RNA sensor MDA5 suppresses LINE-1 retrotransposition by regulating the promoter activity of LINE-1 5'-UTR

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Abstract

**Background:** Type 1 long interspersed elements, or LINE-1, are the only active retroelements in human cells. The retrotransposition process of LINE-1 can trigger the activation of the innate immune system and has been proposed to play a role in the development of several autoimmune diseases, including Aicardi-Goutières syndrome (AGS). In contrast, all known AGS-associated proteins, except MDA5, have been reported to affect LINE-1 activity. Thus, MDA5 is likely to also function as a LINE-1 suppressor.

**Results:** MDA5 was found to potently suppress LINE-1 activity in a reporter-based LINE-1 retrotransposition assay. Although MDA5 is an endogenous RNA sensor able to activate the innate immune system, increased interferon (IFN) expression only weakly contributed to MDA5-mediated LINE-1 suppression. Instead, MDA5 effectively reduced the levels of LINE-1 ORF1p and ORF2p, as a result of the MDA5-mediated downregulation of the promoter activity of LINE-1 5’-UTR, and the subsequent generation of LINE-1 RNA. Interestingly, despite MDA5 being a multi-domain protein, the N-terminal 2CARD domain alone is sufficient to inhibit LINE-1 activity.

**Conclusion:** Our data reveal that MDA5 functions as a promoter regulator and suppresses the promoter activity of LINE-1 5’-UTR. Consequently, MDA5 reduces LINE-1 RNA and protein levels, and ultimately inhibits LINE-1 retrotransposition. In contrast, MDA5-induced IFN expression only plays a mild role in MDA5-mediated LINE-1 suppression. In addition, the N-terminal 2CARD domain was found to be a functional region for MDA5 upon inhibition of LINE-1 replication. Thus, our data suggest that besides being an initiator of the innate immune system, MDA5 is also an effector against LINE-1 activity, potentially forming a feedback loop by suppressing LINE-1-induced innate immune activation.

**Keywords:** LINE-1, MDA5, retrotransposition, 5’-UTR, promoter regulation
**Background**

RNA sensing plays an important role in innate immune activation inside human cells. It senses the presence of non-self RNA (viral RNA, for instance) and activates downstream pathways, leading to the expression of interferons (IFNs) (1). Subsequently, IFN triggers the expression of interferon-stimulated genes (ISGs), many products of which function as suppressors against exogenous pathogens (2, 3). One of the well-studied RNA sensors is melanoma differentiation-associated protein 5 (MDA5). MDA5 belongs to the RIG-I-like receptor (RLR) family and generally detects viral RNA. Once bound to a viral RNA, MDA5 activates the downstream mitochondrial antiviral signalling protein (MAVS) and ultimately triggers the activation of the innate immune system (4). Therefore, it would be easy to imagine that the malfunction of MDA5 can trigger innate immune dysregulation. Mutations in MDA5 have consistently been associated with an autoimmune disease termed Aicardi-Goutières syndrome (AGS) (5, 6).

In addition to MDA5, there are six other proteins that have been linked with AGS (7). Notably, among these proteins, TREX1, SAMHD1, and ADAR1 were found to suppress the replication of the type 1 long interspersed element (LINE-1 or L1) (8-11). However, whether the heterotrimer RNaseH2 formed by the other three AGS-associated proteins inhibits or supports LINE-1 replication remains a topic of controversy (12, 13). In human cells, LINE-1 is the only active retrotransposon that can replicate autonomously (14). A full-length LINE-1 element is 6-kb in length, containing two sense open reading frames (*ORF1* and *ORF2*) that are flanked by the 5’-untranslated region (5’-UTR) functioning as the promoter and the 3’-UTR containing the poly A signal (15, 16). Both coded proteins (ORF1p and ORF2p, respectively) interact with LINE-1 RNA to trigger the assembly of other proteins to form the LINE-1 ribonucleoprotein particle (RNP), which is a fundamental unit for LINE-1 retrotransposition (17, 18). In a recent study, we found that LINE-1 RNP also functions as an endogenous trigger to activate the innate immune system through RNA-sensing pathways (19),
providing direct evidence of the relationship between retrotransposons, innate immune activation, and IFN-based autoimmune diseases.

Interestingly, one of the RNA sensors involved in the above pathways is MDA5 (19). Another intriguing fact is that MDA5 is not only an initiator of increased IFN production, but also an effector of IFN activation (i.e., IFN promotes MDA5 expression) (20). This most likely indicates that, in addition to sensing RNA, MDA5 may possess certain function(s) post-innate immune activation. Given the complex relationship between MDA5 and AGS, LINE-1 and innate immune activation, and IFN and MDA5, we hypothesized that MDA5 may function as a potent LINE-1 suppressor.

Results

MDA5 potently suppresses LINE-1 retrotransposition in cultured cells

To determine whether MDA5 suppresses LINE-1 activity, a widely used EGFP-based LINE-1 retrotransposition assay was introduced (21, 22). This assay involves the use of two plasmids (Fig. 1A). 99 PUR RPS EGFP (L1-RPS) contains a sense LINE-1 element, whose 3’-UTR is interrupted with an antisense EGFP expression cassette, whereas the EGFP signal can only be detected as a post-retrotransposition event (see Methods for details) (Fig. 1B). 99 PUR JM111 EGFP (JM111) was similar to L1-RPS but contained two missense mutations on ORF1p, thus becoming retrotransposition-incompetent (Fig. 1A). With the help of this LINE-1 retrotransposition assay, we found that exogenous MDA5 potently suppressed the replication of L1-RPS in HEK293T cells (Fig. 1C). Parallel tests indicated that MDA5 merely reduced EGFP expression driven by the CMV promoter (Fig. 1D), suggesting that MDA5 indeed targeted LINE-1 for inhibition.

IFN elevation, though unessential, contributes to MDA5-mediated LINE-1 suppression
MDA5 is the initiator of the RNA sensing pathway that triggers the activation of the innate immune system (20, 23). The resulting elevation of IFN may induce the expression of ISGs, some of which have been identified as LINE-1 suppressors (9, 24, 25). To determine whether MDA5 reduced LINE-1 retrotransposition through IFN elevation, we first checked and discovered that exogenous MDA5 expression indeed increased endogenous IFNβ production (Fig. 2A). IFNβ is a secreted protein that is believed to act normally by binding the receptor on the surface of a cell (26). However, the treatment of HEK293T cells with IFNβ did not affect the retrotransposition potency of L1-RPS (Fig. 2B), whereas similar levels of IFNβ significantly reduced HIV infection in THP-1 cells (Fig. 2C). This was consistent with previous observations, wherein the treatment of HEK293T cells with IFNβ did not induce an antiviral effect (27).

Surprisingly, expressing exogenous IFNβ inside HEK293T cells significantly increased the expression levels of ISGs like MX2, OAS2, and OAS3, as indicated in the changes of endogenous mRNA levels with quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 2D). Notably, among these gene products, MX2 has been identified as a potent LINE-1 suppressor (28). As a result, the expression of exogenous IFNβ in HEK293T cells effectively reduced the retrotransposition activity of L1-RPS (Fig. 2E). These data suggest that increasing endogenous IFNβ protein levels in HEK293T cells could inhibit LINE-1 replication, most likely by inducing ISG expression through intracellular pathway(s).

To evaluate the contribution of IFN elevation in MDA5-mediated LINE-1 suppression, we introduced two mutations, MDA5 S88K and K743R, which have been previously reported with reduced efficiency in innate immune activation (29, 30). Through a promoter activity assay based on the expression of firefly luciferase (Fig. 3A), we first confirmed that both MDA5 S88K and K743R were defective in IFNβ elevation in HEK293T cells (Fig. 3B). However, although mildly ineffective, both S88K and K743R still suppressed LINE-1
retrotransposition (Fig. 3C), suggesting that IFN activation may not be the key mechanism in MDA5-mediated LINE-1 suppression. To further test this idea, further mutants were introduced in this study. RNA interaction is essential for the activation of MDA5 and the subsequent pathway to trigger IFN production, whereas amino acid residues such as H927, K950, and K1002 are important for MDA5’s efficacy for binding RNA (31). Consistently, MDA5 mutants like H927E, K950E, or K1002E weakly activated the innate immune system (Fig. 3D). However, similar to S88K and K743R, these three mutants inhibited LINE-1 retrotransposition in HEK293T cells, with a slightly weakened potency compared to that of wild-type MDA5 (Fig. 3E). Therefore, although IFN elevation and the subsequent increased levels of ISG proteins contribute to MDA5-mediated LINE-1 suppression, a more effective mechanism should be applied.

**Levels of LINE-1 proteins are reduced in the presence of MDA5**

Next, we investigated the mechanism underlying the MDA5-mediated suppression of LINE-1. It is worth noting, however, that despite MDA5 interacting with LINE-1 RNA (19), the above results with H927E, K950E, or K1002E mutants indicated that RNA binding may not contribute to MDA5’s potency in LINE-1 regulation. Therefore, we first tested whether MDA5 could affect the LINE-1 protein levels. L1-1FH was constructed based on the LINE-1 sequence, expressing an ORF1p with an HA and a Flag tag at its C-terminus (24) (Fig. 4A). The co-transfection of L1-1FH and MDA5-expressing vector in HEK293T resulted in a reduction of ORF1p levels expressed from L1-1FH (Fig. 4B). This was further confirmed by the observation that exogenous MDA5 expression led to the downregulation of endogenous ORF1p levels (Fig. 4C).

We then introduced another LINE-1 construct, L1-2TAP, into this study to further examine whether MDA5 could affect the expression/stability of ORF2p. Similar to L1-1FH,
L1-2TAP was also constructed based on the LINE-1 sequence, but expressing an ORF2p with a tandem affinity purification (TAP) tag at its C-terminus (Fig. 4A). Again, the expression of exogenous MDA5 protein reduced the levels of both ORF1p and ORF2p expressed from L1-2TAP (Fig. 4D).

To further validate whether the MDA5-mediated reduction of LINE-1 proteins was due to the MDA5-induced production of IFN, similar tests were conducted with MDA5 mutants ineffective in innate immune activation. As shown in Figure 4E and 4F, mutations compromising MDA5’s potency in activating the RNA sensing pathway or binding RNA did not affect the efficacy of MDA5 in suppressing the expression of both ORF1p and ORF2p in HEK293T cells. These results indicate that MDA5 is potent in reducing the levels of LINE-1 ORF1p and ORF2p, to which MDA5-mediated innate immune activation does not contribute.

**MDA5 suppresses the promoter activity of LINE-1 5’-UTR**

It is worth noting that LINE-1 uses a single transcript to express both ORF1p and ORF2p proteins (Fig. 5A). Thus, a simultaneous reduction of both ORF1p and ORF2p expression most likely indicated a change in the levels of LINE-1 RNA. To test this hypothesis, L1-RPS and MDA5 were co-transfected into HEK293T cells, and the levels of L1-RPS mRNA were extracted and reverse transcribed at 12 h post-transfection, as previously reported (32). As shown in Figure 5B, the exogenous expression of wild-type MDA5 significantly reduced the levels of L1-RPS mRNA. Interestingly, MDA5 mutants incapable of binding RNA maintained their potency in reducing L1-RPS mRNA (Fig. 5C). Notably, all the tested wild-type MDA5 and their mutants were effective in suppressing LINE-1 ORF1p and ORF2p expression (Fig. 4). Therefore, these data confirmed that MDA5 compromises the expression of ORF1p and ORF2p by reducing LINE-1 mRNA levels.
Intriguingly, the abovementioned data also suggested that RNA destabilisation may not be the reason for MDA5-mediated LINE-1 mRNA reduction, as MDA5 mutants that were unable to interact with RNA still showed efficiency in downregulating LINE-1 mRNA (Fig. 5C). As such, we focused on the LINE-1 5’-UTR, and tested whether MDA5 could affect the promoter activity of LINE-1 5’-UTR in a firefly luciferase reporter assay (10) (Fig. 5D). The results are shown in Figure 5E, which indicates that the luciferase activity detected in HEK293T cells was lowered in the presence of MDA5, indicating that MDA5 indeed compromised luciferase expression driven by the LINE-1 5’-UTR. Again, MDA5 mutants that were effective in LINE-1 suppression were also competent in 5’-UTR suppression (Fig. 5F), confirming that at least 5’-UTR suppression is major contributor to MDA5-mediated LINE-1 inhibition.

**The 2CARD region is important for MDA5-mediated LINE-1 suppression**

The human MDA5 protein is a 1,025-amino acid-long protein with multiple regions (Fig. 6A). To identify the region that is essential for MDA5-mediated LINE-1 suppression, continuous deletions were made from the N- or C-terminus of MDA5 (Fig. 6A), and the resulting mutants were subjected to LINE-1 retrotransposition assay. Interestingly, C-terminal deletions that left the 2CARD (tandem caspase activation and recruitment domain) region untouched only weakly affected MDA5’s potency in LINE-1 suppression (Fig. 6B), whereas deleting 2CARD alone significantly compromised the efficacy of MDA5 to suppress LINE-1 (Fig. 6C). Consistently, the 2CARD region alone was sufficient to reduce the promoter activity of LINE-1 5’-UTR (Fig. 6D), whereas losing 2CARD almost completely sabotaged the capability of MDA5 to suppress LINE-1 5’-UTR (Fig. 6E). These data suggest that the 2CARD region is important for MDA5-mediated LINE-1 suppression, and further confirmed that suppressing the 5’-UTR is a major mechanism for MDA5 to inhibit LINE-1 retrotransposition.
**Discussion**

Recent studies have increasingly indicated that the activity of endogenous retroelements, such as LINE-1, is associated with autoimmune diseases, such as AGS (8, 19), Sjögren’s syndrome (33), and systemic lupus erythematosus (34), most likely by activating the innate immune system (35-39). Consistently, many AGS-associated proteins, such as TREX1, SAMHD1, and ADAR1, are potent LINE-1 suppressors (8-11). In this study, we identified another AGS-associated protein, MDA5, which is a LINE-1 regulator. Indeed, MDA5 effectively reduced the retrotransposition activity of L1-RPS in HEK293T cells. Further investigations revealed that MDA5 decreased the protein levels of both ORF1p and ORF2p, as the presence of MDA5 lowered the levels of LINE-1 RNA. Interestingly, although MDA5 was found to be capable of binding LINE-1 RNA to induce the activation of the innate immune system (19), RNA binding capacity was not the reason for the MDA5-induced reduction in LINE-1 RNA. Instead, MDA5 suppressed the promoter activity of LINE-1 5’-UTR with its N-terminal 2CARD region, which ultimately resulted in the inhibition of LINE-1 retrotransposition.

As an RNA sensor, MDA5 triggers the expression of endogenous IFN. Surprisingly, despite the widely accepted idea that IFNs are secreted proteins that normally function by binding IFN receptors on the cell surface (26), treating HEK293T cells with extracellular IFNβ only weakly affected LINE-1 replication. Instead, expressing IFNβ inside HEK293T cells significantly suppressed LINE-1 replication, suggesting the existence of intracellular pathway(s) of IFN-inducing ISG expression. Indeed, further examination confirmed that intracellular IFNβ could increase the expression of some ISGs, whereas some of these gene products are potent LINE-1 suppressors. However, it appeared that activating IFN production only had a minor contribution to MDA5-mediated LINE-1 suppression, as MDA5 mutants that failed in trigging innate immune activation had the greatest inhibitory effect on LINE-1 activity.
This, in contrast, correlated with the fact that all these mutants were still fully functional in reducing the promoter activity of LINE-1 5’-UTR, as well as the subsequent expression of LINE-1 proteins, confirming that MDA5 represses LINE-1 retrotransposition mostly through the disruption of the efficacy of LINE-1 5’-UTR.

The abovementioned data suggest that besides sensing RNA and triggering IFN production through the RNA sensing pathway, MDA5 may also have other role(s). In other words, in addition to being an initiator, MDA5 may also be an effector or even a regulator of the innate immune activation, which is consistent with the fact that MDA5 is one of the ISGs. Indeed, in this study, MDA5 potently suppressed the promoter activity of LINE-1 5’-UTR through a mechanism independent of IFN elevation. Considering that endogenous retroelements are believed to be fossils of ancient retroviruses (15), it is possible that, after being upregulated post-innate immune activation, MDA5 could function as a restriction factor to suppress gene expression from exogenous pathogens. It is also likely that elevated MDA5 may affect the levels of host RNA and/or proteins, to either generate additional anti-pathogen defence or regulate the levels of innate immune activation. In fact, MDA5 was first discovered through its ability to induce apoptosis (20, 40), which leads to the elimination of infected cells (41). In contrast, it was previously reported (and confirmed in our study) that expressing exogenous MDA5 alone can increase endogenous IFN levels (42), constituting a feed-forward loop. Our study also suggested another pathway through which, due to lower levels of LINE-1 RNA and proteins, as well as subsequent LINE-1 retrotransposition, MDA5 downregulated LINE-1-induced innate immune activation, forming a feedback control of the innate immune system, at least to some degree.

However, surprisingly, the 2CARD region is important for MDA5-mediated LINE-1 suppression, because the oligomerization of the same region is also essential for the activation of MAVS, as well as the subsequent RNA-sensing pathway, leading to the production of IFN
Indeed, 2CARD by itself is sufficient to reduce the promoter activity of the 5’-UTR, as well as the expression levels of both LINE-1 proteins. Interestingly, it appeared that 2CARD may activate the RNA sensing pathway and suppress LINE-1 activity through different interfaces. The amino acid residue S88 in the first CARD region to function as a switch for the MDA5-mediated RNA sensing pathway, which is abolished when S88 is phosphorylated by phosphatase PP1 or mutated to amino acids, such as aspartic acid (D) and glutamic acid (E), which mimic phosphorylation (29). Consistently, we also confirmed that the S88E mutation significantly compromises MDA5’s ability to activate the IFNB promoter. However, S88E did not affect MDA5-mediated LINE-1 suppression. Indeed, MDA5 S88E is fully capable of reducing the promoter activity of LINE-1 5’-UTR and subsequently decreasing levels of LINE-1 ORF1p and ORF2p. This not only validates the observation that MDA5 could inhibit LINE-1 retrotransposition through an IFN-independent pathway, but also suggests that different critical residues are used by MDA5 to activate the innate immune system or repress LINE-1 activity.

Conclusions

MDA5 was found to be a potent LINE-1 suppressor. As an initiator of the RNA sensing pathway, MDA5 can trigger the production of IFN and subsequent ISGs, some of which function as LINE-1 inhibitors. In contrast, as an effector of innate immune activation, MDA5 can also suppress the promoter activity of LINE-1 5’-UTR in an IFN-independent mechanism, leading to the reduction of LINE-1 RNA and proteins, as well as the inhibition of LINE-1 retrotransposition. Although the same 2CARD region is critical for MDA5-mediated innate immune activation and promoter suppression, different interfaces or at least different amino acid residues are used in both functions. Thus, by uncovering the mechanism of MDA5-mediated LINE-1 suppression, we have revealed that promoter suppression is a novel function
of MDA5, the study of which will extend our understanding of the biological functions of MDA5.

Materials and methods

Cells and plasmids

Human embryo kidney 293T (HEK293T) cells were cultured with DMEM (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA), in an incubator containing 5% CO\textsubscript{2} under 37 °C.

The \textit{IFIH1} (coding MDA5) gene was retrieved from HEK293T cells through reverse transcription and polymerase chain reaction (PCR), and inserted into VR1012 (43), with a myc-tag at the protein's C-terminus. Mutations and deletions of MDA5 were achieved using standard techniques. All mutants constructs were sequence confirmed.

The retrotransposition-competent vector 99 PUR RPS EGFP (L1-RPS) (22), the retrotransposition incompetent 99 PUR JM111 EGFP (JM111) (22), pc-L1-1FH (L1-1FH) (24), pGL3.1-5’-UTR-luciferase (5UTR-Luc) (10), pGL3.1-IFNB-luciferase (IFNB-Luc) (44), and were have been described previously. The pc-L1-2TAP (L1-2TAP) was constructed into pCDNA3.1.

All transfections were performed with Lipofectamine 3000 (Sigma-Aldrich, USA) and Opti-MEM (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

Antibodies

The following antibodies were used to detect protein expression in this study: anti-tubulin (Transgen, China), anti-histone (GenScript, USA), anti-HA (Thermo Fisher Scientific, USA), anti-myc from (Sigma-Aldrich, USA), and anti-IFNb1 from (Abcam, UK). All antibodies were used according to the manufacturer protocols.
**LINE-1 retrotransposition assay**

The LINE-1 retrotransposition assay has been previously described (9, 22). In brief, the LINE-1 plasmid was transfected into HEK293T cells at 2 μg per well in 12-well plates together with VR1012 or one of the test plasmids. The cells were selected by the addition of puromycin (final concentration, 5 μg/ml) at 48 h post-transfection. GFP-positive cells were examined 48 h later by flow cytometry using a FACSCalibur cytometer. Gating exclusions were based on background fluorescence of the retrotransposition-incompetent JM111. At least 20,000 single-cell events per sample were gated and analysed using FlowJo (version x.0.7).

**Luciferase assay**

The dual-luciferase reporter system from Promega was used to detect potential effects on promoter activity. In brief, luciferase-expressing vectors were transfected into HEK293T cells. At 48 h post-transfection, the cells were lysed with TransDetect Double-Luciferase Reporter Assay Kit (Transgen, China) and tested with a Fluoroskan Ascent™ FL Microplate Fluorometer and Luminometer (Thermo Fisher Scientific, USA) according to manufacturer's protocol for luciferase detection. The empty vector pGL3-basic was used as negative control for luciferase activity, and the results were used to remove background noise (and are not shown).

**Western blotting**

Interested cells were lysed in 1× loading buffer (80 mM Tris, pH 6.8, with 2.0% sodium dodecyl sulfate [SDS], 10% glycerol, 100 mM dithiothreitol, and 0.2% bromophenol blue). The samples were boiled for 30 min, and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto nitrocellulose membranes (GE Whatman, UK) and probed with various primary antibodies against the indicated proteins (see the figure legends). Secondary antibodies were alkaline phosphatase-
conjugated anti-rabbit (Jackson, USA) and anti-mouse (Jackson, USA), and staining was carried out with 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Roche, Switzerland) and nitroblue tetrazolium (NBT) (Sigma-Aldrich, USA).

**RNA isolation, reverse transcription, and PCR amplification**

Extraction of total RNA from transfected cell samples was performed using FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, China) according to the manufacturer’s instructions. The cDNA was generated using MonSciptTM RT III All-in-One Mix (with DNase) (Monad, China), according to the provided instructions. PCRs were performed using 2*Phanta Max Master Mix (Vazyme, China) according to the manufacturer’s instructions.

The primers used for PCR were as follows: ACTB, forward (5’-ACCGAGCGCGGTACGTACG-3’) and reverse (5’-CTTAATGTACGCACGATTTCC-3’); L1-1F (5’-CAAAGACCGATATTCTCCTCA-3’) and EFGP-2F (5’-ACTACCTGAGCCACGTTCC-3’).

**Detection of IFNβ, OAS2, OAS3 and MX2 production**

At 48 h post-transfection, the cells were subjected to quantitative real-time PCR (qRT-PCR) to determine the changes in endogenous mRNA levels. RNA from the samples of interest was first extracted with a FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, China) and then subjected to reverse transcription with MonSciptTM RT III All-in-One Mix (with DNase) (Monad, China). The qRT-PCR was performed with TransStart Top Green qPCR SuperMix (Transgen, China) and specific primers. The reaction was performed under the following conditions as suggested by the manufacturer: 94 °C for 30 s, followed by 40 cycles at 94 °C for 10 s and 60 °C for 30 s, followed by a dissociation protocol. Single peaks in the melting curve analysis indicated specific amplicons. The primers used for qRT-PCR were as follows: ACTB, forward (5’-ACCGAGCGCGGTACGTACG-3’) and reverse (5’-CTTAATGTACGCACGATTTCC-3’); IFNB, forward (5’-
CACGACAGCTCTTTCCATGA-3’) and reverse (5’-AGCCAGTGCTCGATGAATCT-3’); OAS2, forward (5’-TTCTGCCTGCACCACTCTCTCAACGA-3’) and reverse (5’-GCCAGTCTTCAGAGCTGTGCCTTTG-3’); OAS3, forward (5’-CCGAACCTGTCCTGGGCCTGATCC-3’) and reverse (5’-CCCATTCCCCAGGTCCCATGTGG-3’); MX2, forward (5’-CAGAGGCAGCGGAATCGTAA-3’) and reverse (5’-TGAAGCTCTAGCTCGGTGTTC-3’). The endogenous mRNA levels of ACTB were used as the loading control and are not shown unless otherwise indicated.

**HIV-1 production and infection**

NL4-3 Δenv EGFP (a generous gift of Dr. R. Siliciano) (45) was co-transfected together with pHEF-VSVG (from Dr. L.-J. Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) into HEK293T cells. The medium was changed at 24 h post-transfection, and the supernatant was collected after an additional 24 h. For viral infection, equal volumes of viruses were used to infect THP-1 cells seeded onto a 12-well plate in the presence of DEAE (Sigma-Aldrich, USA) at a final concentration of 20 µg/ml. The cells were collected and analysed for GFP expression using a FACSCalibur (BD Biosciences, USA); 20,000 single-cell events per sample were gated and analysed using FlowJo (version x.0.7).

**Quantification and statistical analysis**

Flow cytometry data are presented as the means ± SD of three replicates within one experiment and are representative of at least three independent experimental repeats. Data were analysed using unpaired, two-tailed, Student's t-tests. Differences in means were considered statistically significant at p<0.05. Analyses were performed using Microsoft Excel software (Redmond, USA).
Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ Contributions

JY and YZ carried out all kinds of experiments involved in this study, while JD focused on IFNβ-induced LINE-1 suppression, YW on MDA5 deletions and their impacts on MDA5-mediated LINE-1 suppression, SW on firefly luciferase-based promoter activity assays, QW on PCR and qRT-PCR, and XZ on MDA5-mediated reduction of LINE-1 proteins; JY, YZ, JD, WX, and KZ analysed data; WX and KZ designed and supervised the project, and wrote the manuscript with help from all other authors. All authors have read and approved the final manuscript.
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References

1. Roers A, Hiller B, Hornung V. Recognition of Endogenous Nucleic Acids by the Innate Immune System. Immunity. 2016;44(4):739-54.
2. Lazear HM, Schoggins JW, Diamond MS. Shared and Distinct Functions of Type I and Type III Interferons. Immunity. 2019;50(4):907-23.
3. Schoggins JW. Interferon-Stimulated Genes: What Do They All Do? Annu Rev Virol. 2019;6(1):567-84.
4. Dias Junior AG, Sampaio NG, Rehwinkel J. A Balancing Act: MDA5 in Antiviral Immunity and Autoinflammation. Trends Microbiol. 2019;27(1):75-85.
5. Oda H, Nakagawa K, Abe J, Awaya T, Funabiki M, Hijikata A, et al. Aicardi-Goutieres syndrome is caused by IFIH1 mutations. Am J Hum Genet. 2014;95(1):121-5.
6. Rice GI, del Toro Duany Y, Jenkinson EM, Forte GM, Anderson BH, Ariau G, et al. Gain-of-function mutations in IFIH1 cause a spectrum of human disease phenotypes associated with upregulated type I interferon signaling. Nat Genet. 2014;46(5):503-9.
7. Crow YJ, Manel N. Aicardi-Goutieres syndrome and the type I interferonopathies. Nat Rev Immunol. 2015;15(7):429-40.
8. Stetson DB, Ko JS, Heidmann T, Medzhitov R. Trex1 prevents cell-intrinsic initiation of autoimmunity. Cell. 2008;134(4):587-98.
9. Zhao K, Du J, Han X, Goodier JL, Li P, Zhou X, et al. Modulation of LINE-1 and Alu/SVA retrotransposition by Aicardi-Goutieres syndrome-related SAMHD1. Cell Rep. 2013;4(6):1108-15.
10. Li P, Du J, Goodier JL, Hou J, Kang J, Kazazian HH, Jr., et al. Aicardi-Goutieres syndrome protein TREX1 suppresses L1 and maintains genome integrity through exonuclease-independent ORF1p depletion. Nucleic Acids Res. 2017;45(8):4619-31.
11. Orecchini E, Doria M, Antonioni A, Galardi S, Ciafre SA, Frassinelli L, et al. ADAR1 restricts LINE-1 retrotransposition. Nucleic Acids Res. 2017;45(1):155-68.
12. Benitez-Guijarro M, Lopez-Ruiz C, Tarnauskaite Z, Murina O, Mian Mohammad M, Williams TC, et al. RNase H2, mutated in Aicardi-Goutieres syndrome, promotes LINE-1 retrotransposition. EMBO J. 2018.
13. Choi J, Hwang SY, Ahn K. Interplay between RNASEH2 and MOV10 controls LINE-1 retrotransposition. Nucleic Acids Res. 2018.
14. Burns KH, Boeke JD. Human transposon tectonics. Cell. 2012;149(4):740-52.
15. Goodier JL. Restricting retrotransposons: a review. Mob DNA. 2016;7:16.
16. Hancks DC, Kazazian HH, Jr. Roles for retrotransposon insertions in human disease. Mob DNA. 2016;7:9.
17. Wei W, Gilbert N, Ooi SL, Lawler JF, Ostertag EM, Kazazian HH, et al. Human L1 retrotransposition: cis preference versus trans complementation. Mol Cell Biol. 2001;21(4):1429-39.
18. Kulpa DA, Moran JV. Cis-preferential LINE-1 reverse transcriptase activity in ribonucleoprotein particles. Nat Struct Mol Biol. 2006;13(7):655-60.
19. Zhao K, Du J, Peng Y, Li P, Wang S, Wang Y, et al. LINE1 contributes to autoimmunity through both RIG-I- and MDA5-mediated RNA sensing pathways. J Autoimmun. 2018;90:105-15.
20. Kang DC, Gopalkrishnan RV, Wu Q, Jankowsky E, Pyle AM, Fisher PB. mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. Proc Natl Acad Sci U S A. 2002;99(2):637-42.
21. Moran JV, Holmes SE, Naas TP, DeBerardinis RJ, Boeke JD, Kazazian HH, Jr. High frequency retrotransposition in cultured mammalian cells. Cell. 1996;87(5):917-27.
22. Ostertag EM, Prak ET, DeBerardinis RJ, Moran JV, Kazazian HH, Jr. Determination of L1 retrotransposition kinetics in cultured cells. Nucleic Acids Res. 2000;28(6):1418-23.
23. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature. 2006;441(7089):101-5.
24. Goodier JL, Cheung LE, Kazazian HH, Jr. MOV10 RNA helicase is a potent inhibitor of retrotransposition in cells. PLoS Genet. 2012;8(10):e1002941.
25. Li X, Zhang J, Jia R, Cheng V, Xu X, Qiao W, et al. The MOV10 helicase inhibits LINE-1 mobility. J Biol Chem. 2013;288(29):21148-60.
26. Walter MR. The Role of Structure in the Biology of Interferon Signaling. Front Immunol. 2020;11:606489.
27. Bahr A, Singer A, Hain A, Vasudevan AA, Schilling M, Reh J, et al. Interferon but not Mxb inhibits foamy retroviruses. Virology. 2016;488:51-60.
28. Goodier JL, Pereira GC, Cheung LE, Rose RJ, Kazazian HH, Jr. The Broad-Spectrum Antiviral Protein ZAP Restricts Human Retrotransposition. PLoS Genet. 2015;11(5):e1005252.
29. Wies E, Wang MK, Maharaj NP, Chen K, Zhou S, Finberg RW, et al. Dephosphorylation of the RNA sensors RIG-I and MDA5 by the phosphatase PP1 is essential for innate immune signaling. Immunity. 2013;38(3):437-49.
30. Lang X, Tang T, Jin T, Ding C, Zhou R, Jiang W. TRIM65-catalized ubiquitination is essential for MDA5-mediated antiviral innate immunity. J Exp Med. 2017;214(2):459-73.
31. Wu B, Peisley A, Richards C, Yao H, Zeng X, Lin C, et al. Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5. Cell. 2013;152(1-2):276-89.
32. Volkmann B, Wittmann S, Lagisquet J, Deutschmann J, Eissmann K, Ross JJ, et al. Human TRIM5alpha senses and restricts LINE-1 elements. Proc Natl Acad Sci U S A. 2020.
33. Mavragani CP, Sagalovskiy I, Guo Q, Nezos A, Kapsogeorgou EK, Lu P, et al. Expression of Long Interspersed Nuclear Element 1 Retroelements and Induction of Type I Interferon in Patients With Systemic Autoimmune Disease. Arthritis Rheumatol. 2016;68(11):2686-96.
34. Hung T, Pratt GA, Sundararaman B, Townsend MJ, Chaivorapol C, Bhangale T, et al. The Ro60 autoantigen binds endogenous retroelements and regulates inflammatory gene expression. Science. 2015;350(6259):455-9.
35. Ablasser A, Hemmerling I, Schmid-Burgk JL, Behrendt R, Roers A, Hornung V. TREX1 deficiency triggers cell-autonomous immunity in a cGAS-dependent manner. J Immunol. 2014;192(12):5993-7.
36. Gray EE, Treuting PM, Woodward JJ, Stetson DB. cGAS Is Required for Lethal Autoimmune Disease in the Trex1-Deficient Mouse Model of Aicardi-Goutieres Syndrome. J Immunol. 2015.
37. Mackenzie KJ, Carroll P, Lettice L, Tarnauskaite Z, Reddy K, Dix F, et al. Ribonuclease H2 mutations induce a cGAS/STING-dependent innate immune response. EMBO J. 2016.
38. Pokatayev V, Hasin N, Chon H, Cerritelli SM, Sahuja K, Ward JM, et al. RNase H2 catalytic core Aicardi-Goutieres syndrome-related mutant invokes cGAS-STING innate immune-sensing pathway in mice. J Exp Med. 2016;213(3):329-36.

39. Xiao N, Wei J, Xu S, Du H, Huang M, Zhang S, et al. cGAS activation causes lupus-like autoimmune disorders in a TREX1 mutant mouse model. J Autoimmun. 2019.

40. Kovacsovics M, Martinon F, Micheau O, Bodmer JL, Hofmann K, Tschopp J. Overexpression of Helicard, a CARD-containing helicase cleaved during apoptosis, accelerates DNA degradation. Curr Biol. 2002;12(10):838-43.

41. Orzalli MH, Kagan JC. Apoptosis and Necroptosis as Host Defense Strategies to Prevent Viral Infection. Trends Cell Biol. 2017;27(11):800-9.

42. Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, et al. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci U S A. 2004;101(49):17264-9.

43. Hartikka J, Sawdey M, Cornefert-Jensen F, Margalith M, Barnhart K, Nolasco M, et al. An improved plasmid DNA expression vector for direct injection into skeletal muscle. Hum Gene Ther. 1996;7(10):1205-17.

44. Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell. 2005;122(5):669-82.

45. Zhang W, Du J, Evans SL, Yu Y, Yu XF. T-cell differentiation factor CBF-beta regulates HIV-1 Vif-mediated evasion of host restriction. Nature. 2012;481(7381):376-9.
**Figure legends**

**A.** LINE-1 plasmids used in this study. In the retrotransposition-competent plasmid L1-RPS, an anti-sense CMV-EGFP cassette is inserted into the 3’-UTR of LINE-1, whereas the EGFP gene is interrupted by a sense Group I intron. JM111 is based on L1-RPS but contains an R260A/R261A mutation on ORF1p, and is therefore incompetent in retrotransposition. **B.** Schematic representation of the mechanism of the EGFP-based LINE-1 assay. To produce an EGFP signal after transfection, L1-RPS needs to be transcribed through the LINE-1 5’-UTR promoter to generate the RNA, spliced to remove the intron, and then reverse-transcribed and integrated into the genome to finish the replication. Only then can the EGFP signal be generated through the expression of the integrated CMV-EGFP cassette. Any direct transcription of the CMV-EGFP cassette from the plasmid will not produce EGFP because of the un-spliced intron. **C.** Flow cytometry results showing that MDA5 potently suppresses the retrotransposition activity of L1-RPS. HEK293T cells seeded on a 24-well plate were co-transfected with 1 μg of L1-RPS and 25, 75, or 225 ng of MDA5-expressing vector. Cells were collected at 96 h.

**Fig 1. MDA5 suppresses LINE-1 activity in an EGFP-based LINE-1 retrotransposition assay.**
post-transfection to detect EGFP-positive cells through flow cytometry. The western blotting results above indicate the MDA5 protein levels in transfected cells. **D.** Flow cytometry results showing that MDA5 only weakly affected EGFP expression driven by the CMV promoter. HEK293T cells seeded on a 24-well plate were co-transfected with 45 ng of pc-EGFP and 25, 75, or 225 ng of MDA5-expressing vector. Cells were collected at 96 h post-transfection to detect EGFP-positive cells through flow cytometry. Western blotting results above indicate the MDA5 protein levels in transfected cells.

**Fig 2.** Intracellular instead of intercellular IFNβ induces suppressive effects against LINE-1 retrotransposition in HEK293T cells. **A.** qRT-PCR data showing that exogenous MDA5 expression elevates IFNβ expression in HEK293T cells. HEK293T cells were transfected with 45 ng of MDA5-expressing vector and subjected to qRT-PCR at 48 h post-transfection. **B.** Flow cytometry data indicating that the addition of IFNβ in culture medium does not induce LINE-1 suppression in HEK293T cells. HEK293T cells pre-treated with
commercial IFNβ protein (2.5, 25, or 250 U/ml, final concentration) were transfected with 1 μg of L1-RPS. At 96 h post-transfection, the cells were collected to detect EGFP-positive cells through flow cytometry. C. Flow cytometry data showing that IFNβ effectively induces HIV suppression in THP-1 cells. THP-1 cells pre-treated with commercial IFNβ protein (2.5, 25, or 250 U/ml, final concentration) were infected with VSVg-coated NL4-3 Δenv EGFP pseudovirus, and subjected to flow cytometry at 48 h post-infection to detect EGFP-positive cells. D. qRT-PCR results showing that the expression of ISGs can be triggered by intracellular IFNβ. HEK293T cells were transfected with 15, 45, or 135 ng of IFNβ-expressing vector, and subjected to qRT-PCR to detect endogenous RNA levels of MX2, OAS2, and OAS3. Endogenous ACTB mRNA levels were used to equilibrate the results, but are not shown. E. Flow cytometry data suggesting that intracellular IFNβ induces suppressive effects against LINE-1 retrotransposition in HEK293T cells. HEK293T cells seeded on a 24-well plate were co-transfected with 1 μg of L1-RPS and 25, 75, or 225 ng of IFNβ-expressing vector. Cells were collected at 96 h post-transfection to detect EGFP-positive cells through flow cytometry.
Fig 3. IFNβ elevation contributes mildly to MDA5-mediated LINE-1 suppression. A. Schematic representation of the IFNB-Luc plasmid, where the expression of firefly luciferase is driven by the IFNB promoter. B, D. Luciferase activity data indicating the potency of wild-type MDA5 or its mutants in IFNβ elevation. HEK293T cells seeded in a 24-well plate were co-transfected with 100 ng of IFNB-Luc and one of MDA5-expressing vectors (5, 15, or 45 ng). Luciferase activity was tested at 48 h post-transfection. The western blotting results above indicate the MDA5 protein levels in transfected cells. C, E. Flow cytometry results showing the efficacy of MDA5 mutants in LINE-1 suppression. HEK293T cells seeded on a 24-well plate were co-transfected with 1 μg of L1-RPS and one of MDA5-expressing vectors (25, 75, 225 ng). Cells were collected at 96 h post-transfection to detect EGFP-positive cells through flow cytometry. The western blotting results above indicate the MDA5 protein levels in transfected cells.
Fig 4. MDA5 reduces the expression levels of LINE-1 ORF1p and ORF2p. A. Schematic representation of LINE-1 vectors. Both vectors were based on the natural LINE-1 sequence, whereas L1-1FH contains a Flag and HA tag at the C-terminus of ORF1p, and L1-2TAP contains a TAP tag at the C-terminus of ORF2p. B. Western blotting results showing that MDA5 reduces the ORF1p levels expressed from L1-1FH. HEK293T cells seeded in a 24-well plate were co-transfected with 1 μg of L1-1FH and 25, 75, or 225 ng of MDA5-expressing vector, and subjected to western blotting at 48 h post-transfection. C. Western blotting results showing that MDA5 downregulates the endogenous levels of ORF1p. HEK293T cells seeded in a 24-well plate were transfected 25, 75, or 225 ng of MDA5-expressing vector and subjected to western blotting at 48 h post-transfection. D. Western blotting results showing that MDA5 decreases the protein levels of both ORF1p and ORF2p expressed from L1-2TAP. HEK293T cells seeded in a 24-well plate were co-transfected with 1 μg of L1-2TAP and 25, 75, or 225 ng MDA5-expressing vector, and subjected to western blotting at 48 h post-transfection. E, F. Western blotting results showing the efficiency of MDA5 mutants against LINE-1 ORF1p and ORF2p expression.
Fig 5. MDA5 suppresses the promoter activity of LINE-1 3’-UTR. A. Schematic representation of the amplicon region on JM111 in the PCR assay detecting full-length LINE-1 RNA. B. Electrophoresis image showing that MDA5 reduces levels of LINE-1 RNA generated from JM111. HEK293T cells seeded in a 24-well plate were co-transfected with 1 μg of JM111 and 25, 75, or 225 ng of MDA-expressing vector, and subjected to the PCR assay at 12 h post-transfection to detect the levels of LINE-1 RNA from JM111. The western blotting results show the MDA5 protein levels in the transfected cells. C. Electrophoresis image showing the potency of MDA5 in reducing the levels of LINE-1 RNA generated from JM111. HEK293T cells seeded in a 24-well plate were co-transfected with 1 μg of JM111 and one of MDA5-expressing vectors (225 ng) and subjected to the PCR assay at 12 h post-transfection to detect the levels of LINE-1 RNA from JM111. The western blotting results show the MDA5
protein levels in the transfected cells. D. Schematic representation of the 5UTR-Luc plasmid, where the expression of firefly luciferase is driven by LINE-1 5’-UTR. E. Luciferase activity data indicating that MDA5 suppresses the promoter activity of LINE-1 5’-UTR. HEK293T cells seeded in a 24-well plate were co-transfected with 200 ng of 5UTR-Luc and 25, 75, or 225 ng of MDA5-expressing vector. Luciferase activity was tested at 48 h post-transfection. The western blotting results show the MDA5 protein levels in the transfected cells. F. Luciferase activity data indicating the potency of MDA5 mutants in LINE-1 5’-UTR regulation. HEK293T cells seeded in a 24-well plate were co-transfected with 200 ng of 5UTR-Luc and one of MDA5-expressing vectors (225 ng). Luciferase activity was tested at 48 h post-transfection. The western blotting results show the MDA5 protein levels in the transfected cells.
Fig 6. The 2CARD region is critical for MDA5-mediated LINE-1 suppression. A. Schematic representation showing the deletion mutants of MDA5. B and C. Flow cytometry results showing the efficacy of MDA5 mutants in LINE-1 suppression. HEK293T cells seeded on a 24-well plate were co-transfected with 1 μg of L1-RPS and one of MDA5-expressing vectors (25, 75, or 225 ng), and were collected at 96 h post-transfection to detect EGFP-positive cells through flow cytometry. The western blotting results indicate the MDA5 protein levels in the transfected cells. D and E. Luciferase activity data indicating the potency of MDA5 mutants in LINE-1 5′-UTR regulation. HEK293T cells seeded in a 24-well plate were co-transfected with 200 ng of 5′-UTR-Luc and one of MDA5-expressing vectors (25, 75, or 225 ng).
Luciferase activity was tested at 48 h post-transfection. The western blotting results show the MDA5 protein levels in the transfected cells.