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The ER-Associated Protein ZDHHC1 Is a Positive Regulator of DNA Virus-Triggered, MITA/STING-Dependent Innate Immune Signaling

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SUMMARY

Viral DNA sensing within the cytosol of infected cells activates type I interferon (IFN) expression. MITA/STING plays an essential role in this pathway by acting as both a sensor for the second messenger cGAMP and as an adaptor for downstream signaling components. In an expression screen for proteins that can activate the *IFNB1* promoter, we identified the ER-associated protein ZDHHC1 as a positive regulator of virus-triggered, MITA/STING-dependent immune signaling. *Zdhhc1*$^{-/-}$ cells failed to effectively produce IFNs and other cytokines in response to infection with DNA but not RNA viruses. *Zdhhc1*$^{-/-}$ mice infected with the neurotropic DNA virus HSV-1 exhibited lower cytokine levels and higher virus titers in the brain, resulting in higher lethality. ZDHHC1 constitutively associated with MITA/STING and mediates dimerization/aggregation of MITA/STING and recruitment of the downstream signaling components TBK1 and IRF3. These findings support a role for ZDHHC1 in mediating MITA/STING-dependent innate immune response against DNA viruses.

INTRODUCTION

Innate immune response is critical for efficient host defense against viral infection. Upon viral infection, structurally conserved viral components called pathogen-associated molecular patterns (PAMPs) are recognized by pathogen recognition receptors (PRRs) in the cell, which initiates a series of signaling events that lead to the production of type I interferons (IFNs) (including IFN-β and IFN-α family members), proinflammatory cytokines, and other downstream antiviral effecter proteins. These downstream cytokines and effecters act to inhibit viral replication, eradicate virus-infected cells, and facilitate the initiation of adaptive immune response (Akira et al., 2006; Hiscott, 2007; Janeway and Medzhitov, 2002; Paludan and Bowie, 2013; Seth et al., 2006; Wilkins and Gale, 2010).

The host cells have developed different mechanisms for recognition of viral nucleic acids, which is the first step of innate antiviral response. Among PRRs, certain Toll-like-receptors (TLRs) and RIG-I-like receptors (RLRs) are known to detect viral RNA (Akira et al., 2006). For example, TLR3 recognizes viral double-stranded RNA (dsRNA) released by infected cells, whereas RIG-I and MDA5 sense viral RNA in the cytoplasm (Akira et al., 2006). In addition to the viral RNA sensors, several proteins have been reported to recognize viral DNA, including TLR9 (Lund et al., 2003), AIM2 (Bürckstümmer et al., 2009), DAI (Takaoka et al., 2007), RNA polymerase III (Chiu et al., 2009), IFI16 (Unterholzner et al., 2010), DDX41 (Zhang et al., 2011), and LSm14A (Li et al., 2012). Various studies have demonstrated that these proteins are important for innate immune responses against various DNA viruses in distinct cell or mouse models. For example, it has been shown that IFI16 is a sensor for HIV DNA and required for CD4 T cell depletion following HIV infection (Horan et al., 2013; Jakobsen and Paludan, 2014; Unterholzner et al., 2010). However, studies have demonstrated that these proteins are not universally required for detecting viral DNA in distinct cell types or in vivo (Paludan and Bowie, 2013). Recently, a nucleotidyltransferase family member, called cyclic GMP-AMP (cGAMP) synthase (cGAS), has been identified to detect cytosolic DNA in various cell types (Sun et al., 2013). Gene knockout studies demonstrate that cGAS is essential for innate immune response against DNA viruses in cells and mice (Li et al., 2013; Schoggins et al., 2014), suggesting that cGAS is a widely used viral DNA sensor. Recently, studies with *cGas*$^{-/-}$ cells and mice suggest that cGAS also plays a role in innate immune response against the single-stranded RNA (ssRNA) virus West Nile virus (WNV), though it is unknown whether cGAS directly senses viral RNA or regulates basal immune response (Schoggins et al., 2014).

Recognition of viral DNA by cGAS leads to the synthesis of the second messenger cGAMP from ATP and GTP (Civril et al., 2013; Sun et al., 2013; Wu et al., 2013). Synthesized cGAMP binds to MITA (also called STING, MPYS, and ERIS), a critical adaptor in virus-triggered IFN induction pathways and innate antiviral responses (Abe et al., 2013; Ishikawa and Barber, 2008; Ouyang et al., 2012; Shu et al., 2012; Zhang et al., 2013; Zhong et al., 2008). MITA is a 379 amino acid (aa) protein, which is consisted of the N-terminal four transmembrane domains (aa 1–137) and the C-terminal domain (CTD, aa 138–397). MITA anchors itself in the ER, mitochondria and mitochondrial-associated membrane via its N-terminal transmembrane domains, while its CTD hangs in the cytosol to bind the second messenger cGAMP.

Viral infection induces dimerization/aggregation and subsequent signaling to downstream through its C-terminal tail (CTT, aa 340–379), which serves as a scaffold to assemble IRF3 in close proximity to TBK1, leading to TBK1-dependent phosphorylation of IRF3 and induction of downstream genes (Tanaka and Chen, 2012; Zhong et al., 2008). Gene knockout studies demonstrate that deficiency of MITA impairs type I IFN induction triggered by DNA viruses such as herpes simplex virus-1 (HSV-1) and viral clearance in mice (Ishikawa et al., 2009). Although it is widely accepted that MITA is required for type I IFN induction triggered by DNA viruses in mice, whether MITA plays a role in innate immune responses against RNA viruses is still unresolved. Several studies have demonstrated a role of MITA in type I IFN induction triggered by RNA viruses, including Sendai virus (SeV) and vesicular stomatitis virus (VSV) in certain human cell lines (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Sun et al., 2009; Zhong et al., 2008). In addition, induction of type I IFNs triggered by VSV infection is reduced to ~20% in *Mita*$^{-/-}$ mice (Ishikawa and Barber, 2008; Ishikawa et al., 2009). However, induction of type I IFNs in the serum triggered by encephalomyocarditis virus, also an RNA virus, is not affected in *Mita*$^{-/-}$ mice (Ishikawa et al., 2009). It is possible that MITA is involved in innate immune responses against RNA viruses in a virus- and cell-type-specific manner.

In this study, we identified ZDHHC1, an ER-associated protein, as a mediator for type I IFN induction in expression screens. Gene knockout studies suggest that ZDHHC1 deficiency impaired production of type I IFNs and other cytokines in response to infection with DNA viruses. Consistently, *Zdhhc1*$^{-/-}$ mice were more susceptible to lethal infection by HSV-1. ZDHHC1 was constitutively associated with MITA and important for dimerization/aggregation of MITA and recruitment of downstream components TBK1 and IRF3 to MITA. Our findings suggest that ZDHHC1 plays an important role in innate immune response against DNA viruses by modulating MITA activity and provide important insights into the delicate regulatory mechanisms of innate immune response against DNA viruses.

RESULTS

Identification of ZDHHC1 as a Positive Regulator of Virus-Triggered Signaling

Virus-triggered induction of type I IFNs is delicately regulated to ensure proper but not excessive innate immune response. To further elucidate the mechanisms of virus-triggered IFN induction, we performed expression screens to unambiguously identify proteins that can activate the IFN-β promoter. These efforts led to the identification of ZDHHC1, a member of the aspartate-histidine-histidine-cysteine (DHHC) palmitoyl acytransferase family, which could markedly activate the IFN-β promoter in reporter assays (Figures S1A and S1B available online). Further experiments indicated that overexpression of ZDHHC1 activated the IFN-β promoter in a dose-dependent manner in HeLa cells (Figure 1A). Previously, it has been demonstrated that induction of IFN-β requires cooperation of two transcription factors, IRF3 and NF-κB (Maniatis et al., 1998). Consistently, overexpression of ZDHHC1 activated ISRE (an enhancer motif for IRF3) and NF-κB in a dose-dependent manner in reporter assays (Figure 1A). Overexpression of ZDHHC1 also induced IRF3 phosphorylation dose dependently (Figure S1C). Overexpression of ZDHHC1 also dramatically potentiated activation of the IFN-β promoter triggered by transfection of synthetic viral RNA analog poly(I:C) or B-DNA (Figure 1B) or by infection with HSV-1 and SeV in human HeLa cells (Figure 1C). Overexpression of ZDHHC1 also dramatically potentiated ISRE and NF-κB activation triggered by both HSV-1 and SeV (Figure S1D). These results suggest that ZDHHC1 is involved in virus-triggered induction of IFN-β.

ZDHHC1 is a member of the DHHC palmitoyl transferase family. Specific aspartate-histidine (DH) and cysteine (C) residues in the DHHC domain of ZDHHC1 are important for its palmitoyl transferase activity (Korycka et al., 2012; Sharma et al., 2008). To determine whether the palmitoyl transferase activity of ZDHHC1 is required for its ability to mediate IFN-β induction, the conserved D161H162 or C164 of ZDHHC1 was separately mutated. Reporter assays indicated that these mutants activated the IFN-β promoter and potentiated SeV- and HSV-1-triggered activation of the IFN-β promoter to similar levels as wild-type ZDHHC1 (Figure S1E), suggesting that the palmitoyl transferase activity is not required for its function in virus-triggered IFN-β induction.

To determine whether endogenous ZDHHC1 is important for virus-triggered IFN-β induction, we first examined the expression levels of ZDHHC1 in several cell lines. RT-PCR and immunoblot analysis indicated that ZDHHC1 was similarly expressed in all examined cell lines, including HEK293, HEK293T, HeLa, THP1, and *Mita*$^{-/-}$ mouse dendritic cell (DC), except *Zdhhc1*$^{-/-}$ mouse DC (Figure S1F and S1G). In these experiments, MITA was found to be expressed in THP1, HeLa, HCT116, HEK293, and mouse *Zdhhc1*$^{-/-}$ DC but not in *Mita*$^{-/-}$ mouse DC (Figure S1F and S1G). MITA expression was barely detectable in HEK293T cells in these experiments (Figure S1G). In addition, cGAS was also found to be ubiquitously expressed in these examined cells (Figure S1F and S1G). To investigate the functions of ZDHHC1, we performed RNAi-mediated knockdown experiments in HeLa cells (Figure 1D). Reporter assays indicated that knockdown of ZDHHC1 inhibited activation of the IFN-β promoter triggered by transfected poly(I:C) and B-DNA in these cells (Figure 1E). Similarly, knockdown of ZDHHC1 also inhibited HSV-1- and SeV-triggered expression of the *IFNB1* in HeLa cells (Figure 1F). Noticeably, ZDHHC1 knockdown was less effective in inhibition of poly(I:C)- or SeV-triggered than B-DNA- or HSV-1-triggered induction of IFN-β in these experiments. In similar experiments, knockdown of ZDHHC1 had no inhibitory effects on TLR3-mediated activation of the IFN-β promoter triggered by extracellular stimulation of the 293-TLR3 cells with poly(I:C) (Figure S1H). These data suggest that ZDHHC1 specifically mediates virus-triggered and cytoplasmic receptor-mediated induction of IFN-β.

ZDHHC1 Is Essential for DNA but Not RNA Virus-Triggered Signaling in Murine Cells

Human ZDHHC1 is consisted of 485 aa residues and shares 98% sequence identity with its murine ortholog. To investigate the functions of ZDHHC1 in innate antiviral response, we generated ZDHHC1-deficient mice by a conventional gene-targeting method (Figures S2A–S2C). *Zdhhc1*$^{-/-}$ mice were viable, grossly normal, and reproductive, suggesting that ZDHHC1 is

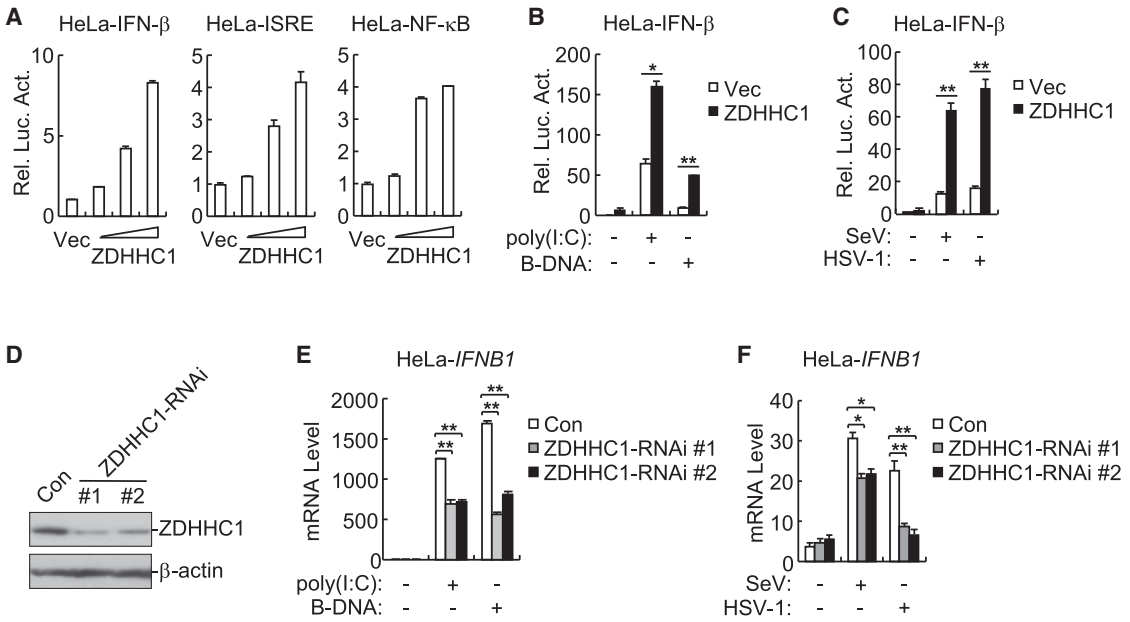

Figure 1. Identification of ZDHHC1 as a Positive Regulator of Virus-Triggered Signaling

(A) ZDHHC1 activates the IFN-β promoter in a dose-dependent manner. HeLa cells (1×10^5) were transfected with the indicated reporter plasmid (0.1 μg) and increased amounts of ZDHHC1expression plasmid (0.05, 0.1, and 0.2 μg). Luciferase assays were performed 24 hr after transfection.

(B) ZDHHC1 potentiates poly(I:C)-, B-DNA-induced activation of the IFN-β promoter. HeLa cells (1×10^5) were transfected with the IFN-β promoter plasmid and the indicated plasmid (0.1 μg) for 20 hr and then further transfected with poly(I:C) (0.5 μg) and B-DNA (0.5 μg) for 18 hr before luciferase assays were performed.

(C) ZDHHC1 potentiates SeV- and HSV-1-induced activation of the IFN-β promoter. HeLa cells (1×10^5) were transfected with the IFN-β promoter plasmid and the indicated plasmid (0.1 μg) for 20 hr, cells were then left untreated or infected with SeV or HSV-1 for 12 hr before luciferase assays were performed.

(D) Effects of ZDHHC1-RNAi plasmids on endogenous ZDHHC1. HeLa cells (1×10^7) were transfected with the indicated RNAi plasmids (10 μg), and immunoblots were performed with the indicated antibodies.

(E) Effects of ZDHHC1-RNAi plasmids on poly(I:C)- and B-DNA-induced activation of the IFN-β promoter. HeLa cells (1×10^5) were transfected with the IFN-β promoter plasmid (0.1 μg) and the indicated RNAi plasmids (1 μg) for 24 hr, followed by further transfection of the indicated nucleic acids for 20 hr before luciferase assays were performed.

(F) Effects of ZDHHC1-RNAi plasmids on SeV- and HSV-1-induced expression of *IFNB1*. HeLa cells (2×10^5) were transfected with the indicated RNAi plasmids (2 μg). Twenty-four hours after transfection, cells were left untreated or infected with SeV or HSV-1 for 12 hr before quantitative PCR (qPCR) analysis.

Graphs show mean ± SD, n = 3. See also Figure S1.

dispensable for growth and development (Figure S2D). The numbers and compositions of major immune cells in lymph nodes, spleen, and thymus were similar between *Zdhhc1*$^{-/-}$ *and Zdhhc1*$^{+/+}$ mice (Figure S2E), suggesting that ZDHHC1 is not essential for development of the examined immune cells.

To investigate the roles of ZDHHC1 in innate immune response, we compared expression of downstream genes induced by synthetic nucleic acids transfected into *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ mouse embryonic fibroblasts (MEFs). Previously, it has been demonstrated that in addition to B-DNA, transfected dsDNA (120-mers) representing the genomes of HSV-1 (HSV120), dsDNA of approximately 90 bp (dsDNA90), and poly(dG:dC) were efficient at inducing the expression of type I IFNs (Abe et al., 2013; Ishikawa et al., 2009). As shown in Figure 2A and 2B, the levels of *Ifnb1*, *Isg56*, and *Il6* mRNAs, as well as the secreted IFN-β, TNFα, and IL6 proteins, were markedly lower in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ MEFs following transfection of the synthetic DNAs. Unexpectedly, the mRNA levels of these downstream genes induced by the synthetic dsRNA analog poly(I:C) were similar in *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ MEFs (Figure 2A). Consistently, the levels of *Ifnb1*, *Isg56*, and *Il6* mRNAs induced by the DNA virus HSV-1 were markedly lower in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ MEFs (Figure 2C). In these experiments, the mRNA levels of these downstream genes induced by the RNA viruses SeV and VSV were similar between *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ MEFs (Figure 2C). These results suggest that endogenous ZDHHC1 plays an important role in DNA- but not RNA-virus-triggered induction of downstream antiviral genes in MEFs. To confirm the roles of ZDHHC1 in DNA-virus-triggered signaling, we examined the activation of downstream signaling components involved in virus-triggered type I IFN induction. As shown in Figure 2D, HSV-1- but not SeV-triggered phosphorylation of TBK1, IRF3, IκBα, and p38, which are hallmarks of activation of virus-triggered IFN induction pathways, were markedly lower in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ MEFs. These results suggest that ZDHHC1 is important for HSV-1- but not SeV-triggered activation of IRF3, NF-κB, and p38, as well as induction of downstream cytokines and other effecters.

We next determined whether ZDHHC1 is essential for virus-triggered induction of downstream genes in immune cells. We obtained mouse bone-marrow-derived macrophages (BMDMs). As shown in Figure 3A, the levels of *Ifnb1*, *Isg56*, and *Il6* mRNAs induced by transfected synthetic DNAs, including B-DNA,

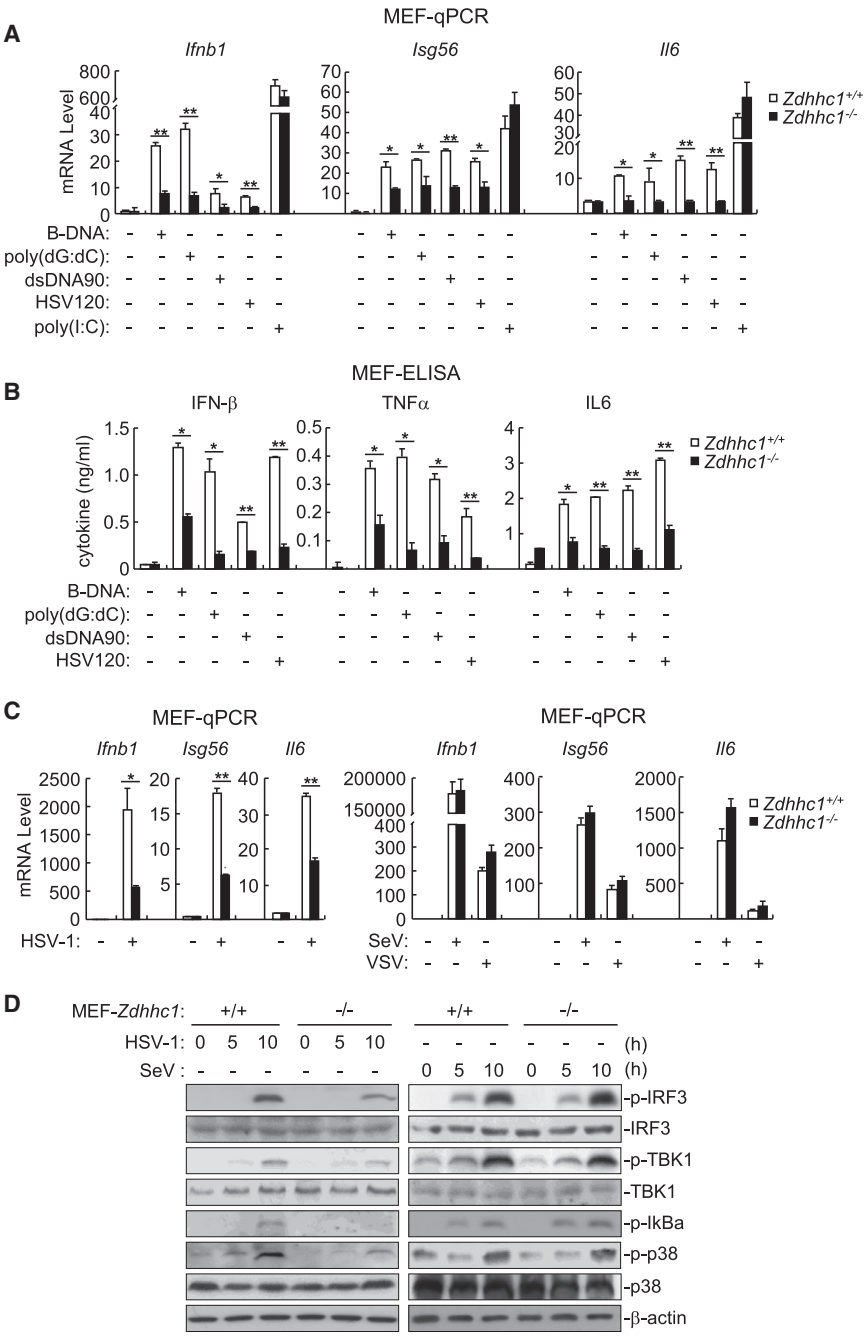

Figure 2. ZDHHC1 Is Essential for DNA but Not RNA Virus-Triggered Signaling in MEFs

(A) Effects of ZDHHC1 deficiency on transcription of *Ifnb1*, *Isg56*, and *Il6* induced by transfected nucleic acids in MEFs. MEFs (2×10^5) were transfected with the indicated nucleic acids (3 μg/ml) for 12 hr before qPCR analysis.

(B) Effects of ZDHHC1 deficiency on secretion of IFN-β, TNFα, and IL6 induced by transfected nucleic acids in MEFs. MEFs (1×10^5) were transfected with the indicated nucleic acids (3 μg/ml) for 24 hr. The culture medium was collected for quantization of the indicated cytokines by ELISA.

(C) Effects of ZDHHC1 deficiency on HSV-1, SeV-, and VSV-induced transcription of *Ifnb1*, *Isg56*, and *Il6* in MEFs. MEFs (2×10^5) were left untreated or infected with HSV-1, SeV, or VSV for 5 hr before qPCR analysis.

(D) ZDHHC1 deficiency impairs HSV-1- but not SeV-induced phosphorylation of downstream components. MEFs (2×10^6) were left untreated or infected with HSV-1 or SeV for the indicated times before immunoblot analysis with the indicated antibodies.

Graphs show mean ± SD, n = 3. See also Figure S2.

poly(dG:dC), dsDNA90, and HSV120, were markedly lower in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ BMDMs. ELISA experiments indicated that the levels of secreted IFN-β, TNFα, and IL6 induced by transfected dsDNA90 and HSV120 but not poly(I:C) were markedly lower in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ BMDMs (Figure 3B). Additionally, we found that HSV-1-induced expression of *Ifnb1*, *Isg56*, *Tnfa*, and *Il6* mRNAs were markedly decreased in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ BMDMs (Figure 3C). In addition to HSV-1, induction of *Ifnb1*, *Il6*, and *Tnfa* by another examined DNA virus, ectromelia virus (ECTV), was also impaired in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ BMDMs (Figure S3). In similar experiments, SeV- and VSV-induced expression of *Ifnb1*, *Isg56*, *Tnfa*, and *Il6* mRNAs were similar in *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ BMDMs (Figure 3C). Consistently, HSV-1-triggered, but not SeV-triggered, secretion of cytokines, including IFN-β, TNFα, and IL6, was decreased in *Zdhhc1*$^{-/-}$ in comparison to wild-type BMDMs (Figure 3D). Thus, ZDHHC1 is essential for DNA- but not RNA-virus-triggered induction of downstream genes in mouse BMDMs.

In addition to BMDMs, we found that transfected synthetic DNAs- or HSV-1- but not SeV- or VSV-triggered expression of *Ifnb1*, *Isg56*, *Tnfa*, and *Il6* genes was impaired in mouse bone marrow-derived DCs of *Zdhhc1*$^{-/-}$ mice (Figure S4). In contrast, expression of *Ifnb1*, *Tnfa* and *Il6* genes was similar between *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ BMDMs and DCs following extracellular treatment with poly(I:C), a ligand of TLR3 (Figure S5). Taken together, these results suggest that ZDHHC1 is essential for DNA but not RNA-virus-triggered induction of downstream genes in mouse immune cells.

ZDHHC1 Is Essential for Host Defense against HSV-1 Infection in Mice

To gain insight into the importance of ZDHHC1 in host defense against viral infection in vivo, we investigated innate antiviral immune response in wild-type and ZDHHC1-deficient mice. We found that serum cytokines induced by HSV-1 infection, including IFN-β, IFN-α, TNFα and IL6, were severely impaired

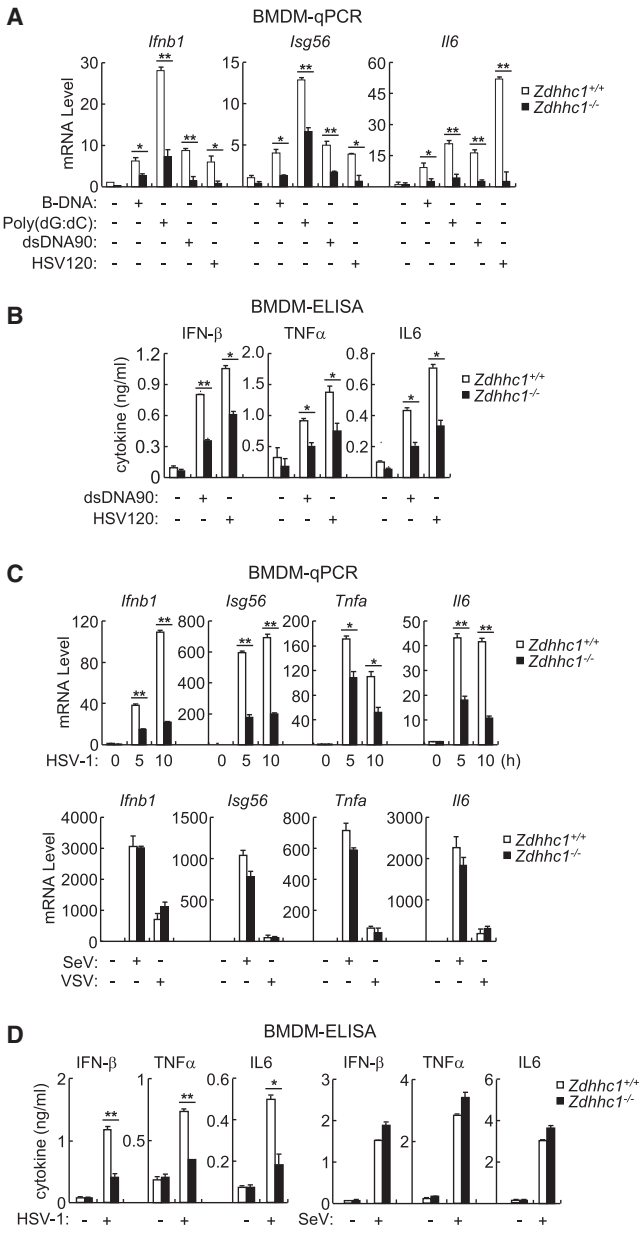

Figure 3. ZDHHC1 Is Essential for DNA but Not RNA-Virus-Triggered Signaling in BMDMs

(A and B) Effects of ZDHHC1 deficiency on expression of the indicated genes in BMDMs. BMDMs (2×10^5) were transfected with the indicated nucleic acids (3 μg/ml) and then qRCR (A) or ELISA (B) experiments were performed at 6 or 24 hr after transfection, respectively.

(C) Effects of ZDHHC1 deficiency on HSV-1-, SeV-, and VSV-induced transcription of *Ifnb1*, *Isg56*, *Tnfa*, *and Il6* in BMDMs. BMDMs (2×10^5) were infected with HSV-1 for the indicated times or with SeV/VSV for 5 hr before qPCR analysis.

(D) Effects of ZDHHC1 deficiency on HSV-1- and SeV-induced secretion of IFN-β, TNFα, and IL6 in BMDMs. BMDMs (1×10^5) were infected with HSV-1 or SeV for 24 hr. The culture medium was collected for quantization of the indicated cytokines by ELISA.

Graphs show mean ± SD, n = 3. See also Figures S3, S4, and S5.

in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ mice (Figure 4A). In parallel experiments, the serum cytokine levels induced by VSV infection were similar between Zdhhc1$^{-/-}$ and *Zdhhc1*$^{+/+}$ mice (Figure 4A). Since HSV-1 is a neurotropic virus and the leading cause of sporadic viral encephalitis, we investigated the effects of ZDHHC1-deficiency on HSV-1-induced expression of downstream genes and viral loads in the brain. The results indicated that after intranasal (i.n.) infection with HSV-1 for 6 days, the expression of *Ifnb1*, *Isg56*, *Rantes* and *Il6* were markedly reduced whereas the viral titers were significantly increased in the brain of *Zdhhc1*$^{-/-}$ mice in comparison to their wild-type counterparts (Figures 4B and 4C). We also compared the survival rates of *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ mice after HSV-1 infection intraperitoneally (i.p.) at a high dose (5×10^7 pfu per mouse). The results indicated that *Zdhhc1*$^{-/-}$ mice were more susceptibility to HSV-1-triggered death than their wild-type counterparts (Figure 5D). In parallel experiments, the survival rates were similar between *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ mice infected with VSV (Figure 5D). Collectively, these data indicated that ZDHHC1 is essential for the induction of downstream antiviral effecters and host defense against DNA but not RNA viruses in mice.

ZDHHC1 Is Associated and Colocalized with MITA

To investigate the molecular mechanisms on the role of ZDHHC1 in innate immune response against DNA viruses, we first determined whether ZDHHC1 is associated with components involved in virus-triggered signaling pathways. The results indicated that human ZDHHC1 was associated with MITA but not other examined components including cGAS, IF116, DDX41, LSm14A, RIG-I, VISA, TBK1, TRAF6 and IRF3 (Figure 5A). Domain-mapping experiments indicated that the N terminus of MITA (aa1-190) and the N terminus of ZDHHC1 (aa1-271), both contain multiple transmembrane domains of the respective proteins, were required for their association (Figure 5B). Since ZDHHC1 and MITA are highly expressed in THP1 cells (Figures S1F and S1G), and knockdown of ZDHHC1 inhibited HSV-1-triggered expression of *IFNB1*, *TNFA* and *IL6* in these cells (Figure S6), we determined whether ZDHHC1 is associated with MITA in these cells. Endogenous coimmunoprecipitation experiments indicated that ZDHHC1 was associated with MITA in THP1 cells and this association was not affected following HSV-1 infection (Figure 6C). In these experiments, ZDHHC1 was not associated with AMFR/gp78, an abundant ER membrane protein that is involved in the final step of endoplasmic reticulum-associated degradation (ERAD) (Chen et al., 2012).

Previously, it has been demonstrated that MITA is localized at the ER and mitochondria. It has also been shown that sensing of viral DNA results in translocation of MITA to Golgi apparatus and eventually to cytoplasmic puncture structures (Burdette and Vance, 2013; Paludan and Bowie, 2013; Saitoh et al., 2009; Saitoh et al., 2010). Since ZDHHC1 contains four transmembrane domains and associates with MITA through their respective N-terminal transmembrane domains, we examined whether ZDHHC1 and MITA colocalize in the cell. Confocal microscopy experiments indicated that ZDHHC1 was colocalized with MITA before and after HSV-1 infection (Figure 5D). ZDHHC1 was mostly localized at the ER and Golgi apparatus but barely at mitochondria (Figure 5E). Interestingly, it seems that more ZDHHC1 was localized at the Golgi apparatus after HSV-1

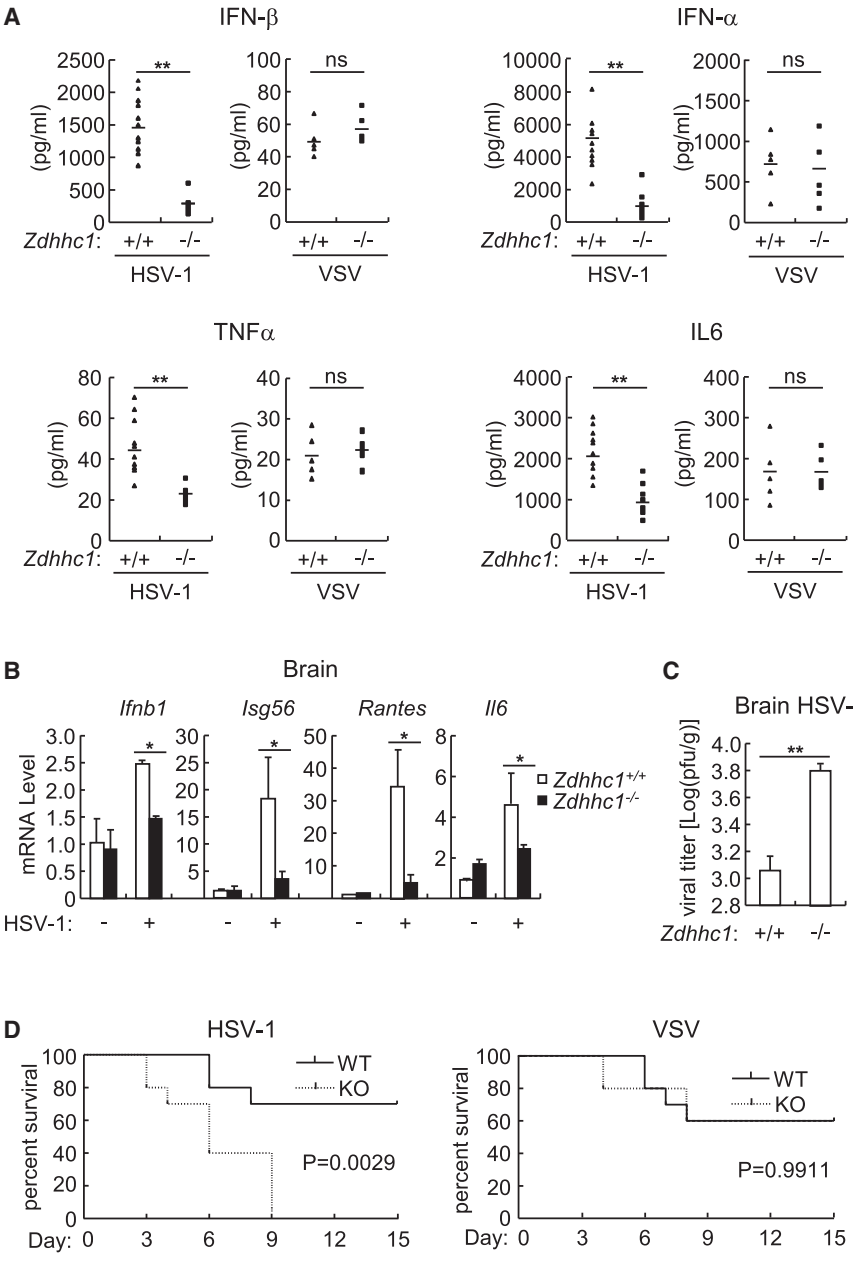

Figure 4. ZDHHC1 Is Essential for Host Defense against HSV-1 Infection

(A) *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ mice were infected i.p. with HSV-1 at 1 × 10^7 pfu per mouse (n = 10/11; 8 weeks old) and VSV at 1 × 10^7 pfu per mouse (n = 5; 8 weeks old) for 6 hr, followed by measurement of the indicated serum cytokines by ELISA.

(B) *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ were infected i.n. with HSV- 1 at 1 × 10^6 pfu per mouse (n = 5; 8 weeks old), and brains were retrieved after 6 days for HSV-1 plaque assays. The viral titer was recorded as pfu per gram of brain.

(C) *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ were infected i.n. with HSV-1 at 1 × 10^6 pfu per mouse (n = 5; 8 weeks old), and brains were retrieved after 6 days for qPCR analysis.

(D) *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ mice were infected i.p. with HSV-1 at 5 × 10^7 pfu per mouse or with VSV at 2 × 10^7 pfu per mouse (n = 10; 8 weeks old). The survival of infected mice was monitored for 15 days.

*p < 0.05; **p < 0.01 (Student's t test). The survival curve was generated by Kaplan-Meier methods followed by log-rank test analysis.

infection (Figure 5E). These data further suggest that ZDHHC1 and MITA are physically associated with each other.

ZDHHC1 Modulates MITA Activity

Since ZDHHC1 is physically associated with MITA, we next determined whether ZDHHC1 regulates MITA activity. Previous studies indicate that cGAS is an universal sensor of viral DNA (Li et al., 2013; Sun et al., 2013), and this sensing leads to production of cGAMP, which is a high affinity ligand for MITA (Ouyang et al., 2012; Wu et al., 2013; Xiao and Fitzgerald, 2013; Zhang et al., 2013). In reporter assays, transfection of B-DNA, cGAMP, cGAS, MITA or ZDHHC1 alone barely or only minimally activated the IFN-β promoter. Co-expression of MITA and ZDHHC1 weakly activated the IFN-β promoter, whereas MITA combines with an upstream signal, such as B-DNA, cGAMP or cGAS, activated the IFN-β promoter to a higher degree (Figure 6A). Importantly, ZDHHC1 greatly potentiated activation of the IFN-β promoter triggered by the combination of MITA and an upstream component such as B-DNA, cGAMP or cGAS (Figure 6A). Furthermore, knockdown of MITA inhibited ZDHHC1- and B-DNA-triggered activation of the IFN-β promoter (Figure 6B), whereas knockdown of ZDHHC1 inhibited B-DNA-, cGAMP plus MITA- but not MITA-triggered activation of the IFN-β promoter (Figure 6C). In addition, cGAMP- or c-di-GMP-induced transcription of *Ifnb1*, *Isg56*, *Tnfa* and *Il6* was impaired in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ DCs, MEFs and BMDMs (Figures 6D and S7). Similarly, cGAMP-triggered production of cytokines, including IFN-β, TNFα and IL6, was also inhibited in *Zdhhc1*$^{-/-}$ cells (Figures 6D and S7). Collectively, these results suggest that ZDHHC1 functions downstream of cGAMP to modulate MITA activity.

Previously, it has been shown that dimerization and aggregation of MITA is important for its activation after viral DNA sensing, which leads to its recruitment of downstream components TBK1 and IRF3 (Ouyang et al., 2012; Tanaka and Chen, 2012; Yin et al., 2012; Zhang et al., 2013). We found that knockdown of ZDHHC1 caused decreased dimerization of MITA (Figure 7A). Furthermore, we determined whether ZDHHC1 deficiency affects the dimerization and aggregation of MITA. As shown in Figure 7B, aggregation of MITA induced by HSV-1 or cGAMP was decreased in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ DCs.

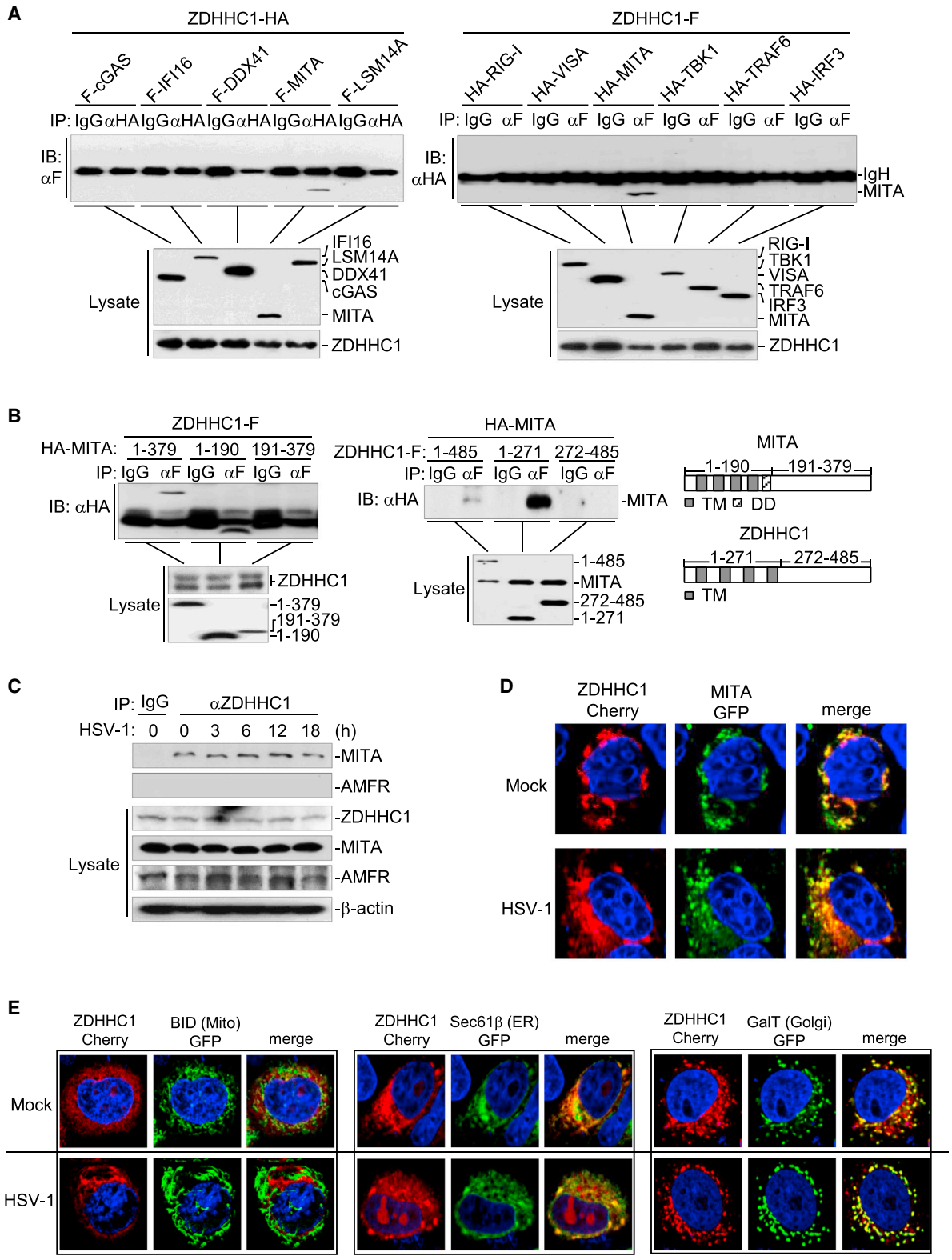

Figure 5. ZDHHC1 Is Associated and Colocalized with MITA

(A) ZDHHC1 is associated with MITA. The 293 cells (2×10^6) were transfected with the indicated plasmids (5 μg each). Coimmunoprecipitation and immunoblot were performed with the indicated antibodies.

(B) Domain mapping of ZDHHC1-MITA association. In the left panel, the experiments were performed similarly as in (A). The right panel shows schematic representations of MITA and ZDHHC1 proteins.

(legend continued on next page)

It has been reported that there are two natural MITA variants at aa232. The prevalent variant contains an arginine (R) whereas a rare variant contains a histidine (H) (Diner et al., 2013; Zhang et al., 2013). Crystal structure studies have demonstrated that R232 from a β sheet of MITA is involved in its binding to cyclic dinucleotides, and is important for the conformational change and functional activation of dimerized MITA (Burdette and Vance, 2013; Zhang et al., 2013). Functional studies have shown that cGAMP potentiates MITA(R232) but not MITA(H232)-mediated induction of IFN-β (Diner et al., 2013; Zhang et al., 2013). Interestingly, we confirmed that cGAMP could potentiate MITA(R232)- but not MITA(H232)-mediated activation of the IFN-β promoter (Figure 7C). However, ZDHHC1 could potentiate both MITA(R232)- and MITA(H232)-mediated activation of the IFN-β promoter (Figure 7C). This data support the hypothesis that ZDHHC1 is important for cGAMP-induced dimerization/aggregation and activation of MITA. Consistently, we found that recruitment of TBK1 and IRF3 to MITA after HSV-1 infection was markedly decreased in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ bone-marrow-derived DCs (Figure 7D). These results suggest that ZDHHC1 is important for the activation of MITA.

DISCUSSION

In recent years, how DNA viruses trigger innate immune response has been extensively investigated. Several recent breakthroughs have helped to outline a model on the induction of type I IFNs triggered by DNA viruses. In this model, cGAS serves as a sensor for viral DNA (Civril et al., 2013; Li et al., 2013; Sun et al., 2013). Upon binding of viral DNA, cGAS catalyzes the production of the second messenger molecule cGAMP, which binds to the adaptor protein MITA (Sun et al., 2013; Wu et al., 2013; Zhang et al., 2013). This induces the dimerization/aggregation and activation of MITA, which in turn recruits downstream TBK1 and IRF3, leading to induction of type I IFN genes (Tanaka and Chen, 2012; Zhong et al., 2008). In this study, we have utilized expression screens to identify ZDHHC1 as a mediator of DNA-virus-triggered IFN induction.

There are several lines of evidences suggesting that ZDHHC1 is important for innate immune response against DNA viruses. First, overexpression of ZDHHC1 activated the promoter of IFN-β and potentiated viral DNA-triggered activation of the IFN-β promoter. Second, induction of type I IFNs (IFN-β and IFN-α) and proinflammatory cytokines (TNFα and IL6) was impaired in *Zdhhc1*$^{-/-}$ MEFs, mouse BMDMs, and DCs upon infection with the DNA viruses HSV-1 and ECTV but not with the RNA viruses SeV and VSV. Third, serum cytokines (IFN-β, IFN-α, TNFα, and IL6) induced by HSV-1 infection were severely impaired in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ mice. In similar experiments, the serum cytokine levels induced by VSV infection were similar between *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ mice. Forth, expression of *Ifnb1*, *Isg56*, *Rantes*, and *Il6* were markedly reduced, whereas the viral titers were significantly increased in the brain of *Zdhhc1*$^{-/-}$ mice in comparison to their wild-type counterparts. In addition, *Zdhhc1*$^{-/-}$ mice were more susceptibility to HSV-1-triggered death than their wild-type counterparts. Collectively, these results suggest that ZDHHC1 is essential for efficient induction of downstream antiviral effecters and host defense against DNA but not RNA viruses in mice.

MITA is an adaptor protein essential for DNA-virus-triggered induction of type I IFNs and proinflammatory cytokines (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Zhong et al., 2008). Our studies suggest that the role of ZDHHC1 in innate immune response against DNA viruses is mediated by its physical and functional association with MITA. Similar to MITA, ZDHHC1 contains four N-terminal transmembrane domains and is predominantly localized at the ER. Coimmunoprecipitation experiments indicated that ZDHHC1 was constitutively associated with MITA before and after HSV-1 infection and this association was mediated by their respective N-terminal transmembrane domains. Confocal microscopy experiments indicate that ZDHHC1 was colocalized with MITA at the ER and both proteins were translocated to the Golgi apparatus and cytoplasmic puncture structures upon HSV-1 infection in HeLa cells.

In addition to a physical association between ZDHHC1 and MITA, several lines of results suggest that ZDHHC1 acts in innate antiviral response by modulating the activity of MITA. First, ZDHHC1 greatly potentiated activation of the IFN-β promoter triggered by the combination of MITA and an upstream component such as B-DNA, cGAMP, or cGAS. Second, knockdown of MITA inhibited ZDHHC1- and B-DNA-triggered activation of the IFN-β promoter, whereas knockdown of ZDHHC1 inhibited B-DNA-, cGAMP plus MITA-, but not MITA-triggered activation of the IFN-β promoter. Third, cGAMP- or c-di-GMP-induced expression of *Ifnb1*, *Isg56*, *Tnfa*, and *Il6* was impaired in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ cells. Forth, cGAMP potentiated MITA(R232)- but not MITA(H232)-mediated induction of IFN-β, whereas ZDHHC1 potentiated both MITA(R232)- and MITA(H232)-mediated induction of IFN-β. Fifth, knockdown of ZDHHC1 caused decreased dimerization of MITA, whereas the aggregation of MITA as well as the recruitment of TBK1 and IRF3 to MITA after HSV-1 infection was markedly decreased in *Zdhhc1*$^{-/-}$ in comparison to *Zdhhc1*$^{+/+}$ DCs. Collectively, these results support the hypothesis that ZDHHC1 is important for cGAMP-induced dimerization/aggregation and activation of MITA upon infection of DNA viruses.

Several studies have demonstrated that MITA is involved in IFN-β induction triggered by poly(I:C) and RNA viruses in certain human cell lines including 293 and HeLa cells (Ishikawa and Barber, 2008; Sun et al., 2009; Zhong et al., 2008). In addition, studies with *cGas*$^{-/-}$ cells and mice suggest that cGAS also plays a role in innate immune response against the ssRNA virus

(C) Endogenous ZDHHC1 is associated with MITA in THP1 cells. THP1 cells (5×10^7) were left untreated or infected with HSV-1 for the indicated times. Coimmunoprecipitation experiments were performed with anti-ZDHHC1, and the immunoprecipitates were analyzed by immunoblots with anti-MITA and anti-KDEL (which recognizes AMFR/gp78). The lysates were analyzed by immunoblots with the indicated antibodies.
(D and E) Colocalization of ZDHHC1 and MITA with the ER and Golgi. HeLa cells (1×10^5) were transfected with GFP-MITA and ZDHHC1-Cherry, as well as GFP-Sec61β (ER marker) or GFP-Galt (Golgi marker), respectively. Twenty hours after transfection, cells were left untreated or infected with HSV-1 for 4 hr before confocal microscopy.
See also Figure S6.

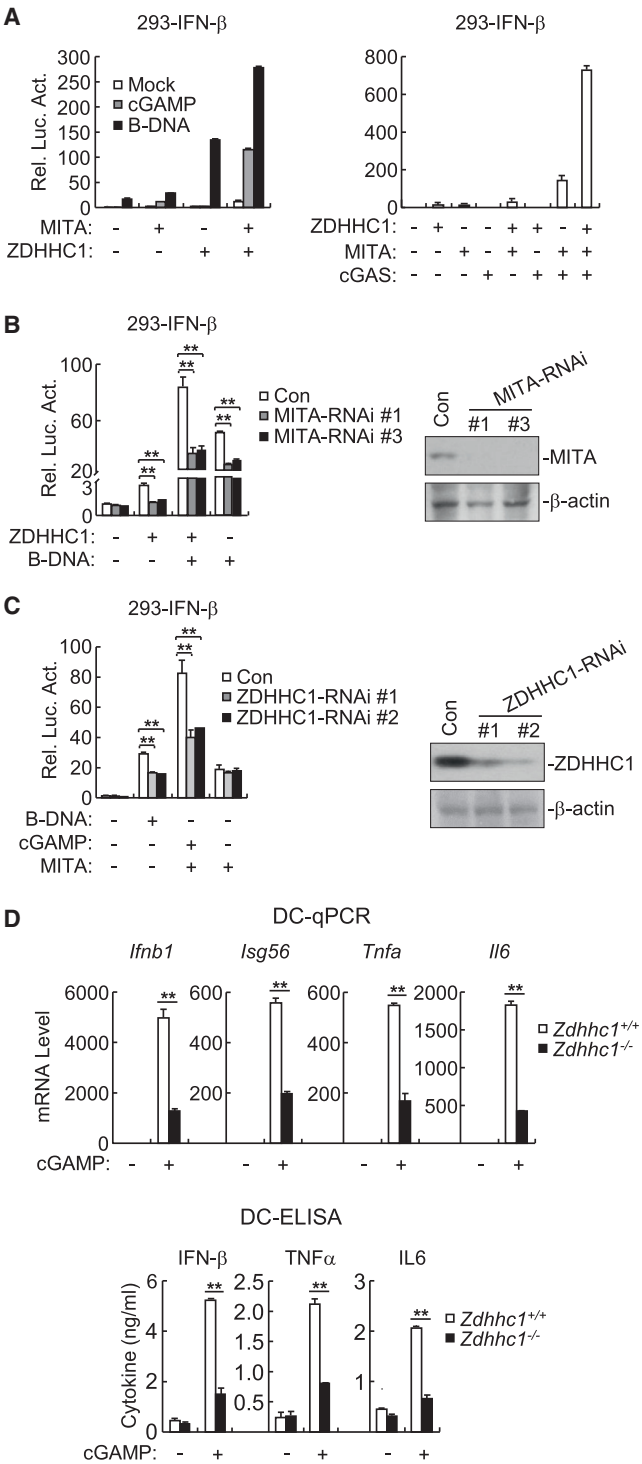

Figure 6. ZDHHC1 Signals through MITA

(A) Effects of ZDHHC1 on activation of the IFN-β promoter mediated by MITA and upstream components. The 293 cells (1×10^5) were transfected with the IFN-β promoter plasmid (0.1 μg) and the indicated plasmids (0.1 μg) for 20 hr or were further transfected with B-DNA (0.5 μg) or cGAMP (0.5 μg) for 16 hr before luciferase assays were performed.

(B) Knockdown of MITA inhibits ZDHHC1-, B-DNA-, and ZDHHC1+B-DNA-induced activation of the IFN-β promoter. The 293 cells (1×10^5) were first transfected with a control or two independent MITA-RNAi plasmids (1 μg). One day later, cells were selected with puromycin (1 μg/ml) for 24 hr and then retransfected with the IFN-β promoter reporter and ZDHHC1 expression plasmid (0.1 μg each) or B-DNA (3 μg/ml). Luciferase assays were performed 24 hr after retransfection. The efficiencies of MITA-RNAi plasmids were shown by immunoblots (left panels).

(C) Knockdown of ZDHHC1 inhibits B-DNA and MITA+cGAMP but not MITA-mediated activation of the IFN-β promoter. The experiments were similarly performed as in (B).

(D) Effects of ZDHHC1 deficiency on cGAMP-induced expression of the indicated genes. DCs were treated with cGAMP (100 nM) in digitonin permeabilization solution at 37°C for 30 min. The cells were then incubated in regular medium for 4 or 12 hr before qPCR or ELISA experiments were performed respectively.

See also Figure S7.

WNV (Schoggins et al., 2014). Interestingly, we found that overexpression of ZDHHC1 potentiated activation of the IFN-β promoter triggered by transfected poly(I:C) or SeV infection, whereas knockdown of ZDHHC1 inhibited activation of the IFN-β triggered by transfected poly(I:C) or SeV infection in HeLa cells. It is possible that, similar as MITA, ZDHHC1 is also involved in RNA-virus-triggered signaling in some human cell lines. This again further supports a physical and functional association between ZDHHC1 and MITA.

Previously, it has been demonstrated that cytokine production triggered by HSV-1 infection is almost completely abolished in *Mita*$^{-/-}$ cells (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Li et al., 2013), which we confirmed (Figure S7C). In this context, it is noticed that cytokine production triggered by HSV-1 in *Zdhhc1*$^{-/-}$ cells and mice was reduced to ~20%–50% in comparison to the wild-type counterparts. Similar observations were also found for cytokine production triggered by cGAMP or c-di-cGMP in *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ MEFs, BMDMs, and DCs. Based on these observations as well as the notion that cGAMP directly binds to MITA, we hypothesize that ZDHHC1 functions as an amplifier for MITA-mediated innate antiviral response. Alternatively, since ZDHHC1 is a member of the DHHC palmitoyl transferase family, it is possible that another member of the family may play a redundant role in MITA-mediated innate antiviral response. Nevertheless, modulation of MITA-mediated signaling by ZDHHC1 provides insight into the complicated and delicate mechanisms on innate antiviral response against DNA viruses.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Poly(I:C), 3′3′-cGAMP, and c-di-GMP (InvivoGen); GM-CSF (peproTech); lipofectamine 2000, and dual-specific luciferase assay kit (Promega); ELISA kit for murine IFN-α and IFN-β (PBL); ELISA kit for murine TNFα and IL6 (Biolegend); mouse antibodies against Flag, β-actin (Sigma), phospho-IκBα, p38, phospho-p38 (CST), and HA (Covance); rabbit polyclonal antibodies against ZDHHC1 (Abcam), KDEL, IRF3 and phospho-IRF3, p65 (Santa Cruz Biotechnology), cGAS/C6orf150 (AVIVA Systems Biology), TBK1, and phospho-TBK1(EPITOMICS) were purchased from the indicated manufacturers. SeV, VSV, HSV-1, and anti-MITA sera were previously described (Li et al., 2012; Zhong et al., 2008). HEK293, HeLa, and THP1 cells were obtained from ATCC. HEK293T cells were originally provided by Dr. Gary Johnson (National Jewish Health). *Mita*$^{-/-}$ mice were generated by conventional gene knockout procedures.

Expression Cloning

The cDNA expression clones encoding ~10,000 independent human cDNAs was obtained from Origene. The clones were individually transfected into

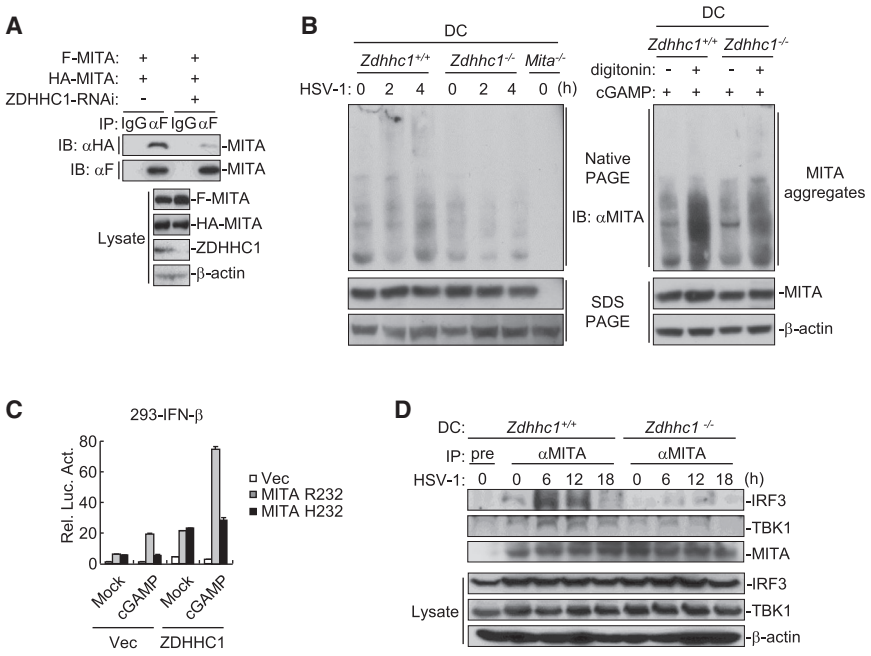

Figure 7. ZDHHC1 Modulates MITA Activity

(A) Knockdown of ZDHHC1 impairs dimerization of MITA. The 293 cells (2×10^6) were transfected with a control or ZDHHC1-RNAi plasmid (10 μg). Twelve hours after transfection, cells were selected with puromycin (1 μg /ml) for 24 hr, then further transfected with FLAG-MITA and HA-MITA for 20 hr before coimmunoprecipitation and immunoblot analysis were performed.

(B) ZDHHC1 deficiency impairs aggregation of MITA induced by HSV-1 or cGAMP. DCs (2×10^7) were infected with HSV-1 for the indicated times (left panels) or treated with cGAMP (100 nM) in control or digitonin-containing solution for 1 hr (right panels). Cell lysates were separated by native or SDS PAGE as indicated and analyzed by immunoblots with the indicated antibodies.

(C) ZDHHC1 potentiates both MITA(R232)- and MITA(H232)-mediated activation of the IFN-β promoter. The 293 cells (1×10^5) were transfected with the IFN-β promoter plasmid (0.1 μg) and the indicated plasmids (0.1 μg) for 20 hr or were further transfected with cGAMP (0.5 μg) for 16 hr before luciferase assays were performed.

(D) ZDHHC1 deficiency impairs HSV-induced recruitment of TBK1 and IRF3 to MITA. DCs (5×10^7) from *Zdhhc1*$^{-/-}$ and *Zdhhc1*$^{+/+}$ mice were left untreated or infected with HSV-1 for the indicated times. Coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies.

293 cells together with an IFN-β promoter reporter plasmid for 16 hr before luciferase assays.

Constructs

IFN-β, ISRE, and NF-κB luciferase reporter plasmids; mammalian expression plasmids for HA-, Flag-tagged MITA, and its mutants; and RIG-I, MDA5, VISA, TBK1, and IRF3 were previously described (Xu et al., 2005; Zhong et al., 2008). The pCMV-ERIS/MITA-HA plasmid was provided by Dr. Zhengfan Jiang (Peking University). pcDNA3.1-Flag-cGAS was provided by Dr. Zhijian Chen (University of Texas Southwestern Medical Center); Flag-tagged DDX41, IFI16, human and murine ZDHHC1, and human ZDHHC1 truncations and point mutants were constructed by standard molecular biology techniques.

DNA Oligonucleotides

The following oligonucleotides were used to stimulate cells:

poly(dG:dC): 5′-CCGCCAGCCCGCGGGCTGGCGCCCCCACTCGGGCCGTCGGGGCCGCGCCTCCCCCGCGAGGCCGCCGGCG-3′;

dsDNA90: 5′-TACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACATACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACA-3′;

HSV120:

5′-AGACGGTATATTTTTGCGTTATCACTGTCCCGGATTGGACACGGTCTTGTGGGATAGGCATGCCCAGAAGGCATATTGGGTTAACCCCTTTTTATTTGTGGCGGGTTTTTTGGAGGACTT-3′;

B-DNA: dsDNA with a sequence of poly(dA-dT)·poly(dT-dA).

Transfection and Reporter Assays

HEK293 cells were transfected by standard calcium phosphate precipitation method. HeLa cells and MEFs were transfected by lipofectamine 2000. To normalize for transfection efficiency, 0.01 μg of pRL-TK (Renilla luciferase) reporter plasmid was added to each transfection. Luciferase assays were performed using a dual-specific luciferase assay kit.

RNAi

Double-stranded oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro-RNAi plasmid (Oligoengine). The following sequences were targeted for ZDHHC1 mRNA: #1-AGGTGGAGTGGGATAGAAA; #2-GTGGAGTGGGATAGAAAGA. MITA-RNAi was as previously described (Zhong et al., 2008).

RNAi-Transduced Stable THP-1 Cells

The 293 cells were transfected with two packaging plasmids (pGAG-Pol and pVSV-G) together with a control or ZDHHC1-RNAi retroviral plasmid. Twenty-four hours later, cells were incubated with new medium without antibiotics for another 24 hr. The recombinant virus-containing medium was filtered and then added to THP-1 cells in the presence of polybrene (8 μg/ml). The infected cells were selected with puromycin (0.5 μg/ml) for 7 days before additional experiments.

Coimmunoprecipitation and Immunoblot Analysis

HEK293 cells (5×10^6), THP1 or DCs (5×10^7) were lysed in l ml NP-40 lysis buffer (20 mM Tris·HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Coimmunoprecipitation and immunoblot analysis were performed as previously described (Xu et al., 2005).

Generation and Phenotyping of *Zdhhc1* Knockout Mice

Strategy of construction of the targeting vector is illustrated in Figure S2A. The linearized targeting vector was electroporated into W4 ES cells, which was then selected in G418 medium. One positive clone was injected into 129S6/SvEvTac blastocysts. Genotyping by PCR was performed using a combination of the following primers: #1-GAGTGTATAGCCCCTGCCAGT, #2-CAACCCTGCACTACCTCACATG, and #3-CTCGAGGGGATCCACTAGTTC. Amplification of the wild-type allele with primers #1 and #2 results in a 459-bp fragment, whereas amplification of the disrupted allele with primers #2 and #3 results in a 264-bp fragment. The primers used for RT-PCR analysis of murine Zdhhc1 mRNA are as follows: ATGAACATCTGCAACAAACCC (forward) and ATGGTGATCGAAGCCGCATAC (reverse).

Preparations of MEFs, BMDMs, and DCs

MEFs were prepared from day 13.5 embryos and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine

serum. Bone marrow cells were isolated from tibia and femur. For preparation of BMDMs, the bone marrow cells were cultured in 10% M-CSF-containing conditional medium from L929 cells for 3–5 days. For preparation of DCs, the bone marrow cells were cultured in medium containing murine GM-CSF (50 ng/ml) and IL-4 (10 ng/ml) for 6–9 days.

Real-Time PCR

Total RNA was isolated for real-time PCR analysis to measure mRNA levels of the indicated genes. Data shown are the relative abundance of the indicated mRNA normalized to that of *Gapdh*. Primer sequences for *Ifnb1*, *Rantes*, *Isg56*, *Tnfa*, *Il6*, and *Gapdh* were as previously reported (Li et al., 2012).

Viral Infection in Mice

Mice were infected with HSV-1 or VSV i.p. or i.n. The viability of the infected mice was monitored for 15 days. The mouse serum was collected at 6 hr after infection to measure cytokine production by ELISA. Viral infection in mice was carried out in an ABSL-2 facility, and the protocols and procedures for mice study were approved by the Institutional Review Board of Wuhan Institute of Virology (WIVA31201301).

Viral Plaque Assay

Brains were weighed and homogenized for 3 s in DMEM medium. After homogenization, the brain suspensions were centrifuged at 1,620 × *g* for 30 min, and the supernatants were used for plaque assays on monolayers of Vero cells seeded in 24-well plates. The cells were infected by incubation for 1 hr at 37°C with serial dilutions of brain suspensions. After 1 hr infection, 2% methylcellulose was overlaid, and the plates were incubated for about 48 hr. The overlay was removed, and cells were fixed with 4% paraformaldehyde for 15 min and stained with 1% crystal violet for 30 min before plaque counting.

Fluorescent Confocal Microscopy

HeLa cells were transfected with the indicated plasmids by lipofectamine 2000 (Invitrogen). At 20 hr after transfection, the cells were infected with HSV-1 for 4 hr and then fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were observed with an Olympus confocal microscope under a 100× oil objective.

Flow Cytometry

Spleen, thymus, and peripheral lymph nodes were obtained from *Zdhhc1*$^{+/+}$ and *Zdhhc1*$^{-/-}$ mice, and single-cell suspensions were prepared. After depletion of red blood cells by ammonium chloride, cells were subject to staining with the indicated antibodies for 15 min followed by flow analysis. The antibodies used in this study were CD4-PerCP (BD), CD8-FITC (BD), CD3- FITC (BD), CD19-PE (BD), CD11c-PE (BD), and CD11b-FITC (BD).

Digitonin Permeabilization of Mouse DCs and BMDMs

DCs or BMDMs were treated with cGAMP (100 nM) in digitonin permeabilization solution (50 mM HEPES [pH 7.0], 100 mM KCl, 3 mM MgCl2, 0.1 mM DTT, 85 mM sucrose, 0.2% BSA, 1 mM ATP, 0.1 mM GTP, and 10 μg/ml digitonin) at 37°C for 30 min. The cells were then incubated in regular medium for 4 or 12 hr before qPCR or ELISA experiments were performed, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.09.006.

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