LETTERS

Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus

Etienne Meylan[1], Joseph Curran[2], Kay Hofmann[3], Darius Moradpour[4], Marco Binder[5], Ralf Bartenschlager[5] & Jürg Tschopp[1]

Antiviral immunity against a pathogen is mounted upon recognition by the host of virally associated structures. One of these viral 'signatures', double-stranded (ds) RNA, is a replication product of most viruses within infected cells and is sensed by Toll-like receptor 3 (TLR3) and the recently identified cytosolic RNA helicases RIG-I (retinoic acid inducible gene I, also known as Ddx58) and Mda5 (melanoma differentiation-associated gene 5, also known as Ifih1 or Helicard)[1]. Both helicases detect dsRNA, and through their protein-interacting CARD domains, relay an undefined signal resulting in the activation of the transcription factors interferon regulatory factor 3 (IRF3) and NF-κB. Here we describe Cardif, a new CARD-containing adaptor protein that interacts with RIG-I and recruits IKKα, IKKβ and IKKε kinases by means of its C-terminal region, leading to the activation of NF-κB and IRF3. Overexpression of Cardif results in interferon-β and NF-κB promoter activation, and knockdown of Cardif by short interfering RNA inhibits RIG-I-dependent antiviral responses. Cardif is targeted and inactivated by NS3-4A, a serine protease from hepatitis C virus known to block interferon-β production. Cardif thus functions as an adaptor, linking the cytoplasmic dsRNA receptor RIG-I to the initiation of antiviral programmes.

Innate immune responses defend the host from invading pathogens by recognizing pathogen-associated molecular patterns. Toll-like receptors are transmembrane receptors that use their leucine-rich repeats to recognize pathogen-associated molecular patterns and initiate intracellular responses through recruitment of TIR-domain containing adaptors (see ref. 2 for a review). TLR3 acts as an antiviral receptor that is capable of recognizing dsRNA. TLR3 transmits cellular responses by specifically recruiting the TIR-containing adaptor protein Trif (also known as TICAM1). Triggering of TLR3 results in a massive type-I interferon (IFN) response, as Trif is able to recruit the essential IRF-3-activating kinases TBK1 and IKKε[3,4]. However, the importance of TLR3 in the generation of antiviral immune responses has been recently challenged, as TLR3 is not required to mount antiviral responses against some viruses[5], suggesting the existence of TLR3-independent dsRNA recognition systems.

Recently, two DexD/H box RNA helicases, RIG-I and Helicard (Mda5), were found to participate in the cytoplasmic recognition of dsRNA[6,7]. Expression of RIG-I and Helicard can be induced by retinoic acid and IFN-β treatment, respectively. Both helicases contain two amino-terminal caspase recruitment domains (CARD) and a C-terminal region that is characterized by the presence of a helicase domain[8,9]. RIG-I (and probably Helicard) is able to interact with cytoplasmic dsRNA[7,10], and we have previously shown that Helicard is cleaved during apoptosis[11]. RIG-I and Helicard initiate antiviral responses by activating the transcription factors IRF3 and NF-κB, resulting in an enhanced transcription of type-I IFN[6,7]. The region responsible for RIG-I-induced downstream signalling was mapped to the N-terminal CARD domains, suggesting the existence of a putative CARD adaptor molecule[7,10] that relays the signal.

In an attempt to identify new CARD-domain-containing proteins, we performed profile searches in human protein databases, which led us to the identification of a protein that we named CARD adaptor inducing IFN-β (Cardif). Cardif contains 540 amino acids and has a N-terminal CARD domain similar to the CARD domains of RIG-I and Helicard (Fig. 1a, b). Cardif corresponds to a gene previously identified in a complementary DNA library screen of potential NF-κB-activating molecules[12]. Polymerase chain reaction with reverse transcription (RT–PCR) of different human cell lines, primary cells and tissues revealed broad expression of Cardif (Supplementary Fig. 1).

Because Cardif is characterized by the presence of a N-terminal CARD domain that is similar to RIG-I and Helicard, we investigated whether the helicases could interact with Cardif upon overexpression of Cardif in human embryonic kidney (HEK) 293T cells. Cardif interacted with RIG-I and showed a weak interaction with Helicard, but did not interact with any of the other tested CARD-domain-containing proteins (Fig. 1c). Cardif oligomerization through its CARD domain was not detected under these experimental conditions.

In subsequent experiments we concentrated on the functional consequences of the RIG-I–Cardif interaction. Overexpression of full-length Cardif in 293T cells resulted in dose-dependent activation of NF-κB- and IFN-β-reporter plasmids, as well as the IFN-stimulated response element (ISRE) of ISG54, an IRF3-dependent promoter (Fig. 2a). Similar activation was also observed in HeLa cells (data not shown). Overexpression of a deletion construct containing only the CARD domain did not result in activation of any of the reporter genes tested. However, a construct lacking the N-terminal CARD domain (ΔCARD) was sufficient to drive activation of the reporter genes, but to a much lesser extent than the wild-type construct. Activation of the ISRE reporter gene was dependent on IRF3 (Supplementary Fig. 2), as a dominant-negative version of IRF3 lacking the N-terminal DNA-binding region (IRF3 ΔN) inhibited Cardif- and also Trif-induced activation of ISRE, but did not prevent NF-κB activation. A non-phosphorylable form of IκB (dominant-negative IκB) inhibited Cardif- and Trif-induced NF-κB activation only. Overexpression of Cardif was sufficient to induce transcription of interferon-β (*Ifnb1*) and of the NF-κB target gene interleukin 6 (*Il6*) in mouse embryonic fibroblasts (MEFs), as well as inducing the activation of endogenous IRF3 by phosphorylation[13] and

[1]Department of Biochemistry, University of Lausanne, BIL Biomedical Research Center, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland. [2]Department of Microbiology and Molecular Medicine, Faculty of Medicine, University of Geneva, 1 rue Michel Servet, CH-1211 Geneva 4, Switzerland. [3]MEMOREC Biotec GmbH, a Miltenyl Biotec company, Stoeckheimer Weg 1, D-50829 Koeln, Germany. [4]Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, Rue du Bugnon 46, CH-1011 Lausanne, Switzerland. [5]Department of Molecular Virology, University Heidelberg, Im Neuenheimer Feld 345, 69120 Heidelberg, Germany.

IRF3-dependent production of RANTES in 293T cells (Fig. 2b, c).

Together, these results suggest that the carboxy-terminal region of Cardif might be implicated in the recruitment and activation of IKK kinase complexes that are essential for the activation of NF-κB and IRF3. Indeed, we found that Cardif interacted with IKKε (which is able to phosphorylate IRF3) and with IKKα and IKKβ (which are known to phosphorylate IκB), but not with TBK1 (Fig. 3a). This recruitment was more evident for the Cardif mutant containing a deleted CARD domain than for the full-length protein. The Cardif–IKKε interaction contrasts with the propensity of Trif to preferentially interact with TBK1 as opposed to IKKε[14]. This suggests that Trif and Cardif use two different IKK kinases to phosphorylate IRF3, although this might be cell-type-specific.

To unambiguously demonstrate that Cardif acts downstream of RIG-I in antiviral signalling, we overexpressed RIG-I in 293T cells to induce activation of the IFN-β luciferase promoter, and monitored the consequence of using siRNA to knock down the expression of Cardif, RIG-I and Trif (Fig. 3b). Western blotting showed that all three siRNAs efficiently reduced the protein levels of their respective targets (Supplementary Fig. 3). As a consequence of Cardif knockdown, RIG-I-induced, but not Trif-induced IFN-β promoter activation was greatly reduced (Fig. 3b). Notably, although Trif siRNA inhibited Trif-induced IFN-β promoter activation, it increased the IFN-β response triggered by the overexpression of RIG-I, suggesting that RIG-I may act as a sensor of siRNA within the cytoplasm.

Next, we sought to determine whether Cardif siRNA can inhibit an IFN response in the context of stimulation with dsRNA or infection with Sendai virus (SenV), a virus that triggers RIG-I-dependent IFN responses[10]. As neither Poly(I:C) transfection nor SenV infection of 293T cells resulted in an activation of the IFN-β promoter *per se*, we

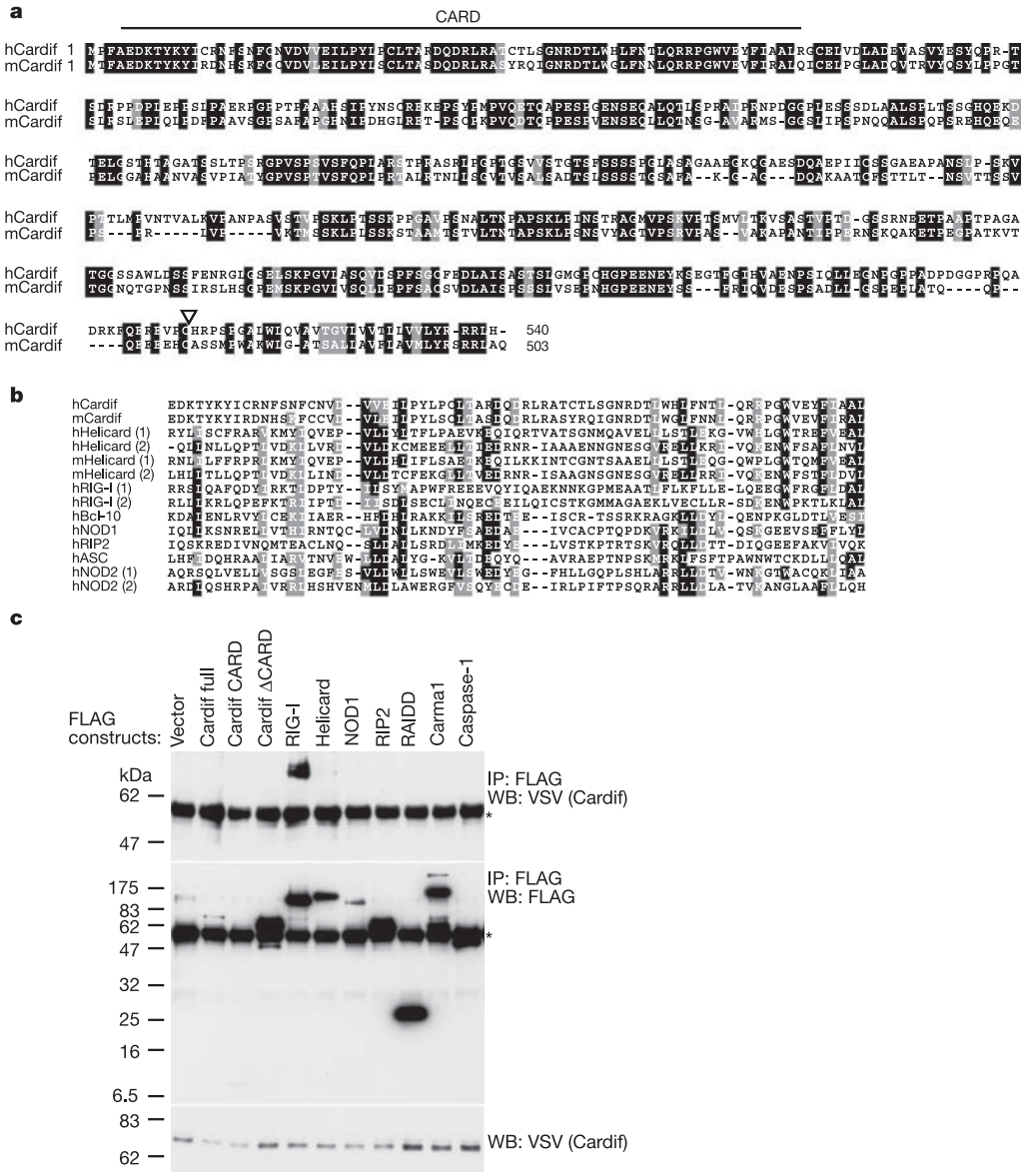

Figure 1 | Cardif is a novel CARD protein that interacts with RIG-I. **a**, Amino acid sequence alignment of human (h) and murine (m) Cardif. Shading indicates 100% amino acid sequence identity (black) or similarity (grey). The N-terminal CARD domain is indicated. The cleavage site targeted by NS3-4A is indicated with an arrowhead. **b**, Sequence alignment of the CARD domains of human and murine Cardif and Helicard (Mda5), and human RIG-I, Bcl-10, NOD1, RIP2, ASC and NOD2. Numbers in parentheses indicate the CARD domain number. Shading indicates ≥50% amino acid sequence identity (black) and similarity (grey). The CARD domain of Cardif shares 37% and 29% similarity with the two CARD domains of Helicard, and 32% and 29% similarity with the CARD domains of RIG-I. **c**, HEK 293T cells were transfected with the indicated FLAG-tagged constructs or an empty plasmid (vector), together with VSV-Cardif. Anti-FLAG immunoprecipitates (IP) and cell extracts were then analysed by western blot (WB). Asterisk indicates immunoglobulin heavy chain.

reasoned that RIG-I, which is an inducible gene, might not be expressed under these conditions. We therefore transfected a small amount of RIG-I, which permitted us to observe a synergistic effect of SenV or Poly(I:C) on IFN activation (Fig. 3c, d). Cardif knockdown inhibited both Poly(I:C)- and SenV-induced IFN-β promoter activation. To confirm these findings, vesicular stomatitis virus (VSV) was used to infect primary MEFs that depend on RIG-I to mount an antiviral response[15]. Cardif knockdown by siRNA inhibited VSV-triggered RANTES expression (Fig. 3e). Together, these results demonstrate the important role of Cardif as an adaptor of RIG-I-dependent antiviral immune responses.

Several human pathogenic viruses, including influenza virus, vaccinia virus and Ebola virus, have evolved strategies to inhibit the early signalling events that lead to IFN production[16]. NS3-4A, a multifunctional protein of hepatitis C virus (HCV) that has serine protease activity essential for the production of mature viral proteins, has recently been shown to block the activation of IRF3 (ref. 17). NS3-4A counteracts TLR3-dependent pathways by targeting Trif for proteolytic cleavage, and also counteracts TLR3-independent pathways by targeting an undefined protein of the RIG-I-dependent pathway[18,19]. IKKε overexpresison was shown to overcome NS3-4A-mediated block of IRF3 activation, suggesting that this viral protease targets an adaptor protein 'upstream' of IKKε[20].

We therefore hypothesized that Cardif might be a substrate for NS3-4A. Indeed, Cardif is cleaved by wild-type but not by a catalytically inactive form of NS3-4A (Fig. 4a). Trif was also cleaved by NS3-4A under the same conditions, as previously reported[18]. The N- and C-terminally tagged cleavage fragments of Cardif allowed us to map the cleavage site to within 5 kDa of the C terminus (Fig. 4a).

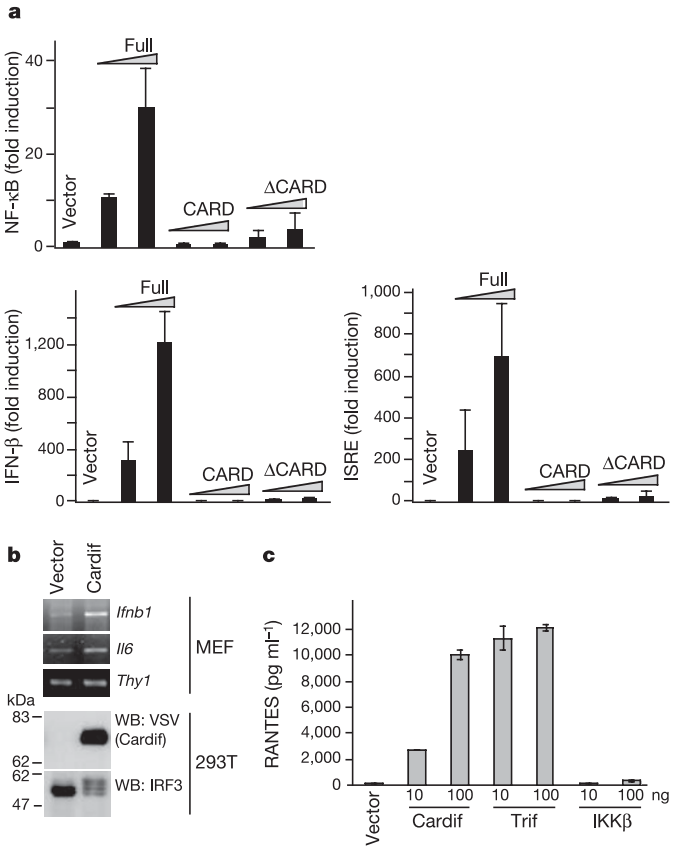

Figure 2 | Cardif activates NF-κB and IRF3. a, 293T cells were transfected with NF-κB, IFN-β or ISRE reporter plasmids, together with an empty vector or increasing concentrations of the indicated Cardif constructs (full-length, CARD or ΔCARD), and analysed for NF-κB-, IFN-β- and ISRE-dependent luciferase activity. **b**, MEFs (top) or 293T cells (bottom) were transfected with an empty vector or VSV-Cardif. Total RNA was isolated from MEFs and assessed for the expression of *Ifnb1*, interleukin 6 (*Il6*) and *Thy1* by RT–PCR. For 293T cells, cell extracts were analysed by western blot. **c**, 293T cells were transfected with an empty vector, or with 10 or 100 ng of Cardif, Trif or IKKβ cDNA. The supernatants were analysed by ELISA for endogenous expression of human RANTES. Error bars in **a** and **c** represent s.d. of triplicate determinations in a single experiment.

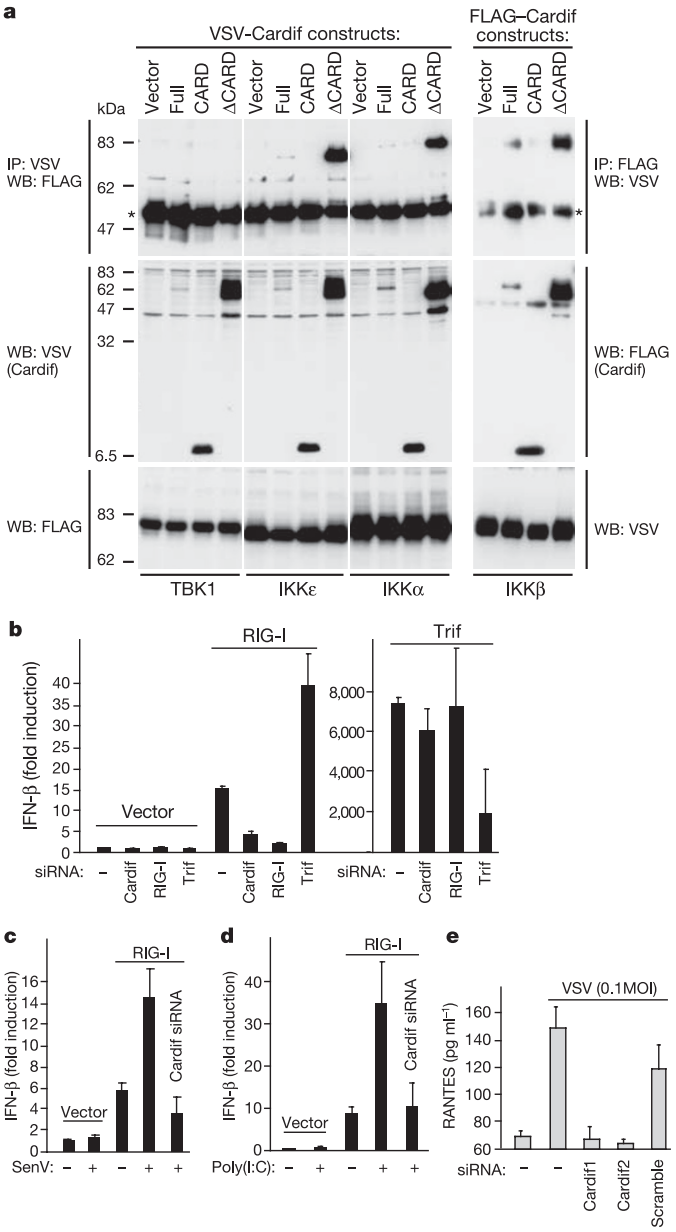

Figure 3 | Cardif links RIG-I to antiviral responses. a, 293T cells were transfected with FLAG–TBK1, FLAG–IKKα, FLAG–IKKε and VSV-Cardif constructs (full-length, CARD or ΔCARD), or VSV-IKKβ and FLAG–Cardif constructs or an empty vector. Tagged Cardif was immunoprecipitated and cell extracts were analysed by western blot. Asterisk indicates immunoglobulin heavy chain. **b**–**d**, 293T cells were analysed for IFN-β-dependent luciferase activity after co-transfection with (1) an IFN-β reporter plasmid, (2) the indicated siRNAs, and (3) RIG-I, Trif or an empty vector (**b**), or after co-transfection as above and infection with Sendai virus (SenV) (**c**), or after co-transfection as above and also with Poly(I:C) (**d**). **e**, Primary MEFs were transfected with two different siRNAs targeting murine Cardif, or a control siRNA (scramble), and were infected 30 h later with VSV for 18 h. The supernatants were analysed by ELISA for endogenous expression of murine RANTES. Error bars in **b**–**e** represent s.d. of triplicate determinations in a single experiment.

As NS3-4A is known to specifically cleave targets after Cys or Thr residues[21], we mutated the most likely target cysteine, located in the C-terminal region of Cardif (Cys 508, see Fig. 1a), to alanine (C508A). This construct showed complete resistance to cleavage by NS3-4A (Fig. 4a, right). Wild-type Cardif, but not the C508A mutant form, was also cleaved when the entire HCV polyprotein was inducibly expressed in a U-2 OS-derived tetracycline-regulated cell line (Fig. 4b). Using a recently described *in vitro* HCV infection system[22–24], we also observed Cardif cleavage in cells harbouring infectious HCV (Fig. 4c). Upon overexpression in 293T cells, both wild-type and C508A mutant Cardif, but not a Cardif construct lacking the C-terminal cleavage-generated end (Cardif 1–508),

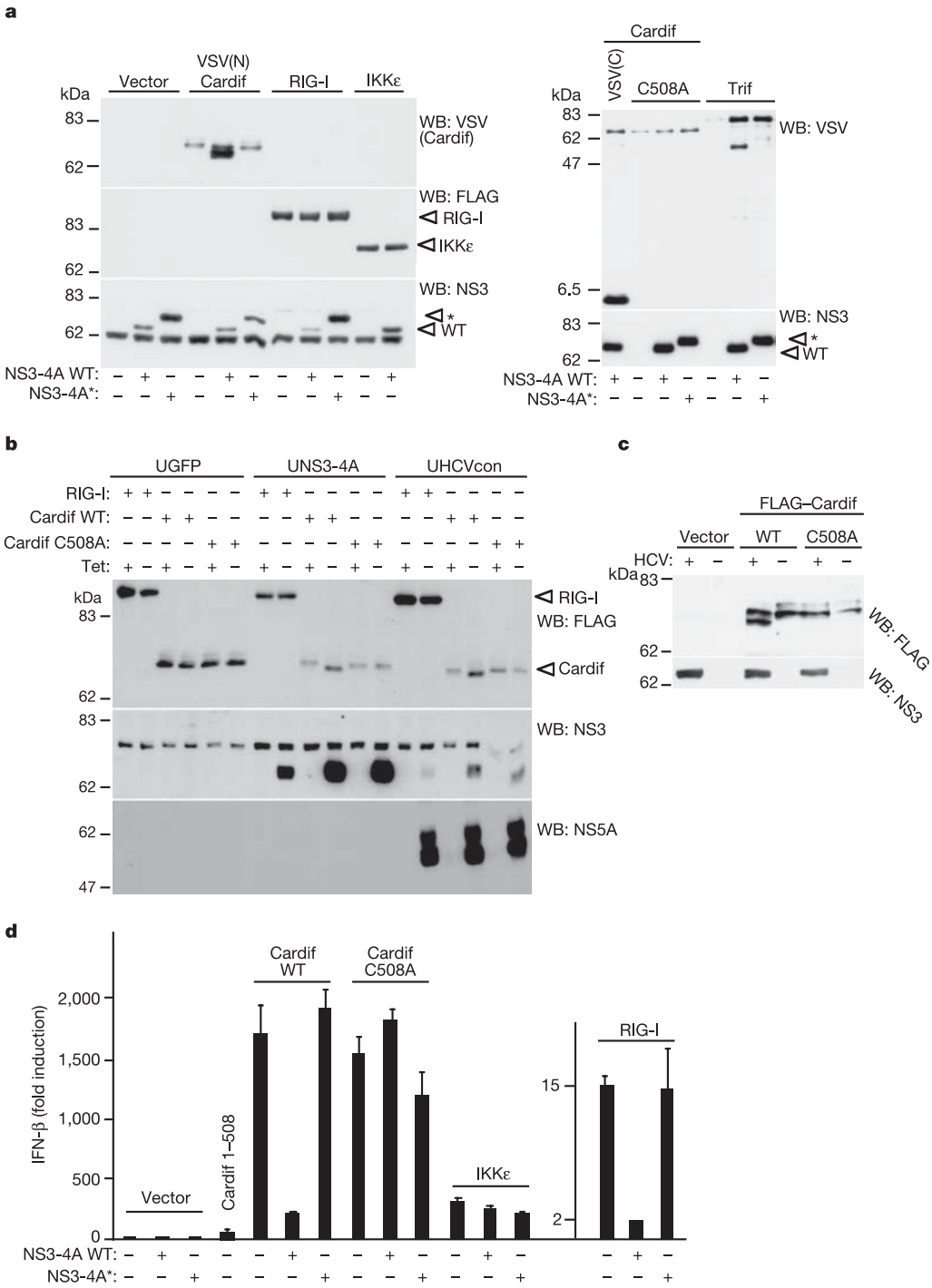

Figure 4 | Cardif is cleaved by the HCV protease NS3-4A. **a**, 293T cells were transfected with FLAG–RIG-I or FLAG–IKKε, VSV-Trif, VSV-Cardif (wild type (WT), either N-terminally VSV(N) or C-terminally VSV(C) tagged, or a mutant Cardif C508A), together with wild-type (WT) or catalytically inactive (*) NS3-4A, and cell extracts were analysed by western blot. **b**, U-2 OS-derived tetracycline-regulated cells were transfected with FLAG–RIG-I and FLAG–Cardif (WT or mutant C508A). After induction of the viral proteins (see Methods), cell extracts were analysed for Cardif cleavage by western blot. **c**, Huh7.5 cells were transfected with wild-type or C508A mutant Cardif. The cells were subsequently infected with HCV Jc1, and analysed for Cardif cleavage by western blot. **d**, 293T cells were co-transfected with (1) an IFN-β reporter plasmid, (2) an empty vector, C-terminally truncated Cardif (1–508), wild-type Cardif, mutant (C508A) Cardif, IKKε or RIG-I, and (3) the indicated NS3-4A constructs, and analysed for IFN-β-dependent luciferase activity. Error bars represent s.d. of triplicate determinations in a single experiment.

triggered an IFN-β response (Fig. 4d). Catalytically active wild-type NS3-4A protease (NS3-4A) potently impaired the ability of wild-type Cardif and RIG-I—but not Cardif (C508A) or IKKε—to mediate signalling (Fig. 4d), demonstrating that NS3-4A-mediated cleavage at Cys 508 results in the inactivation of Cardif.

Millions of people are chronically infected with HCV and are at risk of developing liver disease. HCV has evolved an efficient strategy to block a dsRNA-dependent innate immune response, by specifically cleaving and thereby inactivating two adaptor proteins—Trif and Cardif—that have important roles in the two distinct arms of the IFN response mediated by TLR3 and RIG-I, respectively. The potent antiviral activity of BILN 2061, an NS3-4A inhibitor developed for HCV treatment[25], was initially explained by its inhibition of the NS3-4A-induced maturation of the HCV polyprotein. In addition, BILN 2061 might prevent Cardif and/or Trif inactivation during infection with HCV, thereby maintaining the innate immune response. Interestingly, a mutation in the Cardif-interacting CARD domain of RIG-I was recently found to allow replication of an HCV replicon in otherwise resistant cells[10], supporting an important role for the RIG-I–Cardif pathway in anti-HCV immunity.

A model consistent with our data includes RIG-I (and Helicard) as a crucial sensor for cytoplasmic dsRNA derived from viruses. Upon binding of dsRNA to RIG-I, a conformational change caused by helicase activity could expose its CARD domain, which associates with the CARD domain of Cardif through a homotypic interaction. As a consequence, the C-terminal segment of Cardif exposes binding sites for the IRF3-phosphorylating kinase IKKε and the NF-κB-activating kinases IKKα and IKKβ, triggering a strong antiviral IFN-β and inflammatory response. Bearing in mind that HCV (and possibly other viruses that are known to inhibit IFN production[16]) inactivates the RIG-I–Cardif pathway, identification of therapeutic agents that mimic the CARD-induced conformational change in Cardif may prove a beneficial antiviral strategy.

Note added in proof: Cardif has also recently been identified by two other research groups, and has been named IPS-1 (ref. 31) or MAVS (ref. 32).

METHODS

Expression vectors. The full length Cardif cDNA was amplified from an expressed-sequence-tag (EST) (IMAGE clone 5751684) by standard PCR using Pwo polymerase (Roche). Cardif was subsequently cloned into a derivative of pCR3 (Invitrogen), in-frame with an N- or C-terminal VSV or FLAG tag. Cardif CARD (amino acids 1–95), ΔCARD (amino acids 91–540) and C-terminally truncated (amino acids 1–508) coding sequences were amplified by PCR. Mutation of Cys 508 to Ala (C508A) in Cardif was achieved by site-directed mutagenesis with two sequential rounds of PCR. Mouse *Irf3* lacking the N-terminal DNA-binding domain (ΔN, amino acids 137–419) was amplified from an EST (IMAGE clone 3666172). The fidelity of all PCR amplifications was confirmed by sequencing. FLAG–IKKα, FLAG–IKKε and FLAG–TBK1 were gifts from G. P. Dotto, T. Maniatis, and M. Nakanishi, respectively. FLAG–RIG-I cDNA was provided by T. Fujita. Human TRIF and mouse Helicard (Mda5) expression plasmids have been described[11,26]. Wild-type pCMVNS3-4A (NS3-4A WT) has been described[27], and its catalytically inactive counterpart (NS3-4A*) containing a Ser 139 to Ala mutation will be described elsewhere. NF-κBLuc and IFN-βLuc reporter plasmids were provided by V. Jongeneel and T. Taniguchi, respectively. pISRE-Luc was from Stratagene. The Renilla-luciferase transfection efficiency vector (phRLTK) was purchased from Promega.

PCR with reverse transcription. RT–PCR was performed as described[26]. Human multiple tissue cDNAs (636742, 636743) were from BD Biosciences. Human primary haematopoietic cells and corresponding cDNAs have been described[28]. The following primers were used: Cardif, 5′-ACTTCATTGCGG CACTGAGG-3′ and 5′-TCTGGATTCCTTGGGATGGC-3′; β-actin, 5′-GGCATCGTGATGGACTCCG-3′ and 5′-GCTGGAAGGTGGACAGCGA-3′; mouse *Thy1* 5′-CCATCCAGCATGAGTTCAGCC-3′ and 5′-GCATCCAGGAT GTGTTCTGA-3′; mouse *Ifnb1* 5′-TTCCTGCTGTGCTTCTCCAC-3′ and 5′-GATTCACTACCAGTCCCAGAGTC-3′; mouse *Il6* 5′-ATGAAGTTCCTCTCT GCAAGAGACT-3′ and 5′-CACTAGGTTTGCCGAGTAGATCTC-3′.

Cell culture conditions. The human embryonic kidney (HEK) 293T cell line, primary mouse embryonic fibroblasts (MEFs) and the hepatoma cell line Huh7.5 (a gift from C.M. Rice) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum. U-2 OS human osteosarcoma-derived cell lines that inducibly express green fluorescent protein (UGFP) or NS3-4A upon tetracycline withdrawal, either alone (UNS3-4A) or in the context of the entire HCV polyprotein (UHCVcon), have been described[27,29].

Transfection, immunoprecipitation and immunoblotting. Transfection, immunoprecipitation and immunoblotting assays in 293T cells were performed as previously described[26]. siRNAs (Ambion) and Poly(I:C) (Invivogen) were also transfected into 293T cells using the calcium-phosphate precipitation technique, at final concentrations of 10 nM (siRNA) or 1 ng ml^{-1} (Poly(I:C)). MEFs were transfected using Lipofectamine 2000 (Invitrogen) or, in the case of siRNA, using TransIT-TKO (Mirus), according to the manufacturers' instructions. U-2 OS cells were transfected using the calcium-phosphate precipitation technique. Twenty-four hours after transfection, U-2 OS cells were washed twice with PBS and were then cultured for 24 h in the presence (tet^{+}) or absence (tet^{-}) of tetracycline to allow the expression of GFP (UGFP), NS3-4A alone (UNS3-4A) or the entire HCV polyprotein (UHCVcon). Huh7.5 cells were transfected using Effectene (Qiagen), according to the manufacturer's instructions. Anti-FLAG M2 and anti-VSV P5D4 antibodies were from Sigma. Anti-IRF3 (sc-9082) was from Santa Cruz. Anti-NS3 1B6 and anti-NS5A 11H have been previously described[27,30]. For the HCV infection experiment, a polyclonal anti-NS3 antiserum was used.

Viral infections. Twenty-four hours after transfection, 293T cells were infected for 24 h with Sendai virus (strain H) at a multiplicity of infection (MOI) of 10. Cells were subsequently lysed and assayed for luciferase activity. Thirty hours after transfection with siRNA, primary MEFs were infected for 18 h with VSV at a MOI of 0.1. HCV chimaeric genotype 2a virus, designated Jc1, will be described elsewhere. HCV production was performed as previously described[22]. Six hours after transfection, Huh7.5 cells were infected for 5 h with cell culture supernatants containing infectious Jc1 particles, cultured for another 60 h and harvested in Laemmli sample buffer for analysis by western blot.

Luciferase reporter assays. Luciferase reporter assays were performed as described previously[26].

Analysis of RANTES expression. Forty-eight hours after transfection of 293T cells in 96-well plates, cell supernatants were analysed for human RANTES expression by enzyme-linked immunosorbent assay (ELISA, R&D Systems), according to the manufacturer's instructions. Eighteen hours after infection of MEFs with VSV in 12-well plates, cell supernatants were analysed for murine RANTES expression (murine RANTES ELISA, R&D Systems), according to the manufacturer's instructions.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The DNA sequence of human Cardif has been deposited in GenBank under accession number DQ181928. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.T. (jurg.tschopp@unil.ch).