

# Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif

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Upon recognition of viral infection, RIG-I and Helicard recruit a newly identified adapter termed Cardif, which induces type I interferon (IFN)-mediated antiviral responses through an unknown mechanism. Here, we demonstrate that TRAF3, like Cardif, is required for type I interferon production in response to intracellular double-stranded RNA. Cardif-mediated IFN $\alpha$  induction occurs through a direct interaction between the TRAF domain of TRAF3 and a TRAF-interaction motif (TIM) within Cardif. Interestingly, while the entire N-terminus of TRAF3 was functionally interchangeable with that of TRAF5, the TRAF domain of TRAF3 was not. Our data suggest that this distinction is due to an inability of the TRAF domain of TRAF5 to bind the TIM of Cardif. Finally, we show that preventing association of TRAF3 with this TIM by mutating two critical amino acids in the TRAF domain also abolishes TRAF3-dependent IFN production following viral infection. Thus, our findings suggest that the direct and specific interaction between the TRAF domain of TRAF3 and the TIM of Cardif is required for optimal Cardif-mediated antiviral responses.

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## Introduction

Mammalian cells respond to viral infection by producing and secreting a family of cytokines called type I interferons (IFNs), composed of at least 13 IFN $\alpha$ s and a single IFN $\beta$ . These cytokines are recognized in an autocrine and paracrine

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manner by the IFN $\alpha$ / $\beta$  receptor (IFNAR), thereby inducing the expression of numerous proteins that establish a cellular environment that is highly resistant to viral infection. As a result, type I IFNs comprise an extremely potent and critical component of the innate immune response against viral pathogens (Theofilopoulos *et al*, 2005).

There has been rapid progress in recent years toward furthering our understanding of how mammalian cells initially recognize the presence of a virus. Cells of the innate immune system, including macrophages and dendritic cells, utilize Toll-like receptors (TLRs) to detect and respond to viral infection (Bowie and Haga, 2005). In contrast, fibroblasts, which do not express TLRs, recognize intracellular double-stranded RNA (dsRNA) produced during viral infection through CARD-domain containing RNA helicases such as Helicard/MDA-5 and RIG-I (Andrejeva *et al*, 2004; Yoneyama *et al*, 2004, 2005; Kato *et al*, 2005). These 'intracellular receptors' utilize a newly identified CARD-containing adapter called Cardif/IPS-1/MAVS/VISA to activate downstream signaling pathways (Kawai *et al*, 2005; Meylan *et al*, 2005; Seth *et al*, 2005; Xu *et al*, 2005). Interestingly, Cardif contains multiple TNF receptor-associated factor (TRAF)-interacting motifs (TIMs), suggesting that Cardif may induce antiviral responses through one or more members of the TRAF family (Xu *et al*, 2005).

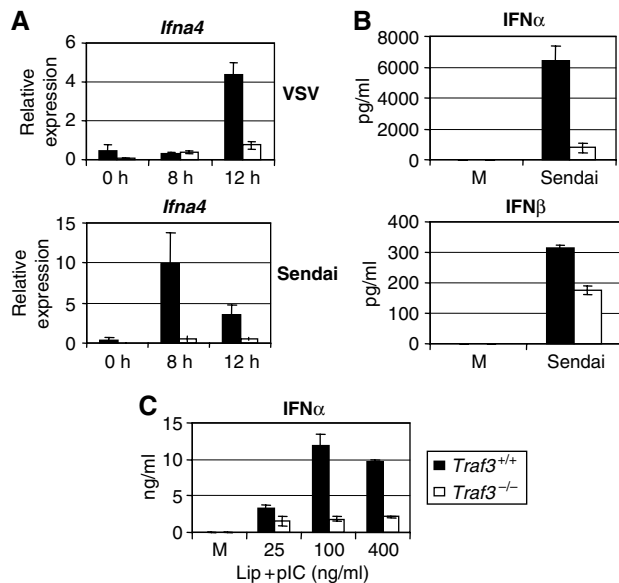
Recently we identified TRAF3 as a critical molecule required for the induction of type I IFNs, but not inflammatory cytokines in response to viral infection and TLR ligation in bone marrow-derived macrophages (BMMs), plasmacytoid dendritic cells (pDCs), and murine embryonic fibroblasts (MEFs) (Hacker *et al*, 2006; Oganessian *et al*, 2006). Accordingly, TRAF3 was specifically required for the activation of IRF3, the transcription factor responsible for type I IFN upregulation, but not NF- $\kappa$ B, which coordinates the transcription of inflammatory cytokines. This finding distinguishes TRAF3 from all other known TRAFs, which predominantly act either as inhibitors or activators of NF- $\kappa$ B.

Despite these findings, it remains unclear how Cardif and TRAF3 are involved in the regulation of type I IFNs. In this report, we show that Cardif induces IFN $\alpha$  through a direct and specific interaction with the TRAF domain of TRAF3, implicating Cardif as the link between cytoplasmic viral receptors and TRAF3.

## Results

### **TRAF3 is required for IFN $\alpha$ production in response to intracellular dsRNA**

We previously found that TRAF3-deficient cells were susceptible to vesicular stomatitis virus (VSV) infection resulting from a defective type I IFN response to this particular virus (Oganessian *et al*, 2006). To examine whether TRAF3 is specifically involved in the recognition of VSV or may be more broadly involved in recognition of RNA viruses, we determined the ability of *Traf3*<sup>+/+</sup> and *Traf3*<sup>-/-</sup> MEFs to induce IFN $\alpha$  in response to infection with both Sendai virus and VSV. Upregulation of *Ifna4* mRNA was almost completely



**Figure 1** TRAF3 is required for IFN $\alpha$  production in response to intracellular dsRNA. (A) mRNA from *Traf3* WT and KO MEFs was assayed for levels of *Ifna4* following infection with Sendai or VSV by Q-PCR. (B) Supernatants from *Traf3* WT and KO MEFs 12 h after infection with Sendai were analyzed for levels of IFN $\alpha$  and IFN $\beta$  by ELISA. (C) IFN $\alpha$  levels in culture supernatants 12 h following liposomal transfection of poly I:C were measured by ELISA.

defective in *Traf3*<sup>-/-</sup> MEFs compared to wild-type cells (Figure 1A). Similarly, TRAF3-deficient cells secreted lower levels of IFN $\alpha$  and IFN $\beta$  12 h after Sendai virus infection (Figure 1B). It is thought that dsRNA produced in the cytoplasm of fibroblasts during viral replication is detected by RNA-binding proteins responsible for transducing the cellular response to viral infection. We therefore tested whether TRAF3 could be involved in signal transduction pathways activated by the presence of intracellular dsRNA by transfecting wild-type and *Traf3*<sup>-/-</sup> MEFs with polyI:C, a synthetic form of dsRNA. When directly transfected into these cells, we found that polyI:C failed to induce a strong IFN $\alpha$  response in fibroblasts that lack TRAF3, indicating that TRAF3 is indeed required for innate antiviral responses to intracellular dsRNA (Figure 1C).

### A structurally intact TRAF3 molecule is required for antiviral activity

Despite these findings, the molecular mechanisms by which TRAF3 is involved in viral response pathways remain unclear. To identify the minimal region required for TRAF3-mediated IFN $\alpha$  production, we reconstituted early passage *Traf3*<sup>-/-</sup> MEFs with a panel of truncation mutants of TRAF3 from the N- and C-terminus (Figure 2A, B, E and F). Transduced cells were then selected with puromycin and infected with VSV or Sendai virus. As shown in Figure 2C, *Traf3*<sup>-/-</sup> MEFs reconstituted with a TRAF3 truncation mutant lacking the TRAF domain could not rescue TRAF3-mediated IFN $\alpha$  production. Furthermore, when infected with a form of VSV that expresses GFP, MEFs reconstituted with a truncation mutant of TRAF3 lacking the TRAF domain exhibited similar levels of GFP<sup>+</sup> cells as *Traf3*<sup>-/-</sup> MEFs reconstituted with vector alone (Figure 2D). We also found that *Traf3*<sup>-/-</sup> cells reconstituted with successive TRAF3 N-terminal truncation

mutants failed to secrete IFN $\alpha$  following Sendai virus infection (Figure 2G). These data indicated that a structurally intact TRAF3 molecule is required for optimal type I IFN-mediated antiviral responses. However, the structural components of TRAF3 that were responsible for its unique biological role in mediating antiviral responses remained unclear.

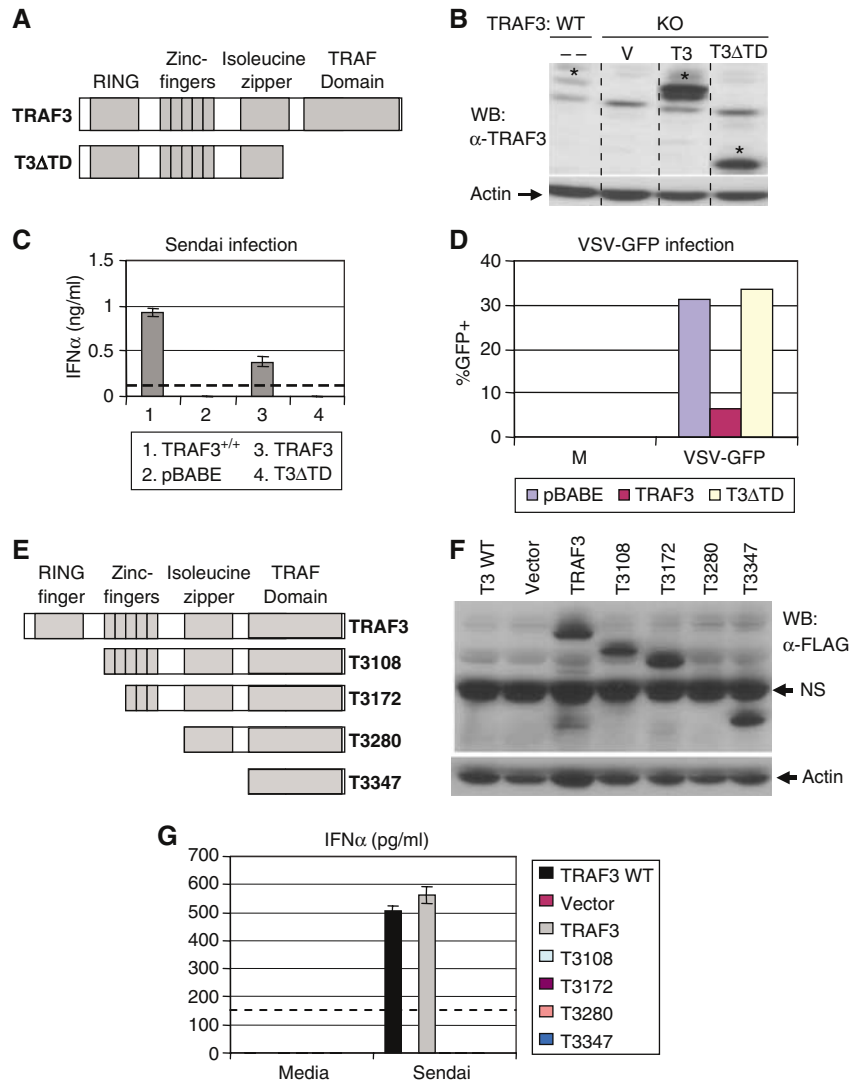
### The TRAF domain of TRAF3 is specifically required for TRAF3-mediated type I IFN production

The secondary structure of TRAF3 is nearly identical to that of TRAF5 (Figure 3A). Nevertheless, TRAF5 could not functionally replace TRAF3 in our reconstitution system (Figure 3C). Therefore, the functional role of TRAF3 appears to be mediated by unique characteristics of its primary amino-acid sequence. To determine which structural features grant TRAF3 its unique function, we generated TRAF3/TRAF5 chimeras in which each of the domains of TRAF3 were replaced with those of TRAF5 (Figure 3A). *Traf3*<sup>-/-</sup> MEFs were then reconstituted with these chimeric molecules and infected with Sendai virus (Figure 3B). Surprisingly, the TRAF3 chimeras containing the RING domain, zinc-fingers, or isoleucine zipper of TRAF5 fully reconstituted TRAF3-dependent IFN $\alpha$  production. In contrast, MEFs expressing the TRAF domain chimera completely failed to reconstitute this function (Figure 3C), suggesting that the TRAF domain is responsible for the functional distinction between these two molecules. To further test whether the TRAF domain of TRAF3 allows for its unique functional role in antiviral responses, we reconstituted *Traf3*<sup>-/-</sup> MEFs with a chimeric molecule in which the entire N-terminus of TRAF5 is fused to the TRAF domain of TRAF3 (Figure 3D and E). As shown in Figure 3F, this chimeric molecule almost completely reconstituted TRAF3-mediated IFN $\alpha$  production. Thus, the TRAF domain but not the RING domain, zinc-fingers, or isoleucine zipper of TRAF3 is responsible for its unique role in mediating the cellular antiviral response.

### Cardif activates IFN $\alpha$ response through TRAF3

These findings suggested that the TRAF domain of TRAF3, but not that of TRAF5, is capable of forming a critical interaction in the type I IFN pathway. Cardif was recently identified as an essential adapter for cytoplasmic dsRNA receptors such as RIG-I and Helicard (Kawai *et al*, 2005; Meylan *et al*, 2005; Seth *et al*, 2005; Xu *et al*, 2005). Interestingly, Cardif contains a well-conserved TRAF-interacting motif (TIM) within its proline-rich region (PRR), which may be capable of forming specific interactions with the TRAF domain of TRAF3 (Xu *et al*, 2005). To assess whether TRAF3 is involved in Cardif-mediated induction of type I IFN, we transfected *Traf3*<sup>-/-</sup> and *Traf3*<sup>+/+</sup> MEFs with IFN regulatory factor 7 (IRF7) and Cardif, together with an *Ifna4* or NF- $\kappa$ B reporter construct. TRAF3-deficient cells have basally high activity of the NF- $\kappa$ B p100 subunit (He *et al*, manuscript in preparation). To control for any effect of this activity in our luciferase assays, a *p100*<sup>-/-</sup> background was used. As shown in Figure 4A, Cardif-mediated *Ifna4* induction was completely dependent on TRAF3. In contrast, Cardif-mediated activation of an NF- $\kappa$ B luciferase reporter was similar in both *Traf3*<sup>+/+</sup> and *Traf3*<sup>-/-</sup> MEFs.

We next sought to determine whether TRAF3 and Cardif interact. Indeed, Cardif associated with TRAF3 in a manner



**Figure 2** A structurally intact TRAF3 molecule is required for antiviral activity. (**A**, **E**) *Traf3*<sup>-/-</sup> MEFs were reconstituted with the indicated constructs. (**B**, **F**) Expression was monitored by immunoblotting. (**C**, **G**) Reconstituted cells were then infected with Sendai virus for 24 h and IFNα levels in the supernatants were assayed by ELISA. (**D**) Cells from (**B**) were infected with VSV-GFP. VSV replication was monitored by flow cytometry. NS designates a nonspecific band. Dotted line indicates the limit of detection for the IFNα ELISA.

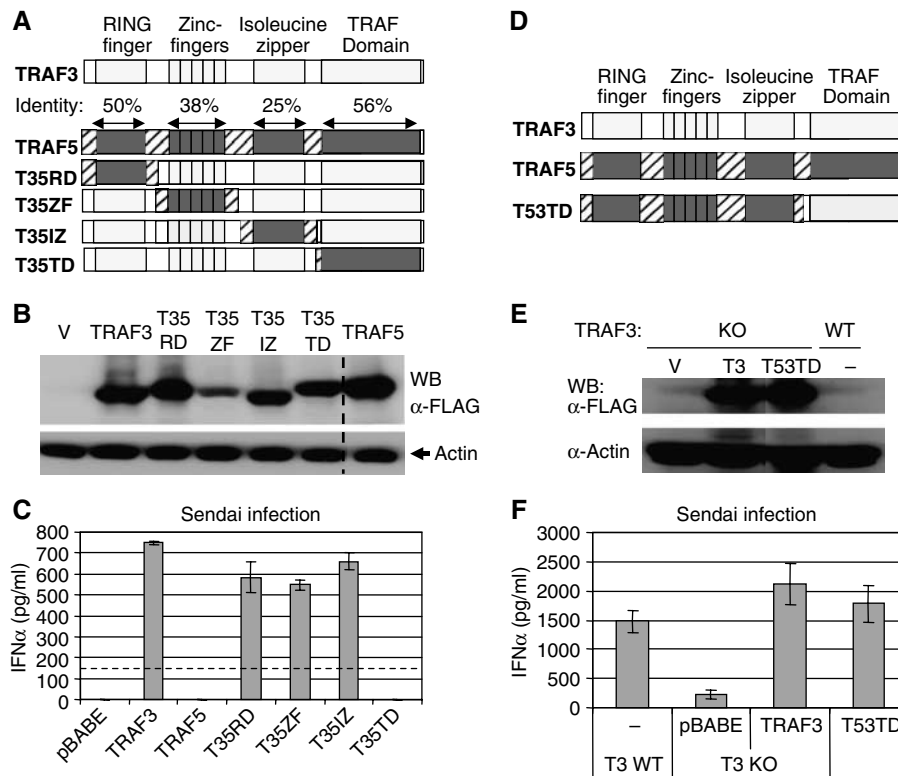
that was independent of its CARD domain and transmembrane region when overexpressed in 293T cells (Figure 4B). Furthermore, constitutive association of TRAF3 and Cardif was also observed at endogenous levels (Figure 4C). Thus, TRAF3 and Cardif may form a complex involved in the regulation of type I IFNs.

**A TIM found within the proline-rich region of Cardif specifically interacts with the TRAF domain of TRAF3**

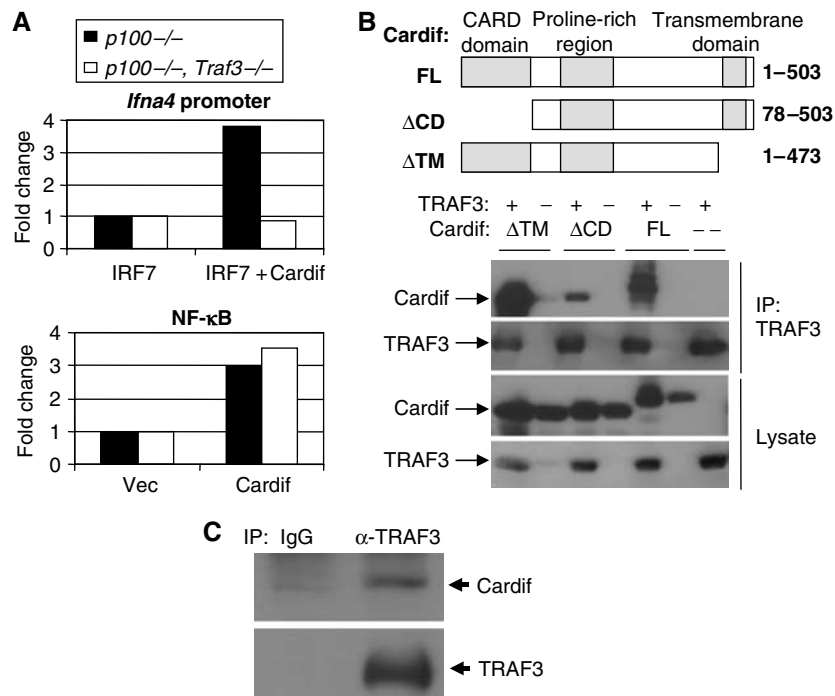
As mentioned above, Cardif contains a well-conserved TIM sequence within its proline-rich region (PRR). To examine whether this TIM could associate with TRAF3, we constructed GST fusion proteins with a 21-amino-acid stretch from the PRR of Cardif containing either the wild-type TIM sequence or one containing a single conserved amino-acid mutation. We then subjected TRAF3 to a GST pull-down assay using these fusion proteins. As demonstrated in Figure 5A, TRAF3 strongly associated with the wild-type but not the mutated TIM of Cardif. Typically, TIMs interact with TRAF molecules through a direct association with the

TRAF domain. Upon investigation of the region of TRAF3 involved in this interaction, we found that the TRAF domain was both necessary and sufficient to associate with the TIM of Cardif (Figure 5B). Therefore, these experiments established the ability of this region of Cardif to act as a classic TIM by binding the TRAF domain of TRAF3.

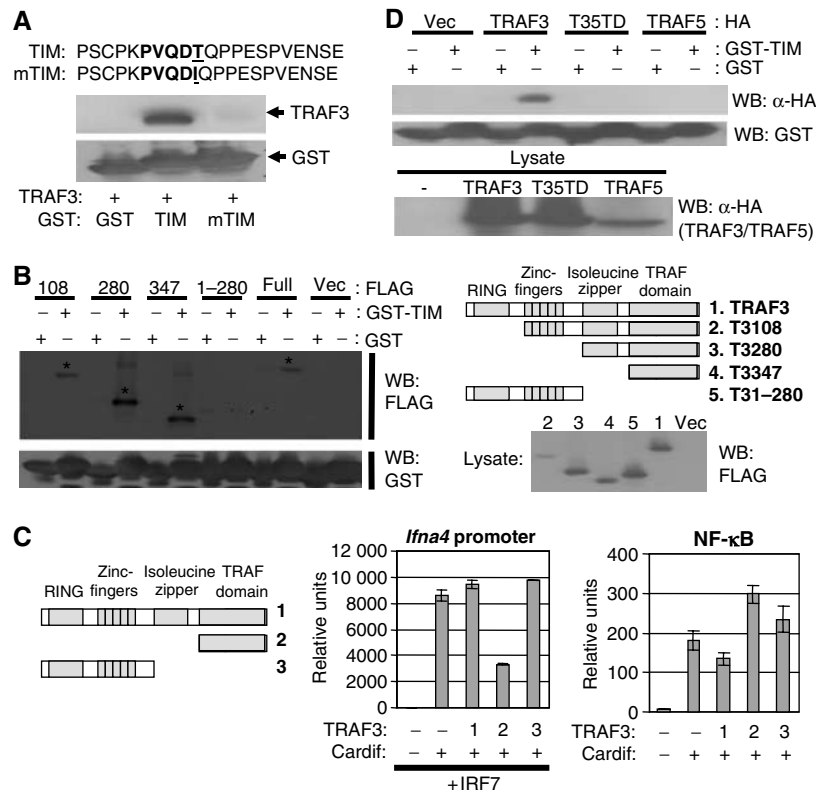
Despite the ability of the TRAF3 TRAF domain to bind the TIM of Cardif, it could not rescue TRAF3-mediated antiviral type I IFN production (Figure 2G). As it lacks any other required functional domains, we wanted to test whether the TRAF domain of TRAF3 would have any dominant negative effect on Cardif-mediated signaling. 293T cells were cotransfected with Cardif, IRF7, and an *Ifna4* promoter or NF-κB luciferase construct along with wild-type TRAF3, the TRAF domain of TRAF3 alone, or an N-terminal segment of TRAF3 as a control. As shown in Figure 5C, coexpression of the TRAF domain of TRAF3, but not wild-type TRAF3 or the TRAF3 N-terminus, significantly abrogated Cardif-mediated activation of the *Ifna4* promoter. In contrast, the TRAF domain of TRAF3 did not impede Cardif-mediated activation of the



**Figure 3** The TRAF domain of TRAF3 is specifically required for TRAF3-mediated type I IFN production. (A, B, D, E) *Traf3*<sup>-/-</sup> MEFs were reconstituted as in Figure 2 with the indicated TRAF3/TRAF5 chimeric molecules and expression was monitored by immunoblotting. (C, F) IFN $\alpha$  production by reconstituted cells 24 h following Sendai infection was monitored by ELISA. Dotted line indicates the limit of detection for the IFN $\alpha$  ELISA.



**Figure 4** Cardif activates IFN $\alpha$  response through TRAF3. (A) *p100*<sup>-/-</sup>, *Traf3*<sup>+/+</sup> MEFs or *p100*<sup>-/-</sup>, *Traf3*<sup>-/-</sup> MEFs were transiently transfected with an NF- $\kappa$ B or *Ifna4* reporter, *IRF7*, and either Cardif or vector control. Luciferase activity was measured 24–48 h later. (B) TRAF3 associates with Cardif independently of the CARD domain and transmembrane region of Cardif when coexpressed in 293T cells by immunoprecipitation assay. 293T cells were transfected with the indicated constructs and immunoprecipitated using  $\alpha$ TRAF3 antibodies. (C) Endogenous association of TRAF3 and Cardif. Whole-cell lysates from ~20 million NIH 3T3 cells were subjected to immunoprecipitation assay using  $\alpha$ TRAF3 or isotype control antibodies, followed by immunoblotting for the presence of Cardif or TRAF3.



**Figure 5** A TIM found within the proline-rich region of Cardif specifically interacts with the TRAF domain of TRAF3. (A) TRAF3 associates with the TIM of Cardif. TRAF3 was subjected to a GST pull-down assay using GST alone or GST fused to the indicated amino-acid sequences as bait. (B) TRAF3 associates with the TIM of Cardif through its TRAF domain. The indicated truncation mutants of TRAF3 were subjected to a GST pull-down assay using GST alone or GST-TIM as bait. (C) The TRAF domain of TRAF3 specifically abrogates Cardif-mediated activation of *Ifna4* promoter. 293T cells were transfected with the indicated constructs at an equal ratio of Cardif to TRAF3 or TRAF3 mutant plasmids and luciferase activity was measured after 24–48 h. (D) The TRAF domain of TRAF3 but not that of TRAF5 interacts with the TIM of Cardif. The indicated TRAF constructs were subjected to GST pull-down assays as above.

NF- $\kappa$ B luciferase construct (Figure 5C). Thus, the TRAF domain of TRAF3 is sufficient to bind Cardif and specifically inhibit its ability to activate the *Ifna4* promoter. This suggests that the TRAF domain of TRAF3 may act as a dominant negative in blocking Cardif-mediated induction of type I IFNs by blocking the ability of Cardif to bind and recruit wild-type TRAF3.

Interestingly, our reconstitution experiments showed that the TRAF domain of TRAF3 is responsible for the functional difference between TRAF3 and TRAF5 in regulating antiviral responses (Figure 3). This result implied that the TRAF3 TRAF domain may mediate specific interactions required for the induction of type I IFNs. To determine if the functional distinction between the TRAF domains of TRAF3 and TRAF5 may be due to a differential affinity for the TIM in Cardif, we subjected wild-type TRAF3, wild-type TRAF5, and our TRAF domain chimera to a GST pull-down assay using the GST-TIM fusion protein as bait. As shown in Figure 5D, the TRAF domain of TRAF3, but not that of TRAF5, specifically bound the TIM of Cardif. Thus, TRAF3 may be specifically recruited to viral detection pathways via a direct and specific interaction with the TIM of Cardif.

#### Mutation of the TIM-binding pocket in TRAF3 abolishes the IFN $\alpha$ response to Sendai virus

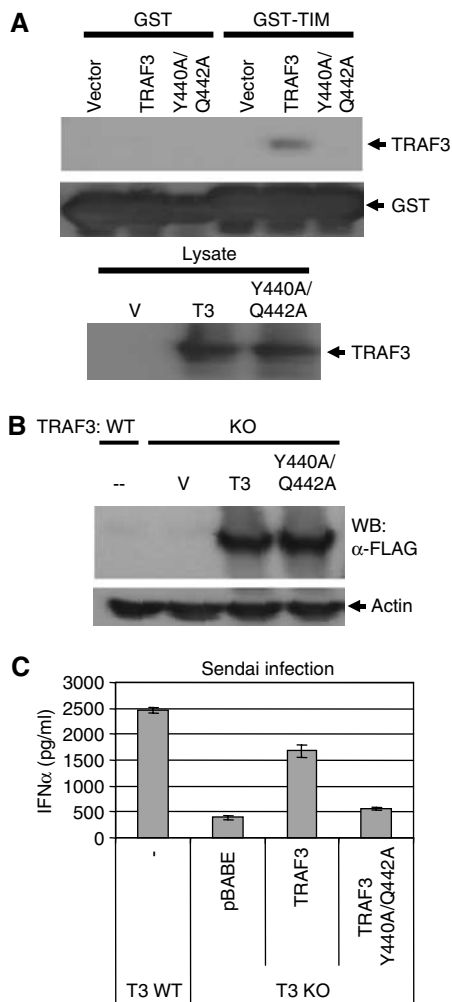
The above experiments demonstrated that the TIM of Cardif specifically bound the TRAF domain of TRAF3. However, we next wanted to test whether the ability of TRAF3 to bind the

TIM of Cardif was actually required for type I IFN production under more physiological conditions. A previous report identified two critical amino acids, Y440 and Q442, in the interaction between TRAF3 and a similar TIM through crystal structure analysis (Ni *et al*, 2004). To confirm the importance of these two amino acids in Cardif-mediated signaling, we created a mutant form of TRAF3 in which both amino acids were mutated to alanine and tested whether this mutant form of TRAF3 could bind the Cardif-TIM. As expected, the Y440A/Q442A TRAF3 mutant had dramatically reduced affinity for the Cardif-TIM as demonstrated by GST pull-down assay (Figure 6A).

This mutant form of TRAF3 then allowed us to test whether the specific interaction between the TIM of Cardif and the TRAF domain of TRAF3 was functionally significant. *Traf3*<sup>-/-</sup> MEFs were reconstituted with vector alone, wild-type TRAF3, or the Y440A/Q442A TRAF3 mutant and infected with Sendai virus (Figure 6B). As shown in Figure 6C, mutation of the TIM-binding pocket almost completely abolished TRAF3-mediated IFN $\alpha$  production in response to Sendai infection. Thus, the ability of TRAF3 to interact with a TIM, such as that found within Cardif, plays a critical role in the antiviral response.

#### Discussion

The mammalian genome encodes multiple mechanisms for the detection of viral infection. In macrophages, dsRNA is



**Figure 6** Mutation of the TIM-binding pocket in TRAF3 abolishes the IFN $\alpha$  response to Sendai virus. **(A)** The Y440A/Q442A TRAF3 mutant no longer binds the Cardif-TIM. The indicated constructs were subjected to GST pull-down assay as above using GST alone or the Cardif-TIM fused to GST as bait. **(B)** *Trif3*<sup>-/-</sup> MEFs were reconstituted with wild-type *Traf3* or the double point mutant of *Traf3*, which abolishes its ability to bind the Cardif-TIM. Expression was monitored by immunoblot. **(C)** IFN $\alpha$  production by reconstituted cells 24 h following Sendai infection was monitored by ELISA.

recognized by TLR3 which utilizes the adapter TRIF to induce type I IFNs, thereby inhibiting viral replication. In plasmacytoid dendritic cells, the most potent producers of type I IFNs, ssRNA and unmethylated DNA are recognized by TLRs 7/8 and 9, respectively. In fibroblasts, which do not express TLRs, dsRNA within the cytoplasm is detected through CARD-containing RNA helicases such as Helicard and RIG-I. Thus, type I IFNs can be upregulated by multiple TLR-dependent and TLR-independent pathways (Kawai and Akira, 2006). Nevertheless, in all of the aforementioned cases, we have now shown that TRAF3 is specifically required for induction of the type I IFN-dependent antiviral response, establishing this adapter as a major convergence point for multiple recognition pathways as well as a key molecular mediator of host defense mechanisms against viral infection (Oganesyan *et al*, 2006).

Cardif was originally identified as a protein capable of activating the NF- $\kappa$ B and type I IFN pathways when over-

expressed. Recent studies indicate that Cardif is, in fact, required for downstream signaling by Helicard and RIG-I via homotypic interactions between their CARD domains. The RNA helicase domain of RIG-I or Helicard most likely acts as an inhibitory domain that is released upon recognition of dsRNA. This recognition event then allows the CARD domains of Helicard/RIG-I to recruit and bind the Cardif CARD domain (Kawai *et al*, 2005; Meylan *et al*, 2005; Seth *et al*, 2005; Xu *et al*, 2005). Although one study by Xu *et al* (2005) suggested that Cardif activates NF- $\kappa$ B through TRAF6, how Cardif induces type I IFN-mediated antiviral responses was not known. Our study resolves this remaining question by showing that Cardif utilizes TRAF3 to activate type I IFN production through a direct interaction by virtue of its well-conserved TIM.

It is interesting to note that Cardif most likely activates NF- $\kappa$ B through TRAF6 while regulating IRF transcription factors through TRAF3 (Xu *et al*, 2005). Indeed, Cardif contains multiple TIMs, some of which would interact preferentially with TRAF3 and others that would interact preferentially with TRAF6. In fact, by reconstituting TRAF3-deficient cells with its close homologue TRAF5, which is a potent activator of NF- $\kappa$ B, we can highlight the specific function of TRAF3. *Traf3*<sup>-/-</sup> fibroblasts expressing TRAF5 failed to produce IFN $\alpha$  in response to Sendai virus infection compared to *Traf3*<sup>-/-</sup> cells reconstituted with TRAF3. Through the reconstitution of *Traf3*<sup>-/-</sup> cells with a series of chimeric molecules between TRAF3 and TRAF5, we further mapped the TRAF domain as the region responsible for the functional difference between these closely related proteins. This is consistent with our observation that only the TRAF domain of TRAF3 but not that of TRAF5 can bind to the TIM of Cardif.

TRAF molecules are at the center of numerous immunological and physiological processes. Although highly homologous, our results suggest that relatively minor structural differences between TRAF family members can have dramatic functional consequences due to highly specific interactions with other signaling molecules. It is now evident that these distinctions between TRAF family members can make the difference between a general inflammatory response and a specific type I IFN-mediated antiviral response. Further analysis into the mechanistic and structural differences between TRAF family members may eventually allow us to guide the immunological response in a manner that more appropriately targets a specific pathogen.

## Materials and methods

### Reagents

Poly I:C was purchased from Amersham Biosciences AB (Uppsala, Sweden). Anti-HA and anti-TRAF3 antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-Myc antibody was obtained from Cell Signalling Technologies (Beverly, MA). Anti-actin antibody was obtained from Sigma Aldrich (St Louis, MO). Anti-Cardif antibodies were obtained from Axxora (San Diego, CA). M2 monoclonal antibody was used to detect Flag-tagged molecules. IFN $\alpha$  and IFN $\beta$  ELISA reagents were obtained from PBL Biomedical Laboratories (Piscataway, NJ). IFN $\alpha$  ELISA was performed according to the manufacturer's recommended protocols. VSV expressing GFP and Sendai virus (SeV; Z strain) were kind gifts from Dr Glen Barber and Dr Debi Nayak, respectively. Lipofectamine reagent was purchased from Invitrogen (Carlsbad, CA). Luciferase assays were performed according to the manufacturer's instructions (Promega, Madison, WI). Rabbit TrueBlot beads and secondary antibodies were obtained from eBioscience (San Diego, CA).

### Constructs

An MGC clone (ID 4190175) containing the full sequence of Cardif was obtained from Invitrogen and sequenced to verify integrity of the insert. Myc-tagged full-length,  $\Delta$ TM, and  $\Delta$ CARD Cardif were generated by PCR using the MGC clone as a template and inserted into pcDNA3.1-TOPO (Invitrogen). GST-TIM and GST-mTIM were constructed as previously described (Li *et al*, 2002). Briefly, synthetic DNA oligos corresponding to the 21-amino-acid region of Cardif containing the TIM or a single isoleucine mutant thereof were annealed and ligated into the *Bam*HI and *Eco*RI sites of pGEX-2T. All TRAF3 deletion mutants were PCR cloned into *Eco*RI/*Sall* into a pBABEpuro retroviral vector with an N-terminal Flag tag. TRAF3/TRAF5 chimeras were constructed using standard overlapping PCR techniques and cloned into pBABEpuro. Double point mutants of TRAF3 were constructed using a Quikchange kit (Stratagene) and wild-type TRAF3 in a pBABE-puromycin vector as a template.

### Quantitative PCR

RNA was isolated and analyzed by real-time quantitative PCR (Q-PCR) as described previously (Doyle *et al*, 2002). *Irfn4* primers were forward, CCTGTGTGATGCAGGAACC; reverse TCACCTCCCA GGCACAGA. All measurements are displayed as relative expression units after normalization to *L32*.

### Cell culture

MEFs were isolated from day 15 embryos. MEFs and HEK 293T cells were cultured in 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) as described previously (Oganesyan *et al*, 2006).

### Reconstitution

To reconstitute *Traf3*<sup>-/-</sup> cells, HEK 293T cells were transfected with a Moloney Murine Leukemia Virus  $\Psi$ A-MLV helper construct plus pBABEpuro alone or pBABEpuro with the indicated construct using standard calcium phosphate methods. *Traf3*<sup>-/-</sup> MEFs were then infected with the filtered 293T cell supernatants followed by selection with 2.5  $\mu$ g/ml of puromycin before infection with either Sendai virus or VSV.

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### Luciferase reporter assay

*p100*<sup>-/-</sup> and *Traf3*<sup>-/-</sup>, *p100*<sup>-/-</sup> cells were transfected via electroporation with pGL2-Basic containing the murine *Irfn4* promoter or 2  $\times$  NF- $\kappa$ B-binding sites and the indicated constructs according to the manufacturer's instructions (Amaxa, Gaithersburg, MD). For 293T cell experiments, 293T cells were transfected with the indicated constructs using standard calcium phosphate methods. After 24–48 h, the cells were lysed and luciferase activity was determined using a luminometer. Measurements are displayed in relative light units (RLU) and are normalized to  $\beta$ -galactosidase levels in each sample.

### Immunoprecipitation and GST pulldown assay

Co-immunoprecipitation experiments and the GST pulldown assays were performed as previously described (Cheng and Baltimore, 1996). In GST pulldowns, glutathione beads (Sigma Aldrich) were incubated with *Escherichia coli*-expressed GST-TIM, GST-mTIM, or GST alone for 2 h. Glutathione beads were then washed and incubated for 1.5 h with lysates of 293T cells expressing the indicated TRAF3 or TRAF5 constructs. After washing, protein was eluted from the beads and separated by SDS-PAGE. Immunoblotting was performed as described previously (Oganesyan *et al*, 2006). Endogenous immunoprecipitation was performed using Rabbit TrueBlot immunoprecipitation system according to the manufacturer's instructions.

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