffer A (Millipore Sigma, DUO82049), and secondary antibody conjugated MINUS and PLUS probes were added for 1 h at 37°C. After washing with 1x buffer A, ligation mix was added for 30 min at 37°C, and then amplification mix was added to each sample for 100 min at 37°C. After washing with buffer B (Millipore Sigma,

Item # DUO82049) cells were mounted with mounting medium (Ibidi Inc, Fitchburg, WI, Item # 19-06-14). Cells were photographed using a WHN10×/22 eyepiece and a 60× objective (field of view is 1.1 mm, and camera correction is 0.63) on an OLYMPUS Microscope System BX53P.

Measurement of co-localization: The measurement of co-localization between fluorophores (Alexa 594 (red) vs Alexa 488 (green)) was performed using the software Image J v1.51, which calculates co-localization based on intensity of the individual fluorophore pixel (Pearson correlation coefficient)⁵⁷.

RNA-protein cross-linking immunoprecipitation (RNA-IP). RNA-IP assays were performed as described by "CLIP-sequencing guidelines and practices" of the ENCODE and modENCODE consortia (<http://encodeproject.org/ENCODE/>) using slight modifications. Briefly, FLAG-tagged OGG1-transfected cells were infected with RSV (MOI = 1) for 24 h, RNA/protein were cross-linked with 1% paraformaldehyde, and cells were harvested using 1 x lysis buffer containing iron chelator desferioxamine⁴¹ (DFO, Millipore Sigma, Item #: 252750). RNA-protein complexes were immunoprecipitated using RNA-IP KIT (Active Motif, Item #: 53024) with "ChIP" quality antibodies (Abs) [anti-FLAG Ab, Item #: F1804, Sigma Millipore (Cambridge, MA); RSV M2-1 (Abcam, ab94805); IgG (Santa Cruz, sc-3887)]. The precipitates were washed three times, de-cross-linked and subjected to qRT-PCR. qRT-PCR reactions were performed in triplicate using SYBR Green PCR Master Mix (Bio-Rad) in a CFX 96 real-time PCR detection system (Bio Rad). The sequence of primers is listed in **Table 1**. qPCR data were normalized using the fold enrichment method. In brief, this method is based on the assumption that the IP using a specific Ab contains both specific signal and background signal, whereas the IP of a negative control-IgG represents the background only. The background was subtracted from the signal, and the remaining value corresponded to the net pull-down of a specific RNA by a given specific Ab normalized to both total RNA and nonspecific (negative control) IP.

Electrophoretic mobility shift assay (EMSA). To evaluate OGG1 and/or M2-1 binding ssRNA or dsRNA with or without oxidative modification to Gua, EMSA was performed. RNA oligos (**Table 3**) were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). For each reaction, 8 pmol 5'-Cy5-labeled probe was incubated with increasing concentrations (0, 2, 4, 8 pmol) of OGG1 (ProSpec, Item #: ENZ-253,) or M2-1 (made as described above) in a total volume of 10 µl containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 1 mM DTT. Binding assays were performed on ice for various lengths of time (1 to 30 min). For competition assays, OGG1 was incubated with RNA for 15 min, then M2-1 was added for increasing lengths

of time (1 to 30 min). For hybridization-coupled EMSA, mRNAs were isolated from RSV (0.1 MOI, 48 h) infected cells using Magnetic mRNA Isolation Kit (NEB, Item #: S1550). One μ g mRNA was mixed with 100 fmol Cy5-labeled probe in hybridization buffer (100 mM Tris (pH 8.0), 50 mM NaCl, 1 mM EDTA) and denatured at 95°C for 5 min and allowed to hybridize overnight. Mung bean nuclease (Item #: M0250S, NEB) was used to digest the single-stranded un-hybridized probes and mRNAs at 30°C for 30 min. 8-oxo(r)Gua-containing hybrids were identified by adding OGG1 (8 pmol) for 30 min on ice. Protein-DNA complexes were resolved on a 6% DNA retardation gel (Invitrogen, Item # EC6365BOX) in 0.25 $\times$ TBE buffer and visualized using AmershamTM Imager 680 (Global Life Sci. Sol. Marlborough, MA). Band intensities were quantified using Image J v1.51 (U. S. NIH, Bethesda, Maryland, USA).

Oligonucleotide excision assay. OGG1 glycosylase activity on 8-oxoGua containing dsRNA or DNA was assayed as described previously⁵³. The sequence of DNA probe: 5'-Cy5/-dAdGdAdGdAdAdGdAdAdGdAdAdGdAdA/8oxodG/dAdGdAdTdGdGdGdTdTdA dTdTdCdGdAdAdCdTdAdGdC-3'). Sequence of RNA probe is 5'-Cy5/-rCrArArArUrGrGrArGrGrU rUrArArUrArU/8oxorG/rGrGrArArUrGrArUrGrGrArA-3'). In brief, 100 fmol Cy5 labeled probes (Integrated DNA Technologies, Coralville, IO) were incubated with increasing concentrations (0.5, 1, or 2 pmol, Item #: ENZ-253, ProSpec) of OGG1 in 10 μ L digestion buffer (10 mM of Tris-HCl (pH 7.5), 10 mM of NaCl, 1 mM of EDTA, 1 mg/mL BSA, and 1 mM of DTT). After incubation for 10 min at room temperature, the reaction was halted by adding 10 μ L loading buffer (containing 8 μ L of formamide, 2 μ L of 10 mM of NaOH) and heated for 5 minutes at 95°C. The cleaved product was separated from the intact probe in a 15% polyacrylamide gel containing 8 M urea in Tris-borate-EDTA buffer (pH 8.4). The separated bands were visualized by using AmershamTM Imager 680.

Cell viability assay. RSV (MOI = 1) and mock-infected cells were treated with increasing concentrations (2.5, 5, 10, 20 μ M) of TH5487, TH2840, TH588, O8 or 0.4% DMSO after virus adsorption every 8 h. Cell culture medium was harvested at 48 h to perform the colorimetric lactate dehydrogenase assay (LDH; Abcam, Item # ab102526). The assay was performed using a 96-well plate according to the manufacturer's instruction. The output of triplicate samples was determined immediately (T1), 10 min (T2), 20 min (T3), and 30 min (T4) at OD 450 nm on a microplate reader (Synergy H1 Hybrid Multi-Mode Reader; BioTek) at 37°C protected from light. Amounts of LDH in the media were calculated using $\Delta A_{450nm} = (A_2 - A_1)$ formula, where: A1 is the sample reading at time T1. A2 is the sample reading at time T2. LDH activity is expressed in nmol of NADH generated by LDH during the reaction time ($\Delta T = T_2 -$

T1). LDH activity = $(BI (\Delta T \times V)) \times D = \text{nmol/min/mL}$, B = amount of NADH in sample, calculated from standard curve (nmol). ΔT = Reaction time (minutes). V = Original sample volume added into the reaction well (mL). D = Sample dilution factor.

Statistical analysis. The data are expressed as the mean $\pm$ SD. Results were analyzed for significant differences using unpaired, two-tailed Student's t-tests using Microsoft Excel procedures. Differences were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Table 1. Primer sequences for PCR

hGAPDH	Forward primer: 5'-ACATCGCTC AGACACCATG -3' Reverse primer: 5'-TG TAGTTGAGGTC AATGAAGGG -3'
RSV NS1 mRNA	Forward primer: 5' - GCTTTGGCTAAGGCAGTGAT -3" Reverse primer: 5'- TGGCATTGTTGTGAAATTGG -3'
RSV N mRNA	Forward primer: 5'- AAGGGATTTTGCAGGATTGTTT -3' Reverse primer: 5'- CTCCCCACCGTAACATCACTTG -3'
RSV G mRNA	Forward primer: 5'- CGGCAAACCACAAAGTCACA -3' Reverse primer: 5'- TTCTTGATCTGGCTTGTTGCA -3'
RSV F mRNA	Forward primer: 5'- AA CAGATGTAAGCAGCTCCGTTATC -3' Reverse primer: 5'- CGATTTTTATTGGATGCTGTACATTT -3'
RSV L mRNA	Forward primer: 5'- CACTCTACAAAACAAAAGACACAATCA -3' Reverse primer: 5'- AGGATGTTGCATTGAACACATT -3'
RSV genomic RNA	Forward primer: 5'- AGTCATAACA ATGAACTAGG -3' Reverse primer: 5'- TTCTTGATCTGGCTTGTTGCA -3"
hOGG1	Forward primer: 5'- GGCTCAACTGTATCACCCTGG -3' Reverse primer: 5'-GGCGAT GTTGTTGTTGGAGGAAC -3'
hNEIL1	Forward primer: 5'-GACAGAGGCAAGTGGCAAAGCA -3' Reverse primer: 5'-GCCTCATTCAAACTGGCTGG -3'
hMTH1	Forward primer: 5'-GTCTTCTGCACAGACAGCATCC -3' Reverse primer: 5'-CTGAAGCAGGAGTGGAACACAG -3'

Table 2. Sequences of siRNA

ON-TARGET plus Human MTH1 (GeneID:4521) siRNA-SMART pool	Target sequence1: 5'- GGGCAAAGUGCAAGAAGGA -3' Target sequence2: 5'- GGAGAGCGGUCUGACAGUG -3' Target sequence3: 5'- GAAAUUCCACGGGUACUUC -3' Target sequence4: 5'- UGUUUUGAGUUCGUGGGCGA -3'
ON-TARGET plus Human OGG1 (GeneID:4968) siRNA-SMART pool	Target sequence1: 5'- CGACAAGACCCCAUCGAAU -3' Target sequence2: 5'- GGACAAUCUUUCCGGUGGA -3' Target sequence3: 5'- GCUCAGAAAUUCCAAGGUG -3' Target sequence4: 5'- UACCCUGGCUCAACUGUAU -3'
ON-TARGET plus Human NEIL1 (GeneID:79661) siRNA-SMART pool	Target sequence1: 5'- UACGAAACCUAGCGGAUAA -3' Target sequence2: 5'- GACCAGAGGUUCUCAAUG -3' Target sequence3: 5'- UGACAUCCCAUCCUUGGAA -3' Target sequence4: 5'- GGACCAAGCUGCAGAAUCC -3'
ON-TARGET plus Non-targeting siRNA	5'- UGGUUUACAUGUCGACUAA -3'

Table 3. Sequences for EMSA

RSV RNA probe - F (genome sense)	5'- UGCGUUGGUCCUUGUUUUUGGACAUGUUUGCAUUUGCCCC-3'
RSV RNA probe - R (mRNA sense)	5'-GGGGCAAUGCAAACAUGUCCAAAAACAAGGACCAACGCA Cy5 -3'
RSV RNA probe - 8- oxo(r)Gua - R (mRNA sense)	5'- GGG G* CAAUGCAAACAUGUCCAAAAACAAGGACCAACGCA Cy5 -3'
DNA probe – F (positive control)	5'-AGAGAAGAAGAAGAA G* AGATGGGTTATTCGAACTAGC-3'
DNA probe – R (positive control)	5'- Cy5-GCTAGTTCGAATAACCCATCTCTTCTTCTTCTTCT-3'
RSV DNA probe – intergenic (genome sense)	5'-Cy5 AATGTTATTGTTAGTCTTGATATCCTAGTTCATTGTTATG-3'
RSV DNA probe – 5'UTR (genome sense)	5'-Cy5 TGCGTTGGTCCTTGTTTTTGGACATGTTTGCATTGCCCC-3'
RSV DNA probe – 3'UTR (genome sense)	5'-Cy5 TTTTAAAGTAACTACTGGCGTGGTGTGTTGGGTGGAGATG-3'

FIGURE 1

Oxidative modification to guanine nucleotide in RSV RNAs in the cytoplasmic inclusion bodies

a. Enrichment of OGG1 on viral RNAs and immunoprecipitation by antibody to 8-oxoGua.

Parallel cultures of OGG1 proficient hSAECs were infected with RSV (MOI = 1), protein-RNA complexes were cross-linked and samples IP-ed using antibodies to OGG1, M2-1 or N proteins. In parallel, RNAs were isolated and then IP-ed with 8-oxoGua-specific antibody. Levels of vRNA in immuno-precipitates were determined by qRT-PCR. The experiment was done three times (n = 3).

b,c,d. Oxidatively modified RNAs co-localized with virus-induced cytoplasmic inclusion bodies.

RSV infected hSAECs were fixed and immunochemically-stained with Abs to nucleoprotein N protein, 8-oxoGua, 3-nitrotyrosine or 2,4-Dinitrophenylhydrazine (protein carbonyls). Randomly

767 selected fields were photographed using an OLYMPUS Microscope System BX53P, with a built-
768 in digital CCD color camera (DP73WDR). Magnification: 60X; scale bar: 40 μ m. The
769 experiments were performed twice with two biological replicates each. IB, inclusion body; 3-NT,
770 3-Nitrotyrosine; DNP, Dinitrophenol, IBs, inclusion bodies, Abs, antibodies, IP-ed,
771 immunoprecipitated
772
773
774

FIGURE 2

Decrease in Gua oxidation or silencing OGG1 expression lowers output of RSV infection

a Decrease in ROS levels by antioxidants lowers 8-oxo(r)Gua-containing mRNA coded by G

gene. RSV-infected hSAEC were mock- or treated with NAC, EUK-8 (concentrations as indicated). mRNAs were isolated, then immunoprecipitated by 8-oxoGua Ab. G mRNA levels in immunoprecipitated RNAs were determined by qRT-PCR. Fold enrichment was calculated relative to IgG control⁷⁴.

b, c, d Decrease in oxidatively modified Gua levels in viral mRNAs lowers RSV output. RSV-infected hSAECs were treated with AOs or with ribavirin (as control, concentrations as indicated in Figs). Levels of mRNAs (b) and viral genome (c) were determined by qRT-PCR. RSV

progeny (d) was determined by plaque assay. NS1, non-structural protein 1; N, nucleoprotein, G, attachment glycoprotein G; F, fusion protein; L, viral RNA-dependent RNA polymerase.

e f g OGG1 silencing led to a decrease in viral mRNA and protein levels. OGG1-silenced hSAECs (e) or OGG1 knockout (CRISPR-Cas9 genomic editing; hSAEC^{OGG1^{-/-}}) and corresponding control (hSAECs and puromycin resistant hSAECs) cells (f) were infected with RSV, and changes in mRNAs levels from NS1, N, G, F and L genes were determined by qRT-PCR (e, f). Protein levels of N, and M2-1 were determined by Wb (g). Inset to panel e and f: OGG1 expression at RNA and protein levels. Right panel to g, quantification of band intensities (n = 3; Image J v1.51). NS1, N, G, F and L, as in legend to b.

h i OGG1-silenced or knockout cells do not support RSV genome replication. OGG1 depleted and knockout hSAECs (and corresponding controls, as in legend to e, f) were infected with RSV and genome levels were determined by qRT-PCR.

j k Decreased yield of RSV in knockout hSAECs is restored by transgenic expression of OGG1. (j) hSAEC transfected with control (NC) or siRNA to OGG1 were RSV-infected. (k) hSAEC^{OGG1^{+/+}}, hSAEC^{OGG1^{-/-}}, and hSAEC^{OGG1^{-/-}} expressing OGG1 (Tg-OGG1^{+/+}) were infected with RSV. Titers were determined by plaque assay. Right panel to k shows levels of OGG1. In Figs b, c, d, e, f, g, j, k, *P* values are of two biological replicates with two parallels.

FIGURE 3

Inhibition of OGG1 binding to 8-oxo(r)Gua in viral RNA, lowers output of RSV infection in cultured cells and lungs

a b c Active site inhibitor of OGG1, TH5487 decreases viral mRNA, protein and genome levels.

hSAECs were RSV-infected and mock- or TH5487-treated (10 μM). mRNA and genome level (a, b) and protein (c) levels were determined by qRT-PCR and Wb analysis, respectively. Right panel to c: band intensities (Image J v1.51). *P* values are from two biological replicates with two parallels. TH5487, 4-(4-Bromo-2-oxo-3H-benzimidazol-1-yl)-N-(4-iodophenyl) piperidine-1-carboxamide⁴⁹. RNA coding for NS1, non-structural protein 1; N, nucleoprotein, G, attachment glycoprotein G; F, fusion protein and L, viral RNA-dependent RNA polymerase.

d OGG1 inhibitor decreases yield of RSV progeny.

RSV-infected hSAECs were treated with solvent or TH5487 (10 μM), TH2840 (10 μM; inactive TH5487 analog), or O8 (10 μM, an inhibitor of OGG1' glycosylase activity). RSV titer was determined by plaque assay (n = 3). TH5487, as in a, TH2840, 4-(2-oxo-2,3-dihydro-1H-1,3-benzodiazol-1-yl)-N-phenylpiperidine-1-carboxamide⁴⁹; O8, 3,4-Dichlorobenzo[b]thiophene-2-carbohydrazide⁵⁰.

e f OGG1 agonist TH5487 decreases RSV replication in lungs.

Mice (n = 6) were RSV-infected by intrapulmonary challenge, and TH5487 was added via intraperitoneal route. At the indicated time points, mRNAs levels from N, G, and F genes (e) as well as genome levels (f) were determined by qRT-PCR. *P* values are from one experiment with six replicates. hpi, hours post-infection. N, G, F, as in legend to a.

5'-UTR of viral mRNAs are the hot-spot of Gua oxidation

a Graphical depiction of G-gene and locations of sequences that served for probe design (sequences are in Table 1).

b "Hot-spot" of 8-oxo(r)Gua generation is at the 5'-UTR of mRNA. RNAs isolated from infected cells were mixed individually with single-stranded Cy5-labeled DNA probes (40 nt long) complementary to genome, 5'-end (containing 5-UTR), 3'-end (containing 3-UTR) of mRNA coding for G-protein and hybridized. Non-annealed sequences were removed by Mung bean nuclease⁴⁸. Cy5-DNA:8-oxo(r)Gua-containing vRNA hybrids were captured by OGG1 (lanes 4,5,6) by using EMSA. RNaseH-treatment degraded DNA:RNA hybrids (DNA:RNA hybrids, lane 7,8,9). There were no interaction between OGG1 and probes (lanes 1,2,3).

c d Concentration-dependent OGG1 binding (c) and its inhibition by TH5487 (d) to DNA:RNA hybrids [(d)C:8-oxo(r)Gua-containing RNA].

e OGG1 binds only to RNA:RNA hybrid-containing 8-oxo(r)Gua (Lanes 6,7 and 8).

f TH5487 prevents binding of OGG1 to dsRNA containing 8-oxo(r)Gua [(r)C:8-oxo(r)Gua].

g OGG1 binding to labeled (Cy-5)dsRNA is competed out by "cold" dsRNA [(r)C:8-oxo(r)Gua].

Arrow: RNA:RNA hybrid [(r)C:8-oxo(r)Gua]. In e,f,g OGG1 was incubated with (r)C:8-oxo(r)Gua RNA-RNA hybrids, and mixtures were subjected to EMSA.

843 **h** Lack of OGG1' enzymatic activity(es) on dsRNA [8-oxo(r)Gua:(r)C]. Excision assays were
844 performed using 8-oxo(r/d)Gua-containing dsDNA and dsRNAs. Lanes 1-4 dsRNA [8-
845 oxo(r)Gua:(r)C]; Lanes 5-8: dsDNA [8-oxo(d)Gua:(d)C]. Representative images are shown in c,
846 d, e, f, g, h (n = 3). In panel b to h images were captured by using Amersham™ Imager 680.
847 Arrows: Cy5-DNA:vRNA hybrid containing 8-oxo(r)Gua.
848

849 **FIGURE 5**

850
851 **Physical interactions between OGG1 and M2-1 in RSV-infected cells and *in vitro***

a b c d e Physical interaction of OGG1 with M2-1. FLAG-tagged OGG1-expressing cells were RSV-infected, lysed and lysates were subjected to IP using Ab to FLAG(OGG1). IP-ed proteins were immunoblotted by using polyclonal antibody to RSV proteins. Lower panel, expression of OGG1 in RSV-infected cells (a). Direct interaction between OGG1 and M2-1 proteins. Equimolar GST-OGG1 and M2-1 were incubated and then immunoblotted using Abs to OGG1 or M2-1 (c, d). Lack of interaction between MTH1 and M2-1 proteins, shown in panel (e).

f Protein-protein interaction between OGG1 and M2-1 occur in IBs *in cellulo*. hSAECs were RSV-infected for increasing length of time, fixed, then immuno-stained with Abs to OGG1 and M2-1. Randomly selected fields were microscopically examined and photographed using an OLYMPUS Microscope System BX53P with a built-in digital CCD color camera. Magnification: 60X; scale bar: 20 μ m. Most-right column: Pearson fluorophores-moment correlation coefficient (Alexa 594 and Alexa 488) was performed using Image J v1.51 software⁵⁷.

g OGG1 accumulates in the cytoplasm of RSV-infected cells. RSV-infected hSAECs were fractionated at the times indicated. Equal amounts of cytoplasmic (CE) and nuclear extract (NE) were subjected to Wb analysis.

h Physical interaction between OGG1 and M2-1 as shown by proximity ligation assays (PLA). RSV-infected cells were processed, and PLAs were performed. Cells were photographed as described in panel f, g, h. Magnification: 60X; scale bar: 20 μ m. Upper panels: PLA signals are localized to cytoplasmic compartments of infected cells. PLA signals were absent when OGG1 (middle panels) or M2-1 (lower panels) specific primary antibodies were separately incubated with cells and then the species-specific secondary antibodies conjugated to DNA oligonucleotides were added.

i Physical interaction between OGG1 and M2-1 within IB as shown by PLA. RSV-infected cells were incubated with Ab to OGG1 and/or M2-1, and ligated DNA oligonucleotides conjugated to secondary Abs were amplified. Cells were photographed as described in panel f, g, h. Magnification: 60X; scale bar: 10 μ m.

FIGURE 6

OGG1 bound to 5'-UTR of nRNA via 8-oxo(r)Gua facilitates M2-1' interaction with transcriptionally active site

a M2-1 lacks ability to recognize 8-oxo(r)Gua in single and double stranded RNAs. Probes $\pm$ 8-oxo(r)Gua were incubated with M2-1, and the magnitude of binding was determined by EMSA.

884 Lanes 1, ssRNA: (r)G); 2, ssRNA containing 8-oxo(r)Gua; lane 3, dsRNA [(r)Gua:(r)C]; lane 4:
885 dsRNA containing 8-oxo(r)Gua, [8-oxo(r)Gua:(r)C]; arrowhead left and right: M2-1 tetramer-RNA
886 complexes. Right panel, graphical depiction of band intensities ($n = 3$, Image J v1.5.1). *** $p <$
887 0.001.

888 **b** Identification of OGG1 and M2-1 8-oxo(r)Gua:(r)C complexes by super-shift assays. OGG1-
889 and M2-1-8-oxo(r)Gua:(r)C complexes were incubated with IgG (lane 1), Ab to OGG1 (lane 2)
890 or Ab to M2-1 (lane 3), and mixtures were subjected to EMSA.

891 **c** M2-1 binding to 8-oxo(r)Gua:(r)C is OGG1 concentration dependent. dsRNA probes were
892 incubated with increasing amounts of OGG1, and then M2-1 (8 pmol) was added. M2-1 binding
893 in the presence of OGG1 to 8-oxo(r)Gua:(r)C was determined by EMSA (left panel). Lanes 1 to
894 5, dsRNA [(r)Gua:(r)C]; lane 6 to 10, dsRNA containing 8-oxo(r)Gua [8-oxo(r)Gua:(r)C]. Right
895 panel: band intensities were quantified by Image J v1.51). P values are as in right panel ($n = 3$).

896 **d** Binding kinetics of M2-1 to 8-oxo(r)Gua:(r)C $\pm$ OGG1. Equimolar amounts of OGG1, M2-1
897 and probe [8-oxo(r)Gua:(r)C] were mixed for various lengths of time, and EMSA was
898 performed. Right panel, graphical depictions of band intensities (Image J v1.51).

899 **e** TH5487 decreases OGG1' ability to facilitate M2-1 8-oxo(r)Gua:(r)C interaction. Increasing
900 concentrations of inhibitor were pre-incubated with OGG1, then 8-oxo(r)Gua:(r)C and M2-1
901 were added before performing EMSA. Right panel, graphical depiction of M2-1 binding ($n = 3$).

902 **f** TH5487 alone has no effect on M2-1' binding. Increasing concentrations of TH5487 were
903 incubated with M2-1. Then probe [8-oxo(r)Gua:(r)C] was added and EMSA was performed.
904 Right panel, graphical depiction of band intensities.

905 **g** OGG1 binds 8-oxo(r)Gua-containing temperature-sensitive secondary structure of viral RNA.
906 OGG1 was incubated with 8-oxo(r)Gua containing ssRNA before and after heat denaturation.
907 Binding was assessed by EMSA. In controls, dsRNA (8-oxo(r)Gua:C) was used. Lane 1 to 4,
908 heat denatured ssRNA; lanes 5 to 8: un-denatured RNA, lane 9-12: 8-oxo(r)Gua:(r)C probe.
909 Right panel, structure of the 40 nt long RNA probe as predicted by "RNAstructure" software⁵⁹.

910 **h** Schematic diagram showing roles of OGG1 in mRNA synthesis. OGG1 recognizes 8-
911 oxo(r)Gua, the ROS-generated epitranscriptomic mark in the 5'-UTR of mRNA, while paired with
912 the 3'-end of the gene and functions as a nucleation site for binding of the transcriptional
913 elongation factor M2-1. OGG1 remain bound to the secondary structure of mRNA (Fig. g).
914 Structure of 5'-end of G-mRNA as predicted as in "g". Secondary structure of G-mRNA is shown
915 Supplementary Fig. 4i. OGG1 agonist (TH5487) inhibits binding of OGG1 to 8-oxo(r)Gua in
916 RNA-RNA hybrid. Lower panel: G-gene is shown. GS, gene start (red) and GE, gene end

(green). OGG1, 8-oxoguanine DNA glycosylase1; TH5487, active site inhibitor of OGG1⁴⁹.

RdRp, RNA dependent RNA polymerase, m7Gppp, methyl guanosine cap.

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