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Selective degradation of plasmid-derived mRNAs by MCPIP1 RNase

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Abstract

Detection and degradation of foreign nucleic acids is an ancient form of host defense. However, the underlying mechanisms are not completely clear. MCPIP1 is an endoribonuclease and an important regulator in both innate and adaptive immunity by targeting inflammatory mRNA degradation. Here we report that MCPIP1 RNase can also selectively detect and degrade the mRNAs encoded by transfected plasmids. In transient transfection, MCPIP1 expression potently degraded the mRNA from exogenously transfected vectors, which is independent on the vector, genes and cell types used. Conversely, the expression of transfected plasmids in MCPIP1-null cells is significantly higher than that in wild-type cells. Interestingly, overexpression of MCPIP1 or MCPIP1 deficiency does not affect the expression of the exogenous genes incorporated into the host genome in a stable cell line or the global gene expression of host genome. This ability is not associated with PKR/RNase L system, as PKR inhibitors does not block MCPIP1-mediated mRNA degradation of exogenously transfected genes. Lastly, expression of MCPIP1 suppressed replication of Zika virus in infected cells. The study may provide a model for understanding the antiviral mechanisms of MCPIP1, and a putative tool to increase the expression of transfected exogenous genes.

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Author Contribution

Y.Q., X.L., R.M. and M.F. performed the experiments; T.W. and S.L. provided the results in Figure 7; H.B.X. and X.H. provided consults; M.F. and T.W. designed and supervised the study and wrote the paper.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

Introduction

Detection and degradation of foreign nucleic acids is an ancient form of host defense. In mammalian, two complementary systems link foreign nucleic acid detection to the interferon-mediated antiviral response. One system consists of several Toll-like receptors (TLRs) including TLR3, TLR5, TLR7 and TLR9 [1]; the other system is exemplified by the cytosolic RNA helicases RIG-1 and MDA5 [2]. Cytoplasmic viral RNA is recognized by RIG-1 and MDA5, which trigger type I interferon (IFN) production. Secreted IFN induces the expression of several nucleases, which degrade viral RNA [3–5]. In addition, double-stranded RNA (dsRNA) produced by viruses or transfected genes also directly activates two types of IFN-induced proteins, PKR (dsRNA-dependent protein kinase) and 2', 5'-oligo A (2–5A) synthetases. 2–5A synthetases produce short, 2', 5'-linked oligoadenylates which activate RNase L, a single-stranded specific endoribonuclease that degrades mRNA and rRNA [6,7].

MCPIP1, also known as ZC3H12A or regnase-1, is a CCCH-zinc finger containing protein that acts as endoribonuclease [8]. Previous studies demonstrated that MCPIP1 selectively degrades the mRNAs that encoding inflammatory cytokines, transcriptional factors and immune modifiers to essentially regulates both innate and adaptive immunity [9–13]. As thus, MCPIP1 deficient mice spontaneously developed systemic inflammatory syndrome and autoimmune response [8,14,15]. Several reports also showed that MCPIP1 has the broad antiviral ability by directly degrading viral RNAs or other unknown mechanisms [16–20].

In this study, we found that MCPIP1 RNase could detect and degrade the mRNAs encoded by exogenously transfected plasmids or infected viruses. This ability is not associated with PKR/RNase L system. The study may provide a model for understanding the antiviral mechanisms of MCPIP1, and a putative tool to increase the expression of transfected exogenous genes.

Experimental

Cells

HEK293 and HeLa cells were obtained from the American Type Culture Collection. These cells were grown as a monolayer in DMEM (Invitrogen) containing 10% FBS, 2 mM L-glutamine, 100 U/ml each of penicillin and streptomycin at 5.0% CO₂. Littermate wild-type and MCPIP1^{-/-} day 13.5 embryos were used to generate mouse embryonic fibroblast (MEF) and maintained in DMEM containing 10% FBS at 5.0% CO₂. HEK293-GFP stable cell line was created by lentiviral transduction of HEK293 with a GFP-expressing construct and maintained in DMEM cultured medium with 10% FBS.

Plasmids

MCPIP1-GFP, Flag-MCPIP1 and Flag-MCPIP1 mutants were described previously [14]. psiCHECK-2 (SV40-Renilla, Promega), pcDNA3-Flag-hZC3H12B and pcDNA3-Flag-hZC3H12C were kindly provided by Dr. Hiroshi Suzuki (University of Tokyo, Japan) and described previously [21]. pcDNA-Flag-USP4 was kindly provided by Dr. Xiongbin Lu (UT MD Anderson Cancer Center). pRK5-HA-Ubiquitin is from Dr. Ted Dawson through

Addgene and described previously [22]. pGL3-Control Vector (SV40-firefly luc.) is from Promega. pEZX-MT01 containing firefly luciferase cDNA under control of SV40 promoter and Renilla luciferase under control of CMV promoter is from GeneCopoeia (Rockville, MD). pEGFP-N1 is from Clontech. pCMV-Flag-ZC3h12D was described previously [23].

Reagents

The MCPIP1 rabbit polyclonal antibody was prepared against the human recombinant MCPIP1 protein as described previously [24]. GFP, GAPDH and actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). pPERK, PERK, pPKR, PKR, pEIF2 α and eIF2 α antibodies were from Cell Signal Technologies; PKR inhibitors C16, anti-renilla and anti-firefly luciferase antibodies were purchased from Sigma.

Transfection

Transient transfection into HEK293 cells was performed using Lipofectamine 2000 according to the manufacturer's instruction. For promoter analysis, HEK293 cells or HeLa cells were seeded into 12-well plates and transfected with FuGENE 6 transfection reagent (Roche Applied Science) following the manufacturer's instruction. The total amount of plasmid DNA was kept constant within each experiment. Luciferase activity was measured by the luciferase assay system (Promega). All transfections were performed in triplicate and repeated at least two times.

RNA isolation and northern blot

Total RNA was isolated from cells using RNA STAT-60 reagent (Tel-Test, Friendswood, TX) following the manufacturer's instruction. An amount of 15 μ g of total RNA was denatured and electrophoresed on 1% agarose-formaldehyde gels. The uniformity of sample loading was verified by UV visualization of the ethidium bromide-stained gel before transfer to Nylon membrane. The cDNA probes for MCPIP1, 2, 3 and 4 were amplified by PCR using individual cDNA clones from ATCC as templates. 32P-labeled cDNA was prepared using the random priming method (Invitrogen). Hybridization was performed using QuickHyb buffer (Stratagene) at 65°C for 2 h or overnight. The membranes were then washed once with 2XSSC and once with 0.1_{SSC}, 1% SDS for 20 min at 65°C.

Quantitative real-time PCR

After removing the genomic DNA using DNase I (Ambion), 2.4 μ g of total RNA was reverse-transcribed to cDNA using a commercially available kit (Applied Biosystems). Quantitative real-time PCR was performed with iCycler Thermal Cycler (Bio-Rad) using 2XSYBR Green master mix (Bio-Rad). Forty cycles were conducted as follows: 95°C for 30 s, 60°C for 30 s, preceded by 1 min at 95°C for polymerase activation. Primer sequences for all genes we measured in this report are available upon request. Quantification was performed by the delta cycle time method, with β -actin used for normalization.

DNA isolation and PCR

The exogenous dCas9 plasmid (0.5 μ g, addgene, no. 44249) or dCas9 (0.5 μ g) combined with MCPIP-HA (2.5 μ g) plasmid were transfected into HEK293 cells for 24 h. Total DNA

was extracted for PCR assay using a commercial kit (QIAGEN), according to the manufacturer's instruction. The PCR protocol consisted of 35 cycles as follows: 95°C for 30 s, 60°C for 1 min and 72°C for 1 min. The PCR products were separated by 1% agarose gel electrophoresis.

Protein isolation and western blot

After washing twice with phosphate-buffered saline (PBS), cells were gently scraped with a rubber policeman into 5 ml of ice-cold PBS and centrifuged at 1000 *g* for 5 min at 4°C. Cells from each 10-cm dish were then resuspended and lysed in 0.5 ml of lysis buffer containing 50 mM NaH₂PO₄, pH 7.6, 250 mM NaCl, 50 mM NaF, 10 mM imidazole, 0.5% Nonidet P-40, 1 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was left on ice for 20 min and then sonicated and centrifuged at 10 000 *g* for 10 min at 4°C. Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce) following the manufacturer's instruction. Protein concentrations were determined by the Bradford method (Bio-Rad) with bovine serum albumin as the standard. For Western blotting, proteins (50 µg) were separated by SDS-PAGE and transferred onto nitrocellulose membranes in transfer buffer containing 0.1% SDS. The membranes were blocked with 5% nonfat dry milk in 0.05% Tween 20 in Tris-buffered saline (TTBS) for 2 h and incubated with the primary antiserum at a 1 : 1000 dilution in the blocking buffer for 1 h. After being washed with TTBS three times for 10 min each, the membranes were incubated with a 1 : 2000 dilution of secondary antibody in TTBS for 1 h. Following three 10-min washes with TTBS, membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to x-ray film.

In vitro mRNA cleavage

mRNA was labeled using P32 by *in vitro* transcription. Radiolabeled mRNA were incubated with human MCPIP1 or mutant proteins at 37°C for 60 min in a 10-µl reaction (18 mM Mg(OAc)₂, 15 mM HEPES pH 7.4, 2.5 mM DTT, pH 7.4). The reaction mixture was resolved by 8% Urea PAGE and exposed to X-ray film.

Virus

Zika virus strains/isolates PRVABC59 (Human/2015/Puerto Rico) and DAK AR 41524 (Mosquito/1984/Senegal) were initially Resources, Manassas, VA) and subsequently propagated in C6/36 cells using our published protocol [25]. The quantification of viral RNA by real-time PCR has also been published [25].

Statistics

Data were expressed as mean ± SD. For comparison between two groups, the unpaired Student's t-test was used. For multiple comparisons, analysis of variance followed by unpaired Student's t-test was used. A value of *P* < 0.05 was considered significant.

Results

MCPIP1 expression suppresses reporter expression in co-transfection experiments

In a transient transfection experiment, we co-transfected a reporter with MCPIP1 into HEK293 cells. Surprisingly, MCPIP1 expression potentially suppressed the reporter activity in a dose-dependent manner, including SV40-driven firefly luciferase reporter and CMV-driven Renilla luciferase reporter (Figure 1A,B). We also repeated the experiment using HeLa cells and the result is similar (Figure 1C). The expression of MCPIP1 in the transfected cells was confirmed by Western blot (Figure 1D). These results suggest that MCPIP1 expression may significantly repress the gene expression from transfected plasmids, which is independent of the promoter used and cell type transfected. To exclude the possibility that the effect is caused by the vector that was used to express MCPIP1, we co-transfected the control reporters with USP4 encoded by same vector. As shown in Figure 1E,F, MCPIP1 expression significantly repressed the reporter activity, but USP4 expression does not, suggesting that this effect is MCPIP1-specific. We also examined the effect of MCPIP1 on the protein and mRNA levels of transfected reporters. As shown in Figure 1G,H, overexpression of MCPIP1 significantly reduced both protein and mRNA levels of transfected firefly luciferase and Renilla luciferase.

MCPIP1 selectively degraded the mRNAs from exogenously transfected plasmids

To further determine the effects of MCPIP1 on the regulation of exogenous gene expression, HEK293 cells were transiently co-transfected with several unrelated plasmids encoding EGFP, LXR α and ubiquitin with MCPIP1 or its mutants. The mRNA expression from the transfected plasmids were examined by Northern blot. As shown in Figure 2A, the full-length of MCPIP1 significantly reduced the mRNA levels from pEGFP, pLXR α and exogenous ubiquitin (e.Ub). Endogenous 28S rRNA, GAPDH mRNA and UBC mRNA were present at constant levels in these experiments. Interestingly, the truncation of N-terminal 80 amino acids of MCPIP1 dramatically increased its activity whereas mutation of MCPIP1 CCCH-ZF impairs its activity. These results were further confirmed by Figure 2B, as 5–100 ng of MCPIP1 dose-dependently decreased exogenous ubiquitin mRNA level, but MCPIP1–C311G did not affect the mRNA expression from transfected ubiquitin, whereas MCPIP1–81–599 showed much stronger effect than wild-type MCPIP1. It was noted that MCPIP1 expression also decreased itself mRNA level as MCPIP1–C311G expressed much higher mRNA level than MCPIP1 wild-type, and MCPIP1–81–599 expressed lower mRNA level than MCPIP1 wild-type. Taken together, these results suggest that MCPIP1 expression selectively degraded the mRNAs from exogenous transfected plasmids.

Exogenous genes are expressed to higher levels in MCPIP1^{-/-} cells

To exclude the possibility that MCPIP1-mediated exogenous gene repression is due to the overexpression of MCPIP1 from transfected plasmid, we generated HEK293 stable cell lines overexpressing MCPIP1. HEK293 cells were transfected with MCPIP1-HA and were screened with G418 (800 μ g/ml) for 2 weeks. Cell clones were screened with HA antibody using western blot. As shown in Figure 3A, the expression of MCPIP1-HA was higher in clones 1# to #6 than that in WT. The 3# cell clone was selected for subsequent experiments. GFP plasmid was transfected into the WT or MCPIP1-stable cell lines. After 24 h, the

transfected cells were harvested and total protein were extracted for western blot. As shown in Figure 3B, GFP expression was significantly decreased in MCPIP1-stable cell lines. To further determine the effect of endogenous MCPIP1 on exogenous gene expression, MCPIP1^{-/-} and wild-type (MCPIP1^{+/+}) MEF cell lines were transiently transfected with SV40-Firefly luciferase reporter or CMV-Renilla luciferase reporter. As shown in Figure 3C, the reporter activities were increased by 72.6 fold and 79.5 fold in MCPIP1^{-/-} cells, respectively. These results further confirmed that MCPIP1 expression significantly suppresses exogenous gene expression.

MCPIP1 RNase did not affect the expression of GFP in GFP-stable cell line

To confirm that MCPIP1 selectively degrades the mRNAs from exogenously transfected plasmids but not from host genomes, we generated GFP-overexpressing HEK293 stable cell lines. Flag-MCPIP1 or Flag-vector were transiently transfected into this cell line. GFP mRNA levels (Figure 4A) and protein levels (Figure 4B) were analyzed by northern blot (Figure 4A) and western blot (Figure 4B), respectively. Interestingly, when GFP gene incorporated into the host genome, MCPIP1 did not recognize and degrade its transcribed mRNA.

MCPIP1-mediated degradation of plasmid-derived mRNAs is not associated PKR activity

To exclude the possibility that MCPIP1 may degrade the plasmid DNA transfected, HEK293 cells were co-transfected MCPIP1 with dCas9 plasmid. After 24 h, total DNA was extracted for PCR assay. As shown in Figure 5A, the plasmid DNA from dCas9 vector was not affected by MCPIP1 expression. To further determine if MCPIP1-mediated repression of exogenous gene expression is associated with PKR activity, HEK293 cells were co-transfected with MCPIP1 and GFP, the transfected cells were treated with or without PKR inhibitor (C16). As shown in Figure 5B, treatment with C16 did not block MCPIP1-mediated repression of GFP expression, but further decreased the expression of both MCPIP1 and GFP. Overexpression of MCPIP1 did not affect the phosphorylation of PERK, PKR and eIF2α.

MCPIP1-mediated degradation of plasmid-derived mRNA is dependent on its RNase activity

As reported previously, MCPIP1 is a protein containing multiple domains [13]. As shown in Figure 6A, an ubiquitin-associated domain (UBA) is located at N-terminal (43–89 aa). The RNase domain (133–270) and CCCH-zinc finger (305–325) are located at the middle and both are critical for its RNase activity. A proline-rich domain (PRR) and a C-terminal conserved region (CRR) are located at C-terminal without known function yet. To further determine the contribution of each domain to the inhibitory effect on exogenous gene expression, HEK293 cells were transiently transfected with different MCPIP1 truncations and mutations with pRK5-ubiquitin or pEZX-MT01 containing firefly luciferase cDNA under control of SV40 promoter. Northern blot and luciferase assay were performed, respectively. As shown in Figure 6B,C, the expression of MCPIP1 wild-type significantly repressed the gene expression from transfected plasmids. Again, the deletion of N-terminal 1–80 aa of MCPIP1 appeared much more potent activity than the MCPIP1 wild-type, suggesting UBA domain may negatively control MCPIP1 enzymatic activity. Further

deletion of RNase domain or mutation of the key amino acids in RNase domain (such as D141N and D225/226A) or CCCH-zinc finger (such as C311G and C306R) abolished the inhibitory effect of MCPIP1 on the gene expression from transfected plasmids. Considering MCPIP1 protein is located in the cytoplasm, it is reasonable to predict that MCPIP1 inhibits gene expression by selectively degrading transcripts generated from exogenous transfected genes. Indeed, MCPIP1 efficiently degraded *in vitro* synthesized RNA, whereas MCPIP1 mutants with D141N, C306R and D225/226A lost the activity (Figure 6D). These results suggest that MCPIP1 represses the gene expression from transfected plasmids by selectively degrading their mRNA in cytoplasm. As MCPIP1 is a prototype member of a gene family which contains other three members, to determine if other members of MCPIP1 protein family also have same activity on exogenous gene expression, HEK293 cells were co-transfected CMV-Firefly luciferase or pEGFP-N1 with MCPIP1, ZC3H12B, ZC3H12C or ZC3H12D, respectively. As shown in Figure 6E, MCPIP1 expression potently repressed exogenous gene expression; ZC3H12C and ZC3H12D also significantly decreased CMV-Firefly luciferase activity; ZC3H12B did not affect the exogenous report activity. To further confirm these results, a GFP expression plasmid was co-transfected with MCPIP1, ZC3H12B, ZC3H12C or ZC3H12D, respectively. As shown in Figure 6F,G, again, MCPIP1 expression potently repressed GFP expression. There were no significant changes of GFP expression when co-transfected with ZC3H12B, ZC3H12C and ZC3H12D, however, we couldn't rule out that the results may be caused by the differences in the levels of protein expressed which are pronounced as shown in Figure 6G.

MCPIP1 expression restricts Zika virus infection

As nucleic acid delivery into mammalian cells by transfection or infection by viruses results in similar forms of cellular response that limits gene expression. Two previously identified cellular enzymes PKR and RNase L that can restrict the exogenous gene expression are critically involved in virus defense [8]. Thus, we assessed the effect of MCPIP1 on virus infection. Zika virus (PRVABC59 strain), was chosen for this part of the study as a representative of single-stranded RNA viruses. Using a doxycycline-inducible A549 clones [17], we found that induction of MCPIP1 expression significantly decreased the viral RNA levels upon infection (Figure 7A,B). These results were consistent with the reports that MCPIP1 exhibited broad-spectrum antiviral activity [16]. The same observation was made using another African Zika virus strain DAK (not shown).

Discussion

Previous studies demonstrated that MCPIP1 is an RNase that directly cleaves mRNAs of inflammatory genes such as IL-6 and IL-12p40, and negatively regulates cellular inflammatory responses [8]. In immune cells, MCPIP1 has its specific mRNA substrates by recognizing some stem-loop-like elements on the 3'-UTR of its target mRNAs [26]. However, in the cell-free system, recombinant MCPIP1 protein can cleave any RNA incubated, suggesting that MCPIP1 RNase activity is not required to be activated by other factors. In this study, we observed that MCPIP1 can selectively recognize and potently degrade the mRNAs from exogenously transfected plasmids, which is not dependent on the vector, gene and cell types used. We also observed that MCPIP1 did not degrade the mRNAs

from exogenous genes incorporated into the host genome in a stable cell line. Overexpression or deficiency of MCPIP1 does not affect the overall gene expression of host genome.

Our results suggest that MCPIP1 can distinguish the mRNAs transcribed from exogenously transfected plasmids with that from the host genome, suggesting that MCPIP1 may be an important component that host defenses the foreign nucleic acids such as viruses. Indeed, our study showed that the cells overexpressing MCPIP1 were resistant to Zika virus infection, which is consistent to several previous reports that MCPIP1 exhibits broad-spectrum antiviral ability, especially RNA-based viruses such as HIV, HCV, CVB3, JEV and DEN [16–20]. However, how MCPIP1 recognizes the foreign nucleic acids from infected viruses or transfected plasmids are still unknown. Transfections and virus infections induce some of the same stress responses possibly because both treatments can result in the production of dsRNA [27]. The synthesis of dsRNA from plasmid DNA can occur as a result of complementary RNA strands generated by transcription from cryptic or bidirectional promoters [27]. It is well documented that RIG-1 and MDA5 recognize cytoplasmic viral RNA by directly binding to dsRNA derived from viruses [28]. Recognition of viral RNA induces K63-linked polyubiquitination of RIG-1, which is essential for RIG-1 activation. The activated RIG-1 is recruited to mitochondrial MAVS and triggers a signal that induces type 1 IFN and proinflammatory cytokine production [29]. In addition, dsRNA also activates PKR and OAS-1. PKR phosphorylate eIF2 α to specially repress the translation of viral RNA, whereas OAS-1 promotes the synthesis of 2–5A, which activates RNase L to cleave viral RNA [30,31]. In our study, overexpression of MCPIP1 does not affect the activation of PKR and phosphorylation of eIF2 α ; furthermore, PKR inhibitor (C16) did not block MCPIP1-mediated repression of exogenously transfected gene expression. It is speculated that the mRNAs transcribed from transfected plasmids may distinctly marked with the mRNAs transcribed from host genome, and MCPIP1 RNase may selectively recognize the plasmid-derived mRNAs. Nevertheless, the mechanisms of how MCPIP1 recognize the mRNAs from exogenously transfected genes need to be further investigated.

MCPIP1 expression also decreased its own mRNA level from transfected MCPIP1 plasmid. Previously, Iwasaki et al. reported that MCPIP1 mRNA was negatively regulated by MCPIP1 itself via a stem-loop region present in the MCPIP1 3' untranslated region [32]. Here we observed that the MCPIP1 mRNA without 3' untranslated region that transcribed from transfected MCPIP1 plasmid was also degraded by MCPIP1 itself, suggesting that a different mechanism may exist for MCPIP1 controlling exogenous gene expression. N-terminal 80 amino acids deletion of MCPIP1 dramatically increased its enzymatic activity, suggesting that MCPIP1 activity may be regulated by an *in vivo* system [33]. Identification of the regulatory mechanisms of MCPIP1 enzyme activity would be extremely interesting topic and has translational potential. Since MCPIP1 potentially decreased the gene expression from transfected plasmids, the MCPIP1 deficient HEK293 cells may allow higher levels of transiently transfected protein expression.

MCPIP1 protein family has four members including ZC3H12A/MCPIP1, ZC3H12B, ZC3H12C and ZC3H12D [34]. The RNase domain is conserved in four ZC3H12 family members. MCPIP1 showed strong inhibitory activity on exogenous gene expression;

ZC3H12C and ZC3H12D exhibited a moderate effect on exogenous gene expression; whereas ZC3H12B had no effect on exogenous gene expression. The mechanisms for this difference need to be further investigated. In summary, we here reported that MCPIP1 functions as an important host factor to limit exogenous gene expression. The study may provide a model for understanding the antiviral mechanisms of MCPIP1, and a putative tool for removing foreign nucleic acid contamination during gene therapy. Knocking-down of MCPIP1 in cells may allow higher levels of transiently transfected protein expression.

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Abbreviations

CCR	C-terminal conserved region
dsRNA	double strand RNA
eIF2α	eukaryotic initiate factor 2 α
FBS	fetal bovine serum
GFP	green fluorescent protein
MCPIP1	monocyte chemotactic protein-induced protein 1
MEF	mouse embryonic fibroblast
PBS	phosphate-buffered saline
TLR	Toll-like receptors
TTBS	tris-buffered saline
Ub	Ubiquitin
UBA	ubiquitin-associated domain

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Figure 1. MCPIP1 suppresses reporter expression in co-transfection experiments.

(A and B) HEK293 cells were co-transfected with different doses of Flag-MCPIP1 as indicated and the reporter of SV40-driven firefly luciferase (A) or CMV-driven Renilla luciferase (B). The reporter activity was measured by luciferase assay system from Promega. The data represent mean $\pm$ SD, $n = 4$; ** $P < 0.01$; (C and D) HeLa cells were co-transfected with different doses of Flag-MCPIP1 as indicated and the reporter of SV40-driven firefly luciferase. The reporter activity was measured as described as above (C); MCPIP1 expression in the transfected cells was detected by Western blot with anti-Flag (D). Actin was served as loading control; (E and F) HEK293 cells were co-transfected Flag-MCPIP1 or Flag-USP4 with the reporter of SV40-driven firefly luciferase (E) or CMV-driven Renilla luciferase (F). The reporter activity was measured as described above; (G and H) HEK293

cells were co-transfected Flag-vector or Flag-MCPIP1 with the reporter of SV40-driven firefly luciferase or SV40-driven Renilla luciferase. The protein and mRNA of firefly luciferase and renilla luciferase was measured by Western blot with anti-firefly luc. or anti-renilla luc. (**G**) and by QPCR (**H**), respectively. All of the experiments were repeated more than two times.

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Figure 2. MCPIP1 selectively degrades the mRNAs from exogenously transfected plasmids.

(A) HEK293 cells were co-transfected Flag-MCPIP1 or its mutants with another plasmid encoding GFP, LXRα, or Ubiquitin. RNA was isolated from the transfected cells and northern blot was performed with indicated probes. 28S was served as a loading control. UBC and UBB are endogenous ubiquitin mRNAs; e.Ub means exogenous ubiquitin mRNA. (B) HEK293 cells were co-transfected 5, 10, 20, 30, 60 and 100 ng per well of Flag-MCPIP1 or its mutants with another plasmid encoding Ubiquitin. RNA was isolated from the transfected cells and northern blot was performed as described above. All of data have been repeated at least twice.

Figure 3. Overexpression or deficiency of MCPIP1 affects the expression of exogenously transfected plasmids.

(A) HEK293 cells were transfected with MCPIP1-HA and were selected with G418 for 2 weeks. Six cell clones as well as wild-type (WT) cells were detected with HA antibody by western blot. (B) The MCPIP1-overexpressing stable cell line (#3 clone) and WT cells were transiently transfected with GFP plasmid. After 24 h, the cell lysates were harvested and analyzed by western blot. Fold-change of the GFP protein levels were determined by densitometry and normalized to GAPDH. Data are presented as the means $\pm$ SD. ($n = 3$). ** $P < 0.01$. (C) The MEFs from MCPIP1-deficient or wild-type embryo were transfected with the reporter of SV40-driven firefly luciferase or CMV-driven renilla luciferase. The reporter activity was measured by luciferase assay system from Promega. The data represent mean $\pm$ SD, $n = 4$.

Figure 4. MCPIP1 does not affect the expression of GFP in GFP-stable cell line. HEK293 stable cell line overexpressing GFP were transiently transfected with Flag-MCPIP1 or Flag-empty vector. After 24 h, the total RNA or cell lysates were isolated and analyzed by northern blot (A) or western blot (B).

Figure 5. MCPIP1-mediated degradation of plasmid-derived mRNAs is not associated with PKR activity.

(A) HEK293 cells were transiently transfected with dCas9 and MCPIP1. After 24 h, total DNA was isolated and analyzed by PCR. (B) HEK293 cells were transiently transfected with MCPIP1 and GFP plasmids. The transfected cells were treated with or without PKR inhibitor C16. After 24 h, the expression of MCPIP1 and GFP were analyzed by western blot. All of the experiments were repeated more than two times.

Figure 6. MCPIP1-mediated inhibition of gene expression from transfected plasmids is dependent on its RNase activity.

(A) Scheme of MCPIP1 protein domains and generation strategy of MCPIP1 mutant constructs. (B) HEK293 cells were co-transfected Flag-MCPIP1 or its mutants with another plasmid encoding Ubiquitin. RNA was isolated from the transfected cells and northern blot was performed as described above. 28S was served as a loading control. e.Ub means exogenous ubiquitin mRNA. (C) HEK293 cells were co-transfected Flag-MCPIP1 or its mutants with the reporter of SV40-driven firefly luciferase. The reporter activity was measured using luciferase reporter assay system from Promega. Data represent mean $\pm$ SD, $n = 4$. * $P < 0.05$, ** $P < 0.01$. (D) In vitro synthesized RNA was labeled by p32 and incubated with purified protein of MCPIP1 and its mutants. The reaction mixture was resolved by 8% Urea PAGE and exposed to X-ray film. (E) HEK293 cells were co-

transfected Flag-MCPIP1, Flag-Zc3h12b, Flag-Zc3h12c, or Flag-Zc3h12d as indicated with pGL3-control vector (SV40-firefly luc.). The reporter activity was measured by luciferase assay system from Promega. The data represent mean $\pm$ SD, $n = 4$. (F and G) HEK293 cells were transfected Flag-MCPIP1, Flag-Zc3h12b, Flag-Zc3h12c, or Flag-Zc3h12d as indicated with pEGFP-N1. RNA was isolated from the transfected cells and Northern blot was performed with GFP probe. 28S was served as loading control (G). Cell lysates were extracted from the transfected cells and Western blot was performed with flag and actin antibodies. Actin was served as loading control (F).

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Figure 7. MCPIP1 expression restricts virus replication.

(**A**) A549 negative control or Doxycycline-inducible MCPIP1 clones were infected with Zika strain PRVABC59 at MOI = 1 for 2 h in the presence or absence of 100 ng/ml Doxycycline. After 48 h incubation, cellular RNA was isolated and quantified by real-time PCR. The relative levels of Zika virus RNA were normalized against that of GAPDH. Data represent mean $\pm$ SD, $n = 3$, ** $P < 0.01$ vs other groups. (**B**) Doxycycline-inducible MCPIP1 clones were left untreated or treated with 100 or 200 ng/ml Doxycycline followed by infection with Zika strain PRVABC59 (MOI 1) for 2 h. After 48 h incubation, cellular RNA was isolated and quantified by real-time PCR as in **A**. The data represent mean $\pm$ SD, $n = 3$, ** $P < 0.01$ vs un-induced group.