Interferon-induced transmembrane protein 1 (IFITM1), also known as interferon-inducible protein 9–27, CD225 and Leu13, is a cell surface molecule that is important for antiproliferative and homotypic adhesion signal transduction in lymphocytes [1,2]. IFITM1 could be highly induced by interferon-α and -γ in response to infection by pathogens, and demonstrated antiviral activities, such as inhibition of influenza A replication and enveloped virus infection [3]. Moreover, IFITM proteins restrict infection of various types of viruses by suppressing viral membrane fusion and infection before the occurrence of hemifusion, through interruption of viral coreceptor function or inhibition of virus entry and replication [4–7]. IFITM1 expression was found to be elevated in cancers of the cervix [8], esophagus [9], ovary [10] and brain [11]. Overexpression of IFITM1 has clinicopathological effects on gastric cancer and is regulated by an epigenetic mechanism [12]. Furthermore, IFITM1 is up-regulated in human colorectal cancer (CRC) and has been

**Abbreviations**

ChIP, chromatin immunoprecipitation; CRC, colorectal cancer; ERV, endogenous retrovirus; H3K9me3, trimethylation of histone H3 on lysine 9; HEF, human embryonic fibroblast; HERV, human endogenous retrovirus; hESC, human embryonic stem cell; IFITM1, interferon-induced transmembrane protein 1; LTR, long terminal repeat; PGC, primordial germ cell; qRT-PCR, quantitative real-time PCR; TERT, telomerase reverse transcriptase; TRF, terminal restriction fragment.
identified as a molecular marker in human colorectal tumors [13]. Recently, IFITM1 was found to be highly expressed in metastatic CRC cell lines as well as colorectal patient-derived tumor samples, and its high expression is associated with a poor prognosis of the disease [14,15], or a more advanced clinical stage [16].

Interestingly, IFITM1 is also expressed in mouse primordial germ cells (PGCs) and is implicated in PGC development [17], and in human naïve pluripotent stem cells [18]. However, it also has been shown that Ifitm genes are not essential for PGC migration and the Ifitm family appears to be functionally redundant during development [19]. The function and implication of IFITM1 expression in pluripotent stem cells remain unclear. Here we investigated the potential roles and the underlying mechanisms of IFITM1 in human embryonic stem cell (hESCs).

Materials and methods

Cell culture

Human embryonic stem cells (RUES2 WT and RUES2 IFITM1-KO) were cultured at 37°C in 5% CO₂ in Essential 8 medium (A1517001, Life Technologies, Carlsbad, CA, USA). Human embryonic fibroblast (HEF) cells were cultured in Dulbecco’s modified Eagle’s medium, 10% FBS, 1% l-glutamine and 1% penicillin/streptomycin. RUES2 IFITM1-KO were cultured at 37°C in 5% CO₂ in Essential 8 medium (A1517001, Life Technologies, Carlsbad, CA, USA). Human embryonic fibroblast (HEF) cells were cultured in Dulbecco’s modified Eagle’s medium, 10% FBS, 1% l-glutamine and 1% penicillin/streptomycin.

Immunofluorescence

Cells were washed twice in phosphate buffered saline (PBS), then fixed in freshly prepared 3.7% paraformaldehyde in PBS for 15 min on ice, washed three times, permeabilized in 0.1% Triton X-100 in blocking solution (3% goat serum plus 0.5% BSA in PBS) for 30 min at room temperature, washed three times each for 5 min, and left in blocking solution (3% goat serum plus 0.5% BSA in PBS) for 2 h. Cells were incubated overnight at 4°C with primary antibodies against IFITM1/2/3 (F12; sc-374026; Santa Cruz Biotechnology, Dallas, TX, USA), IFITM3 (AF3377; R&D Systems, Minneapolis, MN, USA), OCT3/4 (SC-5279; Santa Cruz Biotechnology), or 53BP1 (ab36823, Abcam, Cambridge, MA, USA), washed three times and incubated for 1.5 h with secondary antibodies (goat anti-mouse IgG (H+L) fluorescein isothiocyanate, 115-095-003, Jackson ImmunoResearch Laboratories, West Grove, PA, USA; goat anti-rabbit IgG (H+L) Alexa Fluor®594, S94A-11037, Life Technologies) at room temperature, diluted 1:200 with blocking solution. Samples were washed, and counterstained with 5 μg·mL⁻¹ 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium. Fluorescence was detected and imaged using a motorized fluorescence microscope (Zeiss Axio Imager Z1; Carl Zeiss, Jena, Germany).

Knockout of IFITM1 by CRISPR/Cas9

pSpCas9 (BB)-2A-Puro (PX459) was a gift from Feng Zhang (Addgene plasmid no. 48139). Guide RNAs were designed using the online design tool available at http://crispr.mit.edu. PX459 was digested with Bbs1 and then gel purified. Pairs of oligos including targeting sequences were annealed and cloned into the Bbs1-digested PX459 vector. The primers are listed in Table S1. CRISPR plasmid was nucleofected into RUES2 human ESC using Human Stem Cell Nucleofector Kit 1 (VPH-5012; Lonza, Basel, Switzerland) by Amaxa Nucleofector II (Program B-016; Lonza). 24 h after nucleofection, 0.4 μg·mL⁻¹ puromycin was added into culture medium for 12 h, and single cell clone was selected after limited dilution. Clones were genotyped by PCR and T7E1 assay, verified by sequencing.

Gene expression analysis by quantitative real-time PCR

Total RNA was purified using an RNA Mini Kit (Qiagen, Hilden, Germany) for cells, treated with DNase I (79254; Qiagen). RNA was subject to cDNA synthesis using M-MLV Reverse Transcriptase (28025021, Life Technologies). The PCR reaction was set up in duplicate using the FastStart Universal SYBR Green Master (4913914001; Roche, Basel, Switzerland) and run on the Realplex PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) using primer sets specific for each gene. Primers were designed using PrimerQuest Tool provided by the Integrated DNA Technologies website (http://www.idtdna.com/site) or based on previous publications and confirmed for their specificity by dissociation curves. All reactions (in duplicate) were carried out by amplifying target genes and endogenous glyceraldehyde 3-phosphate dehydrogenase as control in the same plate. Relative quantitative evaluation of target genes was determined by comparing the threshold cycles. cDNA was used as the template for quantitative real-time PCR (qRT-PCR) amplification using the primers in Table S3.

Western blot

The western blot experiment was performed as described previously [20] and the antibodies used were: IFITM1/2/3 (F12; sc-374026; Santa Cruz Biotechnology), NANOG (sc-293121; Santa Cruz Biotechnology), OCT3/4 (SC-5279; Santa Cruz Biotechnology), SOX2 (AB5603, Millipore, Billerica, MA, USA), β-actin (sc1616R; Santa Cruz Biotechnology) and IFITM3 (AF3377; R&D Systems). The protein bands were detected by Amersham ECL Prime western blot detection reagent (RPN2232; GE Healthcare, Chicago, IL, USA).

Telomere measurement by quantitative real-time PCR

Genomic DNA from the cell lines was isolated with DNeasy Blood & Tissue kit (69504; Qiagen). The DNA quality was
assayed using a Nanodrop 2000 spectrophotometer, and the ratio of 260–280 nm was between 1.8 and 2.1. Average telomere length was measured using real-time PCR assay, as previously described [21]. PCR reactions were performed on the Realplex PCR detection system (Bio-Rad Laboratories). For each PCR reaction, a standard curve was made by serial dilutions of known amounts of DNA from human fibroblast cells. The telomere signal (T) was normalized to the signal from the single copy gene (S) human 36B4 to generate a T/S ratio indicative of relative telomere length. Each sample was repeatedly measured at least three times. Primers for the T/S ratio [22] are listed in Table S2.

Fig. 1. Establishment of IFITM1-knockout hES cell lines. (A) Expression levels of IFITM1 by qRT-PCR in hESC lines (RUES2 and WA26) and HEF. Data represent the means and SEM (n = 4). (B) Representative immunofluorescence of IFITM1 in hESC lines (WA26 and RUES2) and HEF cells; HEF cells served as positive control. Nuclei were counterstained by DAPI. Scale bar: 20 μm. (C) Schematic diagram of CRISPR/Cas9-mediated IFITM1 knockout in hESC lines. (D) Western blot analysis showing the acquisition of IFITM1 KO hESC lines. HEF cells served as positive control, and β-actin as loading control. (E) Representative immunofluorescence microscopic images of IFITM3 in hESC at P0. Scale bar: 50 μm. (F) Representative immunofluorescence microscopic images of IFITM3 in hESC at P0. Scale bar: 20 μm.
Telomere terminal restriction fragment analysis by Southern blot analysis

Telomere terminal restriction fragment (TRF) analysis was performed using a commercial kit (TeloTAGGG Telomere Length Assay, 12209136001;Roche Life Science). Genomic DNA from the cell lines was isolated with DNeasy Blood & Tissue kit (69504;Qiagen), and 1.5 μg DNA for each sample was digested using HindIII and RsaI restriction enzymes. Digested DNA underwent electrophoresis through a 0.8% agarose gel (111860;Biowest, Nuaille, Maine-et-Loire, France) for 4 h at 6 V/cm. Gels were denatured, neutralized and transferred to positively charged nylon membranes (RPN2020B, GE Healthcare) overnight. The membranes were hybridized in DIG Easy Hyb Granules (11796895001;Roche Diagnostics, Penzberg, Germany) containing the telomere probe at 70 °C overnight. The TRF length was quantitatively measured according to the kit instructions.

Cell cycle analysis

Cells (WT and IFITM1-KO RUES2 cells) were fixed in freshly prepared 70% ethanol at 4 °C overnight, then centrifuged at 1000 g for 5 min to collect cells and stained with propidium iodide at 37 °C for 30 min in a water bath. Fluorescence-activated cell sorting analysis was used to determine cell cycle phases.

Chromatin immunoprecipitation-qPCR

Chromatin immunoprecipitation (ChIP)-qPCR was performed based on a published protocol [23]. Chromatin extracts were immunoprecipitated using trimethylation of histone H3 on lysine 9 (H3K9me3;ab8898 Abcam) antibody. Input and immunoprecipitation samples were analyzed by qPCR and the primers are listed in Table S3.

Telomerase activity assay

Telomerase activity was determined by the Stretch PCR method according to the manufacturer’s instructions using the TeloChaser Telomerase assay kit (T0001; MD Biotechnology, Xiamen, China). About 2.5 × 10⁶ cells from each sample were lysed, and lysed cells heated at 70 °C for 10 min served as negative control. PCR products of cell lysates were separated on non-denaturing TBE-based 10% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining.

Statistical analysis

Data were analyzed by ANOVA and means compared by Fisher’s protected least-significant difference (PLSD) using STATVIEW software (SAS Institute Inc., Cary, NC, USA). Significant differences were defined as P < 0.05, 0.01 or lower.

Results

Establishment of IFITM1-knockout hESC lines

We analyzed mRNA expression levels of IFITM1 by qRT-PCR in hESCs (WA26 and RUES2) that expressed notably higher mRNA levels of IFITM1 than did human fibroblast cells (HEF; Fig. 1A). We also performed immunofluorescence microscopy of hESCs (WA26 and RUES2) and HEFs. Notably, IFITM1 was localized on the cell surface and cytoplasm and expressed at higher levels in hESCs than HEFs (Fig. 1B). To explore the potential role of IFITM1, we took advantage of CRISPR/Cas9 technology and generated IFITM1-knockout hESCs (Fig. 1C). Both western blot and immunofluorescence validated that IFITM1 protein was undetectable in IFITM1 KO hESCs, comparable to that of HEFs, in contrast to WT hESCs (Fig. 1D,E). IFITM1 antibody may also detect IFITM3 due to similarity of their protein sequences. Therefore, we conducted immunofluorescence staining for IFITM3 in IFITM1 KO and WT hESCs to test the specificity of the sgRNAs used in the CRISPR/Cas9 method. IFITM3 was found in both IFITM1 KO and WT hESCs by immunofluorescence (Fig. 1F), and there was no difference of IFITM3 levels between IFITM1 KO and WT hESCs shown by western blot (Fig. S1), further supporting that the designed sgRNAs were specific to IFITM1, consistent with the gene sequencing data.

Effects of IFITM1 knockout on pluripotency and telomere length in hESCs

ESC colonies were similar in morphology between IFITM1 KO and WT hESCs (Fig. 2A). To elucidate whether high expression level of IFITM1 is required for pluripotency, we examined whether the mRNA levels of OCT4, NANOG and SOX2, which are important for pluripotency, were similar between IFITM1 KO and WT hESCs (Fig. 2B). Also protein levels of NANOG, OCT4 and SOX2 by western blot did not differ between IFITM1 KO and WT hESC lines at various passages (Fig. 2D). In addition, OCT4 immunofluorescence was similar between IFITM1 KO and WT hESCs (Fig. 2C).

Cell proliferation, which was determined by the cell number and cell cycle progression, also did not differ between IFITM1 KO and WT hESCs (Fig. 2E,F). Telomerase is critical for cell proliferation and is a complex of reverse transcriptase comprising two core components: Telomerase reverse transcriptase (TERT) and template RNA TERC (essential RNA component) [24]. Consistent with cell proliferation, IFITM1 deficiency did not alter telomerase activity and the expression levels of TERT and TERC (Fig. 2G,H). Telomere length
IFITM1 suppresses HERV expression in hESCs

Y. Fu et al.
primarily maintained by telomerase and cell dividing times and predicts replicative capacity [25]. Also, IFITM1 KO and WT hESCs at early or late passages presented similar telomere lengths shown as T/S ratio by qPCR (Fig. 2I), and also determined by TRF (Fig. 2J). Besides we performed immunofluorescence analysis of the DNA damage response marker 53BP1 [26] in IFITM1 KO and WT hESCs (Fig. S2A). The number of 53BP1 positive cells and 53BP1 foci number per cell in IFITM1 KO and WT hESCs were quantified, and increased in IFITM1 KO hESCs compared with WT hESCs (Fig. S2B,C).

Expression of human endogenous retroviruses in hESCs and epigenetic regulation by IFITM1

Endogenous retroviruses (ERVs) are transposable genetic elements that comprise nearly 8% of the human genome [27], and can copy and paste their own DNA into the genome [28,29]. ERVs are activated during embryonic development and inactivated during ESC isolation and culture [18]. IFITM1-mediated restriction may be an evolutionarily conserved mechanism protecting both embryos and germ cells from either reinfection of infectious ERVs or exogenous viral infection [18]. We asked whether IFITM1 regulates ERVs in hESCs by examining relative expression of ERVs in IFITM1 KO and WT hESCs (RUES2) using specific primers (Table S3) [30]. With increasing passages (by P15), HERVK, HERVH, LTR7Y and LTR12D-1 were highly up-regulated in IFITM1 KO hESCs, compared with WT hESCs (Fig. 3A,B). Human endogenous retroviruses (HERVs), especially HERVH, are expressed preferentially in hESCs [31], and these elevated further in IFITM1 KO hESCs.

Endogenous retrovirus containing long terminal repeats (LTRs) are silenced through H3K9me3 by ERG-associated protein with SET domain (ESET; also known as SETDB1 or KMT1E) in mouse ESCs [32]. We tested whether H3K9me3 regulates HERV expression through analysis of the level of H3K9me3 on HERVs by ChIP-qPCR. Enrichment of H3K9me3 was reduced at HERVK, HERVH, LTR7Y and LTR12D-1 in IFITM1 KO hESCs compared with WT hESCs (Fig. 3C). Decreased levels of H3K9me3 at these HERV foci could partly explain the elevated expression of HERVs in the IFITM1 KO hESCs, but how IFITM1 reduces H3K9me3 enrichment at HERVs remains to be determined.

Discussion

IFITM1 family members were described as interferon-induced genes, and they are also classical naive-state and PGC markers in the mouse, which nonetheless appear to be dispensable for development [19]. IFITM1 has an essential part in regulating viral infection [33]. It was found to be present as a tight junction protein induced by type 1 interferon in hepatocytes, and it acts by interacting with viral coreceptors to prevent viral entry into cells [34]. Tight junction proteins can interact with adapter proteins and subsequently mediate cell signaling pathways and transcription [35,36]. Since IFITM1 is a tight junction protein, it is likely that it may act by cooperating with other membrane proteins to activate or repress downstream regulators and in turn affect the epigenetic status of ERVs as well. IFITM1 could also be activated by ERV expression. Expression of HERVK in pluripotent cells could further precisely activate IFITM1 and restrict viral infection, but many other interferon-induced genes are not upregulated or expressed [18]. For example, we found the expression of IFITM3 was not upregulated in IFITM1 KO hESCs in which HERVs were increased, suggesting a feedback loop between HERV expression and IFITM1-mediated defense of viral infection and also suggesting that other IFITM proteins may not respond to the upregulated HERVs, though we could not state that they do not regulate HERVs in hESCs. Interestingly, we found that knockout of IFITM1 further promotes the expression of HERVs in hESCs by reducing the level of H3K9me3 at HERV loci, although it has minimal impact on cell proliferation and pluripotency.
HERVs, such as HERVH, play an important role in maintaining pluripotency in hESCs [37,38]. Overexpression of HERVs could result in upregulation of adjacent genes and might link to unbalanced chromosomal translocations [39]; aberrant expression of HERVs can lead to cancer, which is also viewed as a ‘genomic disease’ [14]. We found that DNA damage was increased in IFITM1 KO hESCs in which HERVs were increased, which suggests that IFITM1 plays a role in repressing excessive activation of HERVs and thus may play important roles in genome maintenance. Together, our data suggest that IFITM1 may participate in suppressing HERVs by regulating level of H3K9me3 at HERV loci in hESCs, which may maintain stability of hESCs. It suggests that IFITM1-mediated restriction may be an evolutionarily conserved mechanism protecting cells from reinfection from infectious ERV infection.

**Acknowledgements**

We thank Haifeng Fu, Haiying Wang and Jian Mao for assisting experiments, and David Keefe and Fang Wang for providing hESC lines. This study was supported by China Ministry of Science and Technology Program of International S&T Cooperation (2014DFA30450).

**Author contributions**

YDF designed and conducted the most experiments and analyzed data. ZCZ, HW, PG, RPG and JMW performed some experiments and provided technical support. XYL discussed and interpreted results and revised the manuscript. FQ and LL interpreted results and wrote and revised the manuscript.

**References**

1. Lewin AR, Reid LE, McMahon M, Stark GR and Kerr IM (1991) Molecular analysis of a human interferon-inducible gene family. *Eur J Biochem* **199**, 417–423.
2. Deblandre GA, Marinx OP, Evans SS, Majjaj S, Leo O, Caput D, Huez GA and Wathelet MG (1995) Expression cloning of an interferon-inducible 17-kDa membrane protein implicated in the control of cell growth. *J Biol Chem* **270**, 23860–23866.
3. Brass AL, Huang I-C, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, Van Der Weyden L and Fikrig E (2009) The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* **139**, 1243–1254.
4. Lin T-Y, Chin CR, Everitt AR, Clare S, Perreira JM, Savidis G, Aker AM, John SP, Sarlah D and Carreira EM (2013) Amphotericin B increases influenza A virus infection by preventing IFITM3-mediated restriction. *Cell Rep* **5**, 895–908.
5 Yang J, Guo R, Wang H, Ye X, Zhou Z, Dan J, Wang H, Gong P, Deng W, Yin Y et al. (2016) Tet enzymes regulate telomere maintenance and chromosomal stability of mouse ESCs. Cell Rep 15, 1809–1821.

6 Hatano H, Kudo Y, Ogawa I, Tsunematsu T, Kikuchi A, Abiko Y and Takata T (2008) IFN-γ induces transmembrane protein 1 protein invasion at early stage of head and neck cancer progression. Clin Cancer Res 14, 6097–6105.

7 Thompson PJ, Macfurlan TS and Lorincz MC (2016) Long terminal repeats: from parasitic elements to tumorigenic accomplice? FEBS Open Bio 7, 1102–1110 © 2017 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
32 Matsui T, Leung D, Miyashita H, Maksakova IA, Miyachi H, Kimura H, Tachibana M, Lorincz MC and Shinkai Y (2010) Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. Nature 464, 927–931.
33 Smith S, Weston S, Kellam P and Marsh M (2014) IFITM proteins-cellular inhibitors of viral entry. Curr Opin Virol 4, 71–77.
34 Wilkins C, Woodward J, Lau DT, Barnes A, Joyce M, McFarlane N, McKeating JA, Tyrrell DL and Gale M Jr (2013) IFITM1 is a tight junction protein that inhibits hepatitis C virus entry. Hepatology 57, 461–469.
35 Guillemot L, Paschoud S, Pulimeno P, Foglia A and Citi S (2008) The cytoplasmic plaque of tight junctions: a scaffolding and signalling center. Biochim Biophys Acta 1778, 601–613.
36 Matter K and Balda MS (2003) Signalling to and from tight junctions. Nat Rev Mol Cell Biol 4, 225–236.
37 Lu X, Sachs F, Ramsay L, Jacques PE, Goke J, Bourque G and Ng HH (2014) The retrovirus HERVH is a long noncoding RNA required for human embryonic stem cell identity. Nat Struct Mol Biol 21, 423–425.
38 Wang J, Xie G, Singh M, Ghanbarian AT, Rasko T, Svetnik A, Cai H, Besser D, Prigione A, Fuchs NV et al. (2014) Primate-specific endogenous retrovirus-driven transcription defines naive-like stem cells. Nature 516, 405–409.
39 Elsässer SJ, Noh K-M, Diaz N, Allis CD and Banaszynski LA (2015) Histone H3.3 is required for endogenous retroviral element silencing in embryonic stem cells. Nature 522, 240–244.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Table S1. Primers used for knockout of IFITM1 by CRISPR/Cas9 system.
Table S2. Primers for T/S ratio.
Table S3. Primers for qRT-PCR and ChIP-qPCR.
Fig. S1. Impact of IFITM1-knockout on protein level of IFITM3.
Fig. S2. Impact of IFITM1-knockout on DNA damage.