CircDDX17 enhances CVB3 replication by N6-methyladenosine regulation through miR-1248/NOTCH2 targeting

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Abstract

N6-methyladenosine (m6A) is the most prevalent internal mRNA modification in eukaryotes. m6A is necessary for RNA metabolism and function, and influences RNA viruses replication. Methyltransferase-like protein 3 (METTL3) is a “writer” to regulating m6A. Coxsackievirus B3 (CVB3), belonging to the genus *Enterovirus* of the Picornaviridae family, are species of non-enveloped, positive single-stranded RNA virus. CVB3 infection reduces circular RNA_DDX17 (circDDX17) expression level in HeLa and HL-1 cells, as in viral myocarditis (VM). CircDDX17 enhanced CVB3 replication in HeLa and HL-1 cells and regulated NOTCH Receptor 2 (NOTCH2) by targeting microRNA-1248 (miR-1248) as microRNAs (miRNAs) sponge. NOTCH2 interaction with METTL3, through m6A proteins like methyltransferase-like protein 14 (METTL14), AlkB Homolog 5 (ALKBH5), and YTH N6-Methyladenosine RNA Binding Protein 1 (YTHDF1) to improve CVB3 replication in host cells. Taken together, this study suggests that circDDX17 targets miR-1248 to regulate NOTCH2 expression and regulates METTL3 to enhance CVB3 replication in cells by m6A relation protein.

Introduction

Coxsackievirus B3 (CVB3) is an RNA virus belonging to the *Enterovirus* genus of Picornaviridae. CVB3 has been identified as the most common pathogen for viral myocarditis (VM). CVB3 infections are spread worldwide and the clinical manifestations are mainly asymptomatic or mild infections, cold-like symptoms, but newborns and children are more likely to have severe diseases, such as pancreatitis, myocarditis, encephalitis, and type 1 diabetes [1, 2].

Circular RNAs (circRNAs) are newly recognized non-coding RNAs considered small endogenous RNAs with a wide distribution, considerable variety, and multiple regulatory functions [3]. CircRNAs are circularized by connecting a 5' splice site with the 3' splice site of an upstream exon or intron by a back-splicing reaction [4]. Studies have revealed that circRNAs act as microRNAs (miRNAs) sponges and regulate the splicing and translation of target genes [5]. Some circRNAs serve as a novel diagnostic biomarker in hepatitis B virus derived hepatocellular cancer [6]. In influenza, a virus-induced lung injury [7], and Corona Virus Disease 2019 infected peripheral blood has many differentially expressed circRNAs [8]. These researches showed that circRNAs play an important role in infectious diseases. However, the relationship between circRNAs and CVB3 infection remains unknown.

N6-methyladenosine (m6A) is intimately associated with three categories of molecular compositions: “writers”, “readers”, and “erasers” [9]. Writers are m6A methyltransferases like the methyltransferase-like protein 3 (METTL3) and methyltransferase-like protein 14 (METTL14). Erasers are m6A demethylase, like the fat mass and obesity-associated (FTO) and AlkB Homolog 5 (ALKBH5) [10]. Readers are m6A recognition factors, including the YT521-B homology (YTH) domain family of proteins (YTHDF1, YTHDF2, YTHDF3, and YTHDC1) [10]. In previous researches, m6A methylation negatively regulates the production of hepatitis C virus and flavivirus Zika [11]. In other research, METTL3 inhibits Enterovirus 71 by autophagy regulation [12], decreases syndrome coronavirus clade 2 viral load and viral gene

expression in host cells [13], and promotes Epstein-Barr virus infection of nasopharyngeal epithelial cells [14].

NOTCH1 to 4 are transmembrane receptors that determine cell fate. The NOTCH Receptor 2 (NOTCH2) has been reported to exert distinct functions in regulating tissue homeostasis and cell fate determination [15, 16]. In infectious diseases, NOTCH2 is possibly involved in regulating the Epstein-Barr virus latent/lytic status [17], and 4.3% hepatitis C virus positive cells diffuse large B-cell lymphoma have NOTCH2 mutations [18]. Previous studies have shown that NOTCH2 has some relationship with METTL3, like the Notch signaling pathway as an important downstream target of METTL3 in muscle stem cells [19].

Here, we examine the prevalence, regulation, and functional roles of circDDX17 in CVB3 infection. CVB3 infection could decrease the expression level of circDDX17, and circDDX17 up-regulation the CVB3 replication in HeLa and HL-1 cells. CircDDX17 regulation NOTCH2 by target miR-1248. NOTCH2 and METTL3 expression have a positive correlation; NOTCH2 could regulate CVB3 replication by METTL3, and m6A methylation associated proteins.

Results

Result 1. Characterization of the existence and subcellular distribution of circDDX17 in HeLa cells.

To study circDDX17, the circBank showed that circDDX17 is formed by reverse splicing of the linear transcript of exons 2-5 of DDX17 gene with a length of 451 nucleotides. To further characterize circDDX17, Sanger sequencing was performed to confirm head-to-tail splicing (Fig. 1a-b). A nuclear separation assay (Fig. 1c-d) was conducted to determine the subcellular localization of circDDX17 in HeLa cell lines.

To investigate the roles of circDDX17 in CVB3 infection, HeLa and HL-1 cells were infected with CVB3, and circDDX17 levels were examined. At the early stages of infection (0–2 h), no significant change of circDDX17 expression was detected between CVB3-infected and mock control cells. At 4–7 h post-infection, the circDDX17 expression was significantly decreased compared to MOCK cells (Fig. 1e). CVB3 infection could inhibit circDDX17 in HL-1 cells (Fig. 1f). In VM mice, circDDX17 expression level was lower than MOCK mice (Fig. 1g).

Results 2. CVB3 infection reduced circDDX17 expression level, and circDDX17 promotes CVB3 replication.

HeLa cells (Fig. 2a-b) and HL-1 cells (Fig. 2c) overexpressing circDDX17 and infected with CVB3 were collected for RNA and total protein to analyze CVB3 encodes structural proteins VP1 expression. The results show that circDDX17 increase VP1 expression after CVB3 infection. HEK-293T cells co-transfected with pcicR-3.0-circDDX17 (pcircDDX17) and GFP-CVB3, cells overexpression circDDX17 GFP-positive cell number were observed more than cells co-transfected with pcicR-3.0 (pcicR) and GFP-CVB3 (Fig. 2d). CircDDX17-promoted CVB3 replication was further confirmed by viral plaque assay, showing a significant

increase in viral particle release in circDDX17-expressing cells compared to the supernatants of cells transfected pcicR (Fig. 2e).

CircDDX17-silenced HeLa cells showed VP1 suppress expression at CVB3 5 h and 7 h post-infection (Fig. 2f-h). HEK-293T cells co-transfected with siRNA-circDDX17 (si-circDDX17) and GFP-CVB3 showed lower GFP-positive cells than the cells transfected siRNA-circDDX17-NC (si-circDDX17-NC) and GFP-CVB3 (Fig. 2i), and silencing of circDDX17 could reduce virus release (Fig. 2j).

Results 3. CircDDX17 promotes CVB3 replication by targeting miR-1248 and miR-1248 down-regulate NOTCH2 expression.

CircRNAs have been shown to act as a miRNA sponge to regulate gene expression [20]; therefore, the potential miRNAs associated with circDDX17 were investigated. First, through bioinformatics analysis, three miRNAs (miR-885, miR-1248, miR-1279) were identified from the overlap of three databases (circBank, miRanda, and CircInteractome) as possible targets for circDDX17. RT-qPCR of HeLa cells transfected with pcircDDX17 or si-circDDX17 showed that circDDX17 down-regulates the miRNAs expression (Fig. 3a-c). To analyze the role of CVB3 on miRNAs expression, total RNA from CVB3 infected cells was collected for RT-qPCR. miR-885, miR-1248, and miR-1279 expression increase with virus infection (Fig. 3d-f). To investigate the role of miRNAs in CVB3 infection, HeLa cells were transfected with miRNAs mimics, and the results showed that miR-885, miR-1248, and miR-1279 could inhibit the replication of CVB3, among which miR-1248 had the most obvious effect (Fig. 3g), so we chose miR-1248 as the object of study. The dual-luciferase reporter assay was performed to confirm the direct interaction between circDDX17 and miR-1248. The circDDX17-wt and circDDX17-mu full-length sequences without miR-1248 binding sites were cloned into the luciferase vector. Subsequently, luciferase reporter assays confirmed that miR-1248 markedly reduced the luciferase activity of psiCHECK-2-circDDX17-wild-type (circDDX17-wt) but not that of psiCHECK-2-circDDX17-mutant (circDDX17-mu) compared to the miR-NC group (Fig. 3h). To investigate the signal pathways contributing to the miR-1248 effect on CVB3 replication, we sought to identify its target genes. Bioinformatic analyses using TargetScan, miRDB and miRWalk programs showed that NOTCH2 is one of the predicted targets (Fig. 3i). HeLa cells were transfected with microRNA-1248 mimics (miR-1248) or microRNA-1248-inhibitor (miR-1248-in), and the results showed that miR-1248 has a negative regulatory role on NOTCH2 expression (Fig. 3j).

In previous researches, NOTCH2 has a relationship between METTL3 and DNA-methylation [21]; therefore, HeLa cells were transfected with specific siRNA-NOTCH2 (si-NOTCH2) or pcDNA-3.0-NOTCH2 (pNOTCH2) to silence or overexpressed NOTCH2. The Western blot results show that NOTCH2 regulates METTL3 expression, METTL3 increases with increasing expression level of NOTCH2 and decreases with NOTCH2 decreasing expression level (Fig. 3k-l).

Results 4. CircDDX17 promotes CVB3 replication by targeting the DNA-methylation pathway.

To elucidate the mechanism of CVB3 upregulation of host circDDX17, the expressions of DNA-methylation-associated proteins METTL3, METTL14, ALKBH5 and YTHDF1 were examined. With CVB3

infection, NOTCH2 declined gradually, METTL3 and METTL14 were elevated, and ALBK5 and YTHDF1 as the virus infection gradually decreased (Fig. 4a-b). To understand the roles of NOTCH2 in the circDDX17-mediated DNA-methylation, we examined the downstream effector genes expression after CVB3 infection in HeLa cells overexpressing or silencing circDDX17. Western blot analysis showed that overexpression of circDDX17 upregulated the expression level of NOTCH2, METTL3, METTL14, ALBK5 and YTHDF1 (Fig. 4c-e). Conversely, circDDX17 silencing decreases NOTCH2, METTL3, METTL14, ALBK5 and YTHDF1 expression levels (Fig. 4f-h).

Results 5. CircDDX17 promotes CVB3 replication by targeting miR-1248.

To study the effect of CVB3-induced miR-1248 expression on host-virus interactions, cells were transfected with miRNA mimics and miRNA inhibitor, infected with CVB3, and their effect on CVB3 replication, NOTCH2, METTL3, METTL14, ALBK5 and YTHDF1 expression was examined. Western blot results showed that miR-1248 decreased VP1 expression, while inhibit miR-1248 increased VP1 expression (Fig. 5a-c). CVB3 infection enhances METTL3, METTL14, inhibiting NOTCH2, ALBK5 and YTHDF1 expression. NOTCH2 is a miR-1248 target, miR-1248 play a negative role on NOTCH2 expression independent of CVB3 infection, as NOTCH2 expression levels decreased, so did METTL3 and METTL14 expression levels. MiR-1248 reduce ALBK5 and YTHDF1 expression and miR-1248-in restore their expression after CVB3 infection. However, this phenomenon was not obvious without CVB3 infection.

To further confirm that miR-1248-mediated circDDX17 upregulation improves CVB3 replication, circDDX17 was overexpressed in the presence of miR-1248 (Fig. 5d-f). Western blot results showed that compared with miR-NC + PcicR, overexpression of miR-1248 inhibited VP1, NOTCH2, and METTL3 expression. However, the impact of miR-1248 and pcircDDX17 co-transfection in METTL14, ALBK5 and YTHDF1 expression is not obvious.

HEK-293T cells were co-transfected with pcircDDX17, miR-1248, and GFP-CVB3 for 48 h. Cells transfected pcircDDX17 + miR-NC GFP-positive cell number were observed more than cells transfected pcicR + miR-NC, and pcircDDX17 + miR-1248 co-transfected could improve GFP-positive cells number than cells co-transfected pcicR + miR-1248 (Fig. 5g). HeLa cells overexpressing miR-1248 reduce CVB3 replication 7 h post-infection, and cells silencing miR-1248 increased CVB3 replication 7 h post-infection (Fig. 5h-i). At the same time, miR-1248 decreased CVB3 released as detected by viral plaque assay (Fig. 5j).

Results 6. MiR-1248 inhibits CVB3 replication by targeting NOTCH2 through methylation-related pathway.

To understand the roles of NOTCH2 in circDDX17 and miR-1248-mediated methylation-related pathways, we examined the downstream effector genes expression after CVB3 infection. We first confirmed NOTCH2 function on methylation-related proteins (Fig. 6a-c). Western blot data shows that independent of CVB3 infection, METTL3, METTL14, ALBK5 and YTHDF1 expression were reduced by si-NOTCH2 transfection and induced in pNOTCH2 transfection. In HeLa cells, VP1 expression was decreased by si-NOTCH2 and increased by pNOTCH2 expression. Furthermore, in HL-1 cells, overexpression of NOTCH2 increase VP1 expression compared to the negative control (Fig. 6d). NOTCH2 could significantly increase

VP1 expression level, NOTCH2 deficiency repressed VP1 expression (Fig. 6e-f), and overexpression NOTCH2 increased viral release (Fig. 6g).

To further confirm miR-1248 regulates CVB3 replication by targeting NOTCH2, HeLa cells overexpression miR-1248 and NOTCH2 by co-transfection (Fig. 6h-j). The Western blot analysis data shows that HeLa cells overexpressed NOTCH2 when transfected with pNOTCH2. Without CVB3 infection, cells transfection miR-NC + pNOTCH2 the METTL3 and METTL14 expression levels were higher than cells transfection miR-1248 + pNOTCH2. Seven hours post CVB3 infection, miR-NC + pNOTCH2 increased the production of VP1, METTL3, and ALBK5 compared with miR-NC + pcDNA. Co-transfection of pNOTCH2 and miR-1248 reduced the production of VP1, METTL3, and ALBK5 compared to miR-NC + pNOTCH2.

HEK-293T cells co-transfected with miR-1248, pNOTCH2, and GFP-CVB3 for 48 h, cells transfected miR-NC + pNOTCH2 led GFP-positive cell number observe more than miR-NC + pcDNA, miR-1248 + pNOTCH2 co-transfected could reduce GFP-positive cells number than its miR-NC + pNOTCH2 (Fig. 6k).

Discussion

This study reported that circDDX17 promotes CVB3 replication by regulating miR-1248 and NOTCH2, through METTL3 and other m6A associated proteins. MiR-1248, a target miRNA of circDDX17, plays a negative role in the replication of CVB3 in host cells. NOTCH2 is a fascinating target gene because it has positively correlated with METTL3. NOTCH intercellular signaling is essential to cell fate during differentiation, controlling proliferation, survival, and homeostasis in many cell types [21]. NOTCH signaling played a regulatory role in viral infections, like hepatitis B virus, hepatitis C virus, Epstein-Barr virus, human immunodeficiency virus type 1 and human papillomaviruses [17, 22-27]. Currently, there are no studies between NOTCH2 and CVB3 infection. We found that CVB3 infection decreased NOTCH2 expression, while NOTCH2 increased CVB3 replication.

CVB3 is the commonest pathogen for acute and chronic myocarditis [1, 28]. After CVB3 entry into the cardiomyocytes, the virus replicates and induces cell damage, triggering the host immune responses. If the virus cannot be eliminated, myocarditis can become chronic, triggering extensive myocardial fibrosis and the development of dilated cardiomyopathy [29, 30]. In addition, NOTCH2 regulates heart development, is involved in cardiac fibrosis, and regulates multiple antiviral immune responses, and NOTCH2 mutations result in multiple cardiac disease and vascular anomalies [31]. Currently, the NOTCH2 and CVB3-induced myocarditis remains unknown, and further research is required.

m6A is a conserved internal modification found in almost all eukaryotic nuclear RNAs [32], and in the viral RNA. m6A is dynamic methylation involved in RNA metabolism, splicing, and decay [33, 34]. METTL3 could modulate the NOTCH signaling pathway [35]. In our study, CVB3 infection increased METTL3 and reduced NOTCH2 expression. Furthermore, there is a positive correlation between NOTCH2 and METTL3. METTL3 could negatively regulate type I interferon response by dictating the fast turnover of interferon mRNAs for antiviral [36], and METTL3 boosted Enterovirus 71 replication [37], which might explain how NOTCH2 regulates viral replication. In another way, m6A modification is dynamically and reversibly

regulated by “writers” complex (METTL3 and METTL14) [38], and removed by “erasers” (ALKBH5) [39, 40], it functions by interacting with m6A binding proteins “readers” (YTHDF1, YTHDF2, YTHDF3, and YTHDC1)[41]. Our study analyzed METTL3, METTL14, ALKBH5 and YTHDF1 as targets indicating m6A modification changes and function in cells overexpressing or silencing circDDX17 infected with CVB3. The results showed that CVB3 infection could increase the METTL3 and METTL14 expression. METTL14 plays an important role in the transcription of IFNs and inflammatory cytokines, and regulates antiviral innate immunology response [42]. However, in this research, we have not studied the effect of METTL14 on the replication of CVB3. The ALKBH5 could remove methyl groups, although it is no study focusing on the function of ALKBH5 in CVB3 infection. In others research, silencing ALKBH5 and YTHDF1 increased flavivirus Zika RNA expression [43]. In our study, with the extension of CVB3 infection, the expression of ALKBH5 and YTHDF1 decreases.

MiRNAs and genes regulating CVB3 replication are common. In our previous study, miR-324-3p inhibits CVB3 replication by targeting the tripartite motif 27, but there are fewer studies on circRNA regulation of CVB3 replication. CircRNA can play a role as a miRNA sponge to influence miRNA expression and regulate gene function. We identified circDDX17 as a novel regulator of CVB3 replication. miR-1248 is the specific target miRNA of circDDX17. Moreover, NOTCH2 is miR-1248 target gene, which is distributed in m6A modification proteins. During viral infection, circDDX17 improved the formation and release of viral particles to initiate new infections by increasing miR-1248 and inhibiting NOTCH2 and the interaction of NOTCH2 with METTL3. These findings enrich our understanding of the functional roles of circRNA in viral replication and provide novel insights into the development of therapeutic strategies.

Conclusion

Taken together, our study highlighted circDDX17 as a crucial regulator of CVB3 replication in host cell. We identified miR-1248 as target miRNA of circDDX17 and NOTCH2 as target gene of miR-1248, the results showed circDDX17 regulated CVB3 replication by miR-1248/NOTCH2, and through METTL3 and other m6A genes like METTL14, ALKBH5 and YTHDF1. CircDDX17 and its target miRNA and genes may, thus, constitute attractive therapeutic targets in the fight of CVB3 infection related diseases.

Materials And Methods

Cells

HeLa and HEK-293T cells were a gift from Dr. Huaiqi Jing (National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention). HL-1 cells were stored at School of Medicine, Jiangsu University. Cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA), supplemented with 8% fetal bovine serum (FBS, Gibco, USA), 100U/ml penicillin, and 100µg/ml streptomycin in 5% CO² at 37°C.

Virus

CVB3 (Nancy) [44] was a gift from Professor Ruizhen Chen (Department of Cardiology, Zhongshan Hospital, Shanghai, China). GFP-CVB3, expressing the enhanced green fluorescence protein (GFP), were constructed previously based on pMKS1, a plasmid containing the full-length CVB3 genomic complementary DNA (a gift from Professor Zhaohua Zhong, Harbin Medical University, Harbin, China) [45, 46].

Myocarditis

CVB3 (10^5 PFU/mouse) was injected to 3-week-old BALB/c male mice intraperitoneally. CVB3 was diluted in 100 μ L phosphate buffer saline (PBS, Gibco, USA) for injection, and an equal volume of PBS was injected into the blank control mice (3 mice per group).

Plasmid, miRNA, siRNA, and transfection

PcicR-3.0 (pcicR) was a gift from Professor Songfeng Zhou (Nantong University, Nantong China), pcicR-3.0-circDDX17 (pcircDDX17) for overexpression circDDX17. PcDNA-3.0-NOTCH2 (pNOTCH2) for overexpression NOTCH2, used pcDNA-3.0 (pcDNA) for its negative control. MiR-1248 mimics (miR-1248), negative control miR-NC; miR-1248 inhibitor (miR-1248-in), negative control (NC-in); siRNA-NOTCH2 (si-NOTCH2), negative control (si-NOTCH2-NC) were all synthesized by GenePharma Co., Ltd. (Suzhou, China). Plasmid and oligonucleotide transfection of cells in 12-well plates (1.5×10^5 /well) were transfected using Lipofectamine 3000TM (Invitrogen, USA).

RT-qPCR

The total RNA was isolated using Trizol reagent (Invitrogen, USA). PrimeScript RT Reagent Kit (Takara, Japan) was used for reverse transcription RNA, and quantitative real-time PCR was performed using TB Green Premix Ex TaqII (Takara, Japan). The divergent primer was synthesized by Sangon (China). For RNase treatment, 2 mg of total RNA was incubated with or without 3 U/mg RNase R for 30 min at 37 °C.

Western blot

The total proteins of the cells were extracted using the RIPA lysis buffer (Sigma, USA). Samples were subjected to 12% SDS-PAGE and transferred to polycylinidene fluoride (PVDF) membranes (Millipore, USA). The primary antibodies against NOTCH2 (1:1000, Sangon, China), VP1 (1:1000, Genetex, USA), METTL3 (1:2000, CST, USA), METTL14 (1:2000, CST, USA), ALBKH5 (1:1000, CST, USA), YTHDF1 (1:1000, CST, USA), and GAPDH (1:20000, Genetex, USA). Membranes were blocked, incubated with secondary antibody (1:20000, Jackson, USA) and detected by electrochemiluminescence (ECL, Millipore, USA).

Luciferase Reporter Assays

HEK-293T cells (1×10^5 /well) were plated in 24-well plates. Cells were co-transfected with miR-1248 and psiCHECK-2 luciferase reporter to generate psiCHECK-2-circDDX17-wild-type (circDDX17-wt) or psiCHECK-

2-circDDX17-mutant (circDDX17-mu) constructs after 48 h. Luciferase activity was determined following a dual-luciferase reporter assay detection kit (Promega, WI, USA).

Plaque assay

Sample supernatants were collected at CVB3 7 h post-infection, serially diluted and added onto HeLa cells in 24-well plates (1.0×10^5 cells/well). After incubation for 1 h, cells were rewashed with PBS three times, overlaid with 0.7 % soft agar medium, and incubated for 3 days. Cells were fixed with glacial acetic acid for 30 min and stained with % crystal violet. All the assays were conducted at least in triplicate.

Statistical Analysis

Statistical analysis was performed using the Graph Pad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). The group difference was evaluated by student's t test, error bars represent mean $\pm$ SD and the difference was statistically significant when $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

Declarations

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Author Contributions

HXS and HW conceived and designed the experiments. TJL, YHL, XRQ and YW performed the experiments. HXS, HW and QRY analyzed the data. TJL, HXS, HW, JT and XTD contributed reagents/materials/analysis tools. TJL, HXS and HW wrote the paper.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. Data and material will be made available on reasonable request.

Conflict of interest The authors declare no conflict of interests.

Ethics approval This study was conducted in strict accordance with the recommendations in the Guide to the Care and Use of Experimental Animals-Chinese Council on Animal Care. All protocols were approved

by the Animal Care Committee of University Jiangsu, protocol number: UJS-IACUC-AP-20190307087.

Consent to participate All authors agreed to participate.

Consent for publication All authors read the manuscript and agreed to submission.

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Figures

Figure 1

Characterization and subcellular distribution of circDDX17 in HeLa cells.

a and b. Sanger sequencing confirmation of the head-to-tail splicing of circDDX17. **c and d.** nuclear mass separation assay show that the sub-cellular distribution of circDDX17 was mainly in cytoplasmic. **e.** RT-qPCR analysis of circDDX17 expression in HeLa cells 7 h after CVB3 infection. **f.** RT-qPCR analysis of circDDX17 in CVB3-infected HL-1 cells (100 MOI and 200 MOI) for 24 h. **g.** RT-qPCR analysis of circDDX17 in viral myocarditis, MOCK mice were as control.

Figure 2

CircDDX17 promotes CVB3 replication.

a and b. Western blot and RT-qPCR of VP1 expression levels in CVB3-infected HeLa cells overexpressing circDDX17 at 7 h (Western blot and analyzed using ImageJ software). **c.** Western blot to analysis expression of VP1 after HL-1 overexpression circDDX17. **d.** HEK-293T cells co-transfected pcircDDX17 and GFP-CVB3, observed GFP-positive cell number after 48 h. **e.** Viral plaque assay. Viral titer was determined by plaque assay using the supernatants collected at 7 h (n=3). **f.** RT-qPCR of circDDX17 in si-circDDX17 transfected HeLa cells. **g and h.** Western blot and RT-qPCR of VP1 expression in HeLa cells transfected with the si-circDDX17 and infected CVB3 (10 MOI) for 7 h. **i.** HEK-293T co-transfected with si-circDDX17 and GFP-CVB3 show higher GFP-positive cell number after 48 h. **j.** Viral titers were determined by plaque assay using the supernatants. (n=3).

Figure 3

CircDDX17 promotes CVB3 replication by targeting miR-1248, and miR-1248 targeting NOTCH2.

a, b, and c. Relative expression levels of miR-885, miR-1248, and miR-1279 in HeLa cells were determined by RT-qPCR after cells overexpression or silence the circDDX17. **d, e, and f.** RT-qPCR to detected expression of miR-885, miR-1248, and miR-1279 in CVB3 (10 MOI) infected HeLa cells. **g.** HeLa cells transfected with miR-885, miR-1248, and miR-1279 used the miR-NC for control, infected the CVB3 (10 MOI) for 7 h. Western blot analysis of the VP1 expression. **h.** The putative miR-1248 binding site in circDDX17 (circDDX17-wt) and the designated mutant sequence (circDDX17-mu) are illustrated. Validation of circDDX17 targeting on miR-1248 luciferase assay. **i.** MiR-1248 has a putative binding site for NOTCH2 in three databases. **j.** HeLa cells transfected miR-1248 or miR-1248-in, used the miR-NC and NC-in for negative control, indicated signals were detected by Western blot. **k.** HeLa cells transfected si-NOTCH2, indicated signals were detected by Western blot. **l.** NOTCH2 overexpression in HeLa cells. Indicated signals were detected by Western blot.

Figure 4

CircDDX17 regulates NOTCH2 expression and influences methylation-related pathways after CVB3 infection.

a and b. HeLa cells infected CVB3 (10 MOI) for 7 h, signals were detected by Western blot. **c, d, and e.** HeLa cells overexpression circDDX17 by pcircDDX17, used the PcicR as control, cells infected CVB3 (10 MOI) for 7 h, indicated signals were detected by Western blot. **f, g, and h.** HeLa cells silenced circDDX17 by si-circDDX17, used the si-circDDX17-NC as control, cells infected CVB3 (10 MOI) for 7 h, indicated signals were detected by Western blot.

Figure 5

a, b, and c. HeLa cells transfected miR-1248 or miR-1248-in, used the miR-NC or NC-in for control, cells infected CVB3 (10 MOI) for 7 h, indicated signals were detected by Western blot. **d, e, and f.** HeLa cells co-transfected miR-1248 and pcircDDX17, used the miR-NC and PcicR for control, cells infected CVB3 (10 MOI) for 7 h, indicated signals were detected by Western blot. **g.** HEK-293T cells co-transfected the miR-1248 and pcircDDX17, used the miR-NC and PcicR for control, cells transfected the GFP-CVB3 at the same time, observed GFP-positive cell number after 48 h. **h.** HeLa cells transfected the miR-1248, used miR-NC as a negative control, infected CVB3 (10 MOI) for 7 h, RT-qPCR to detect the VP1 expression. **i.**

HeLa cells silenced miR-1248 and infected CVB3 (10 MOI) for 7 h, RT-qPCR to detect the VP1 expression. j. Viral titers were determined by plaque assay using the supernatants of HeLa cells overexpression miR-1248 (n=3).

Figure 6

MiR-1248 regulates CVB3 replication by targeting NOTCH2 through methylation-related pathways.

a, b, and c. HeLa cells transfected with pNOTCH2 or pcDNA and infected with CVB3 (10 MOI) for 7 h. Signals were detected by Western blot and analyzed using ImageJ software. **d.** HL-1 cells transfected the pNOTCH2, used the pcDNA for control, infected CVB3 (100 MOI) for 24 h, Western blot to analysis the VP1. **e.** HeLa cells transfected pNOTCH2, used pcDNA as a negative control, infected CVB3 (10 MOI) for 7 h, RT-qPCR to detect the VP1 expression. **f.** HeLa cells silenced NOTCH2 by special siRNA and infected CVB3 (10 MOI) for 7 h, RT-qPCR to detect the VP1 expression. **g.** Viral titers were determined by plaque assay using the supernatants (n=3). **h, i, and j.** HeLa cells co-transfected miR-1248 mimics and pNOTCH2, used the miR-NC and pcDNA for control, cells infected CVB3 (10 MOI) for 7 h, indicated signals were detected by Western blot. **k.** HEK-293T cells co-transfected the miR-1248 and pNOTCH2, used the NC and pcDNA for control, and cells transfected the GFP-CVB3, observed GFP-positive cell number after 48 h.