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Linear ubiquitination of NEMO negatively regulates the interferon antiviral response through disruption of the MAVS-TRAF3 complex

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Running Title: Linear ubiquitinated NEMO inhibits RIG-I signaling

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Summary
The RIG-I/Mda5 sensors recognize RNA virus infection through their ability to bind intracellular viral RNA and trigger the host antiviral response. In the present study, we investigated the role of linear ubiquitin assembly complex (LUBAC) - consisting of the E3 ligases HOIL-1L, HOIP, and the accessory protein SHARPIN- in the differential regulation of the RIG-I pathway. LUBAC downregulated virus-mediated interferon (IFN) induction by targeting NEMO for linear ubiquitination. Linear ubiquitinated NEMO associated with TRAF3 and disrupted the MAVS-TRAF3 complex, leading to the inhibition of IFN activation while stimulating NF-κB dependent signaling. In SHARPIN deficient MEFs, vesicular stomatitis virus replication was decreased as a consequence of increased IFN production. Linear ubiquitination thus switches NEMO from a positive to a negative regulator of RIG-I signaling that dissociates the MAVS-TRAF3 complex and contributes to the negative regulation of the IFN antiviral response.

Highlights
• LUBAC-mediated linear ubiquitination of NEMO inhibits RIG-I/Mda5 signaling
• Linear ubiquitinated NEMO binds TRAF3 and competes with MAVS for TRAF3 binding
• Displacement of TRAF3 attenuates antiviral signaling
• SHARPIN-deficient MEFs exhibit an increased antiviral response post-VSV infection
INTRODUCTION

The innate immune response represents the first line of defense against microbial pathogens and results in the production of immunomodulatory cytokines and the mobilization of innate immune cells. Central to the early host defense against viral infection is the production of interferons (IFN) and the synthesis of antiviral IFN stimulated genes (ISGs) that contain virus dissemination and activate the adaptive immune response (Liu et al., 2011; Sadler and Williams, 2008; Sen and Sarkar, 2007). Viral nucleic acids represent pathogen-associated molecular patterns (PAMPs) that are recognized by Pattern Recognition Receptors (PRRs) to trigger the type I IFN - IFNA and IFNβ – production (Belgnaoui et al., 2011; Loo and Gale, 2011; Takeuchi and Akira, 2010; Yoneyama and Fujita, 2010). PRRs responsible for the detection of RNA viruses (and some DNA viruses) include both the membrane-bound Toll-like receptors (TLRs) and cytosolic sensors, such as retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) (Belgnaoui et al., 2011; Beutler, 2009; Kumar et al., 2011; Loo and Gale, 2011; Takeuchi and Akira, 2010; Wilkins and Gale, 2010; Yoneyama and Fujita, 2010).

Early viral RNA replicative intermediates are mainly detected by RIG-I or Mda5 (melanoma differentiation-associated gene 5) – two characterized cytosolic viral RNA receptors belonging to the DExD/H box RNA helicase family (Kato et al., 2006; Yoneyama et al., 2004). The functions of RIG-I and Mda5 are non-redundant as RIG-I specifically detects intracellular double-stranded (ds) viral RNA bearing 5′ triphosphate and panhandle structures (Rehwinkel et al., 2010; Schlee and Hartmann, 2010; Schlee et al., 2009; Yoneyama and Fujita, 2008), leading to RIG-I interaction with the downstream adapter protein MAVS (also known as Cardif/IPS-1/VISA) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005); Mda5 recognizes dsRNA structures such as synthetic poly (I:C) (>2kb) and also signals through MAVS. Strategically localized at the outer mitochondrial membrane or on peroxisomes, MAVS assembles a signaling platform that triggers the IFN antiviral response, via activation of the transcription factors NF-kB and IFN regulator factors (IRF)-3 and -7 (Belgnaoui et al., 2011; Dixit et al., 2010; Scott, 2010). Crucial to MAVS complex formation and downstream signaling is the recruitment of the Tumor Necrosis Factor (TNF) Receptor-Associated Factors (TRAFs), a family of cytoplasmic signaling adapter proteins (Hacker et al., 2011). Several TRAF family
members - TRAF-2, -3, -5 and -6 - directly bind to MAVS via TRAF-interacting motifs (TIM) in the N- and C-terminal regions of MAVS (Paz et al., 2011; Saha et al., 2006; Tang and Wang, 2010; Xu et al., 2005). Interaction of MAVS with TRAF-2 or -6 is involved in IKK-dependent NF-κB activation (Xu et al., 2005), whereas TRAF3 is specifically involved in TBK1-dependent IRF-3 or -7 activation (Paz et al., 2011; Saha et al., 2006).

In addition to the adapters, kinases and accessory proteins of the MAVS complex, the NF-κB modulator protein NEMO (IKKγ) also interfaces with the RIG-I pathway, and forms a regulatory bridge between the canonical IKKα/β kinases and the non-canonical kinases TBK1/IKKε via the TANK adapter (Chariot et al., 2002; Zhao et al., 2007). NEMO activity is regulated by numerous post-translational modifications, including ubiquitination, phosphorylation and sumoylation (Liu and Chen, 2011; Sebban et al., 2006). Multiple ubiquitination signals have been identified - mono, multiple- or polyubiquitination – that control protein fate and turnover (Bhoj and Chen, 2009; Dikic and Dotsch, 2009; Ikeda et al., 2010; Kirisako et al., 2006; Malynn and Ma, 2010; Weissman et al., 2011). Lys48 and Lys63 linkages are the best characterized types of polyubiquitination, with Lys48-linked polyubiquitination leading to ubiquitin-dependent degradation by the 26S proteasome (Dikic and Dotsch, 2009; Gallastegui and Groll, 2010). In contrast, Lys63-linked ubiquitination is associated with activation of protein kinases, protein-protein interactions, DNA repair and endocytosis (Bergink and Jentsch, 2009; Bhoj and Chen, 2009).

Recently, a new form of polyubiquitin chain formation was identified in association with NEMO; unlike Lys63- or Lys48-linked polyubiquitin chains that link ubiquitin moieties via a lysine residue of the previously attached ubiquitin, linear ubiquitination consists of head-to-tail linked ubiquitin moieties (Tokunaga et al., 2009). Upon TNFα stimulation, the E3 ligase linear ubiquitin chain assembly complex (LUBAC), composed of the two RING-IBR-RING (RBR)-containing E3 ligases, HOIL-1L (also known as RBCK1 and RNF54) and HOIP (also known as ZIBRA and RNF31), mediates the formation of linear ubiquitin chains on NEMO (Tokunaga et al., 2009), which functions to activate NF-κB signaling downstream of the TNF receptor. LUBAC activity is also dependent on a third accessory protein SHANK-associated RH domain
interactor (SHARPIN) that stabilizes the E3 complex (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011).

Linear ubiquitination also modulates the RIG-I antiviral pathway but has not been reported to affect the Mda5 receptor. TRIM25, an E3 ligase that regulates RIG-I activation via Lys63-linked ubiquitination (Gack et al., 2007), was characterized as a unique target of the LUBAC complex (Inn et al., 2011), with linear ubiquitination of TRIM25 resulting in its proteasomal degradation. HOIL-1L or HOIP independently mediated Lys48-linked polyubiquitination and proteasomal degradation of TRIM25 (Inn et al., 2011).

In the present study, we demonstrate that the LUBAC complex contributes to negative regulation of the type I IFN response via linear ubiquitination of NEMO. Using chimeric forms of NEMO, modified by the addition of different linear ubiquitin moieties, we demonstrate that NEMO-Ub with 2 or more linear ubiquitin moieties, but not unmodified NEMO, bound to TRAF3 and dissociated the MAVS-TRAF3 complex. Furthermore, in SHARPIN deficient cpdm mouse embryonic fibroblasts (MEFs), Vesicular Stomatitis Virus (VSV) replication is decreased and the IFN antiviral response increased, highlighting the crucial role of linear ubiquitination in the negative regulation of the antiviral response.

RESULTS

LUBAC inhibits the IFN antiviral response downstream of the adapter MAVS. To examine the effect of linear ubiquitination on the antiviral response, LUBAC was co-expressed in HEK 293 cells in the presence of NF-κB and IFN stimulated response element (ISRE) reporter genes. LUBAC activated the NF-κB promoter more than 50-fold but inhibited Sendai virus (SeV)-mediated activation of the ISRE promoter by 3-fold (Fig 1a). To determine at what level in the pathway LUBAC blocked ISRE expression, the active CARD domain containing form of RIG-I (ΔRIG-I) (Yoneyama et al., 2004), the Mda5 sensor, MAVS, TBK1 kinase or the active form of IRF-3 (IRF-3(5D)) were expressed in the presence or absence of LUBAC. All expression constructs resulted in a 300- to 1000-fold induction of ISRE-Luc reporter activity (Fig 1b, white bar). ISRE activation driven by Mda5, ΔRIG-I, or MAVS was inhibited more than 85% by
LUBAC, whereas ISRE induction by TBK1 or IRF-3(5D) was not affected by LUBAC co-expression (Fig 1b, compare black to white bars). The same expression constructs also induced ISG15 protein expression (Fig 1c, compare lanes 3, 5, 7, 9, and 11), and the addition of LUBAC inhibited ISG15 induction by Mda5, ΔRIG-I, or MAVS (Fig 1c, lanes 4, 6 and 8), but not by TBK1 or IRF-3 (5D) (Fig 1c, lanes 10 and 12). To confirm the inhibitory effect of LUBAC on IFN signaling, expression of HOIL-1L was knocked down by small interfering RNA (siRNA) in A549 cells, followed by SeV challenge for 4, 8 and 12h. IRF-3 phosphorylation (4h) and subsequent degradation (12h) was increased in cells with decreased levels of HOIL-1L (Fig 1d). Inhibition of HOIL-1L expression also led to an increase in ISRE promoter activation by Mda5, ΔRIG-I, or MAVS, but not by TBK1 or IRF-3 (5D) (Fig 1e). Similar experiments using the TLR3 and TLR4 adaptor protein TRIF demonstrated that LUBAC did not affect MAVS-independent ISRE promoter activation (data not shown). These results indicate that LUBAC inhibits the IFN antiviral response downstream of MAVS and upstream of TBK1.

**Linear ubiquitination of NEMO inhibits IFN induction.** The association of the IKK adapter NEMO and the TBK1 adapter TANK coordinately regulates NF-κB and IRF-3 signaling downstream of RIG-I (Chariot et al., 2002; Zhao et al., 2007). To investigate whether LUBAC-mediated linear ubiquitination of NEMO was involved in the control of type I IFN signaling, we generated NEMO chimeric constructs fused at their C-terminus with linear ubiquitin chains of variable sizes, consisting of 1, 2 or 4 linear ubiquitin moieties, termed NEMO-Ub1, NEMO-Ub2 and NEMO-Ub4, respectively. The different NEMO-Ub chimeras stimulated NF-κB reporter gene activity without any additional stimulation, whereas unmodified wild type NEMO (wt-NEMO) failed to activate the NF-κB reporter gene (Fig 2a). When the chimeric constructs were tested with the ISRE promoter after SeV infection or expression of Mda5, ΔRIG-I, MAVS, TBK1 or IRF-3(5D), neither wt-NEMO nor NEMO-Ub chimeras activated the ISRE promoter alone; in contrast NEMO-Ub2 and NEMO-Ub4 inhibited SeV, Mda5, ΔRIG-I or MAVS-driven promoter activity by >75%, compared to wt-NEMO or NEMO-Ub1 (Fig 2b and 2c). Similar to the results obtained with LUBAC, the NEMO-Ub constructs did not inhibit TBK1- or IRF-3(5D)-driven ISRE activity. MAVS-independent activation of the ISRE promoter by TRIF was not affected by the different NEMO-Ub constructs (data not shown). Furthermore, NEMO-Ub2
inhibited expression of various ISGs - ISG15, ISG56 and RIG-I - whereas, wt-NEMO or NEMO-Ub1 had no effect on ISG expression (Fig 2d, compare lane 5, 8 and 11, top, second and third panels). Interestingly, NEMO-Ub2 also failed to inhibit ISG expression after IFNα treatment (Fig 2d, lane 12), suggesting that the inhibition of the type I IFN signaling pathway occurred at an early stage of signaling, prior to IFN release.

**Linear ubiquitinated NEMO interacts with TRAF3.** Because both TRAF3 and NEMO are positive regulators of type I IFN signaling downstream of MAVS, we investigated the possibility that linear ubiquitination of NEMO regulated the formation of the MAVS-TRAF3 complex. In co-precipitation experiments, TRAF3 co-precipitated with NEMO-Ub2, weakly with NEMO-Ub1 (Fig 3a, compare lane 4 to 3, top panel) and not with wt-NEMO (Fig 3a, lane 2, top panel). Next, NEMO and TRAF3 were expressed in the presence or absence of LUBAC. NEMO alone did not interact with TRAF3, but the addition of LUBAC led to the formation of the NEMO-TRAF3 complex (Fig 3b, compare lane 5 to 6, second panel). In the same experiment, using a NEMO specific antibody, modified forms of NEMO were detected in the presence of LUBAC, thus suggesting that linear ubiquitinated NEMO interacted with TRAF3 (Fig 3b, lane 6, top panel). To demonstrate that LUBAC E3 ligase complex formation was required for this interaction, HOIL-1L and/or HOIP were expressed in the presence of TRAF3 and NEMO (Fig 3c). A NEMO-TRAF3 interaction was readily detected when both LUBAC components were present, but not when individual components were expressed (Fig 3c, compare lane 7 to lanes 8 and 9), thus demonstrating that NEMO-TRAF3 interaction was dependent on the presence of the LUBAC complex.

**Linear ubiquitinated NEMO competes with MAVS for TRAF3 binding.** To examine whether linear ubiquitination of NEMO affected IFN signaling at the level of MAVS-TRAF3 interaction, MAVS-TRAF3 association was determined in the presence of LUBAC and/or NEMO. Expression of MAVS led to an interaction with TRAF3 (Fig 4a, lane 4); when individually expressed, NEMO and LUBAC co-expression modestly disrupted this interaction, by 2.4 and 1.7 fold respectively (59% and 41%, respectively) (Fig 4a, lane 5 and 7), but in the presence of both LUBAC and NEMO, the MAVS-TRAF3 complex was decreased more than 5-fold (81%) (Fig
Conversely, the LUBAC-induced TRAF3-NEMO interaction was disrupted in the presence of MAVS (Fig 4b, compare lane 8 and 9) and the reciprocal co-precipitation generated similar results (Fig 4c). To determine whether linear ubiquitinated NEMO and MAVS compete for TRAF3 binding, NEMO was precipitated in the presence of constant amounts of LUBAC and TRAF3, but increasing amounts of MAVS. Increasing MAVS disrupted the LUBAC induced NEMO-TRAF3 complex in a dose dependent manner (Fig 4d). Taken together, these results indicate that the MAVS-TRAF3 interaction is disrupted by linear ubiquitinated NEMO.

**Increased antiviral response and decreased VSV replication in cpdm MEFs.** To test the endogenous role of linear ubiquitination in the RIG-I pathway, *cpdm* MEFs, deficient in the accessory protein SHARPIN that functions to stabilize the LUBAC complex (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011) were used to measure replication of a recombinant VSV expressing green fluorescent protein (VSV-GFP) (Fig 5a). In *cpdm* MEFs, at 24 and 48h after VSV infection, fewer cells were positive for GFP fluorescence, compared to wt MEFs (~ 70% GFP-positive wt MEFs, compared to ~40% GFP-positive *cpdm* MEFs) (Fig 5b). Additionally, VSV replicated in *cpdm* compared to wt MEFs, as reflected by VSV glycoprotein (VSV-G) expression (Fig 5c, second panel). Furthermore, an enhanced antiviral response was observed in *cpdm* MEFs compared to wt MEFs following VSV infection, as reflected by an increase in IFNβ promoter activity (~200-fold induction in wt MEFs compared to ~600-fold increase in *cpdm* MEFs) and IFNα4 promoter (~50-fold induction in wt MEFs compared to ~250-fold in *cpdm* MEFs) (Fig 5d). RIG-I protein expression was also increased in *cpdm* MEFs, thus demonstrating a stronger induction of IFN production (Fig 5c, top panel). Because NF-κB activation by TNFα was shown to be impaired in *cpdm* MEFs (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011), we also examined whether NF-κB activation was defective in the context of VSV infection. NF-κB and IL6 promoter activation were attenuated in *cpdm* MEFs after VSV infection (from ~20-fold to ~ 5-fold and from ~ 9-fold to ~3-fold respectively) (Fig 5e). Because SHARPIN was shown to have an anti-apoptotic role upon TNFα activation (Gerlach et al., 2011; Ikeda et al., 2011), the level of apoptosis in wt and *cpdm* MEFs was assessed after virus infection. An increase in apoptosis, as measured by Annexin V/propidium iodide (PI) positive cells (45% in *cpdm* MEFs compared to 15% in wt MEFs), and an
accumulation of cleaved caspase 3 in *cpdm* compared to wt MEFs were detected (Fig 5f and 5g), demonstrating that SHARPIN deficiency rendered the cells more sensitive to virus-induced cell death.

**Linear ubiquitinated NEMO interacts with TRAF3 and dissociates the MAVS-TRAF3 complex.** Next, using a linear ubiquitin chain specific antibody (Tokunaga et al., 2009), we demonstrated that endogenous NEMO was targeted for linear ubiquitination after VSV infection (Fig 6a). In wt MEFs, NEMO was linearly ubiquitinated 48h after infection (Fig 6a, top panel, lane 5), concomitant with its interaction with TRAF3 (Fig 6a, second panel, lane 5). In contrast, NEMO did not undergo linear ubiquitination in *cpdm* MEFs and did not bind TRAF3 (Fig 6a, lanes 6-10 top and second panel). The reciprocal co-immunoprecipitation experiment in which TRAF3 was immunoprecipitated, confirmed the strong interaction between TRAF3 and NEMO at 48h in wt MEFs (Fig 6b). This association was decreased by more than 6-fold in *cpdm* MEFs, indicating that TRAF3-NEMO association was enhanced in a linear ubiquitin-dependent manner (Fig 6b, compare lane 5 to 10). In parallel, an increased association of MAVS-TRAF3 was observed in *cpdm* MEFs compared to wt MEFs, particularly at 48h after infection (~5-fold increase, Fig 6c compare lane 5 to 10), arguing that linearly ubiquitinated NEMO dislodges TRAF3 from MAVS by physical interaction. This dissociation from MAVS did not occur when NEMO was not linear ubiquitinated.

The expression of antiviral IFN stimulated genes - *IRF7, DDX58* (RIG-I) and *OAS1* were also highly induced in *cpdm* MEFs (Fig 7a, black bars) after virus infection, compared to wt MEFs (white bars), due to intact signalling as a consequence of MAVS-TRAF3 complex formation in *cpdm* MEFs (Fig 6c). *IRF7* mRNA levels peaked at ~150-fold in *cpdm* MEFs compared to ~15-fold in wt MEFs; similarly, *DDX58* peaked at ~13-fold and *OAS1* peaked at ~250-fold; the values in wt MEFs were ~ 3-fold and ~30-fold respectively. By ELISA, a 3-fold increase in IFNα and IFNβ release into the supernatant of *cpdm* cells was detected compared to wt MEFs (Fig 7b). Strikingly, VSV infection of *cpdm* MEFs failed to induce *IL6* promoter (Fig 7c, black bars), illustrating the functional impact of NF-κB inhibition on inflammatory cytokine gene expression. IL6 release post-VSV infection was also 3-fold lower in *cpdm* compared to wt MEFs (Fig 7d). Altogether, these results demonstrate that linear ubiquitination of NEMO following
virus infection facilitates the formation of a NEMO-TRAF3 heterodimer, that sequesters TRAF3 away from the MAVS complex and limits RIG-I dependent signaling (Fig 7e). Linear ubiquitination of NEMO thus negatively regulates production of type I IFN and multiple ISGs, while positively regulating activation of NF-κB and inflammatory gene expression.

**DISCUSSION**

In the present study, we demonstrate an essential role for LUBAC-mediated linear ubiquitination of NEMO in the negative regulation of the RIG-I antiviral pathway through sequestration of TRAF3 from the MAVS adapter. LUBAC and NEMO-Ub constructs inhibited RIG-I signaling downstream of MAVS and upstream of TBK1; linearly ubiquitinated NEMO interacted physically with TRAF3, and disrupted the MAVS-TRAF3 complex, thus providing a mechanistic explanation for the downregulation of RIG-I signaling. Using SHARPIN deficient cpdm MEFs, we observed on the one hand, an increased and prolonged antiviral response, while on the other hand, an impaired NF-κB activation, indicating that linear ubiquitination is required for NF-κB activation downstream of RIG-I. Interestingly, an increase in apoptotic cell death was also detected in SHARPIN-deficient cpdm MEFs after VSV infection, potentially attributable to the absence of the anti-apoptotic activity of NF-κB.

These studies reveal a novel negative feedback mechanism used by host cells to regulate the IFN antiviral response. The formation of the MAVS-TRAF3 complex is a crucial step of the IFN response to RNA viruses and early after virus infection, TRAF3 acts as bridging adapter in the assembly of the active MAVS-TRAF3-TBK1 signaling complex that also includes NEMO (Paz et al., 2011; Zeng et al., 2010). Previously, we demonstrated the positive regulatory role of NEMO as a bridge between the canonical IKKα/β kinases and the non-canonical kinases TBK1/IKKe via the TANK adapter (Zhao et al., 2007). Here, we show that at 24-48h post-infection, linear ubiquitination of NEMO by LUBAC facilitates its interaction with TRAF3, an association that sequesters TRAF3 away from MAVS (Fig 7e). Linear ubiquitination thus switches NEMO from a positive mediator of RIG-I signaling to a negative regulator that dissociates the antiviral signaling complex through a competition mechanism. There are several possibilities that may explain the increased ability of linear ubiquitinated NEMO to dissociate
TRAF3 from MAVS, including a direct binding of TRAF3 to linear ubiquitin chains or a high affinity of TRAF3 for the conformationally altered form of NEMO.

Regulation of RIG-I signaling through ubiquitination has been extensively studied. TRIM25 and RNF135 (REUL) activate the pathway by mediating Lys63-linked polyubiquitination of RIG-I (Gack et al., 2007; Oshiumi et al., 2010). Conversely, inhibition of the pathway is achieved through the Lys48-linked polyubiquitination and subsequent proteasomal degradation of RIG-I and MAVS by RNF125, and TRAF3 by Triad3A (Arimoto et al., 2007; Nakhaei et al., 2009). The ubiquitin editing protein A20 and several deubiquitinases such as DUBA, OTUB and CYLD also negatively regulate RIG-I signaling (Kayagaki et al., 2007; Li et al., 2010; Lin et al., 2006; Wertz et al., 2004; Zhang et al., 2008b); CYLD was shown to inhibit RIG-I signaling by removing Lys63-linked ubiquitin chains from both RIG-I and TBK1 (Zhang et al., 2008b), while DUBA inhibits the pathway by cleaving Lys63 chain from TRAF3, leading to its dissociation from TBK1 (Kayagaki et al., 2007).

These multiple non-redundant mechanisms of regulation of the early antiviral response illustrate the requirement to maintain appropriate regulatory homeostasis of the IFN pathway. Many examples of the pathological consequences of dysregulation of antiviral and inflammatory responses to pathogens exist. The IFIHI gene encoding Mda5 has been associated with several types of autoimmune diseases such as type I diabetes, Grave’s disease and systemic lupus erythematosus (SLE) (Gateva et al., 2009; Smyth et al., 2006; Sutherland et al., 2007). Activation of TLR7 and TLR9 on B cells and plasmacytoid dendritic cells by self-nucleic acid is also crucial step in SLE pathogenesis (Barrat and Coffman, 2008; Guiducci et al., 2010). Production of TLR mediated anti-nuclear antibodies and type I interferon correlated with the severity of the disease (Banchereau and Pascual, 2006; Hahn, 1998). Perhaps unsurprisingly, IRF7, a downstream effector of both RLR- and TLR-signaling, has been linked to SLE onset (Xu et al., 2012). Lastly, the involvement of the ubiquitin regulatory TRIM proteins in various autoimmune and autoinflammatory disorders (including multiple sclerosis, SLE and Sjögren’s syndrome) has been established (Jefferies et al., 2011). How these negative regulatory mechanisms are disrupted...
in various pathological states is critical to the understanding of conditions involving chronic antiviral and inflammatory responses.

**EXPERIMENTAL PROCEDURES**

*Plasmid construction.* Plasmids encoding GFP-MAVS, MYC-MAVS, GFP-ΔRIG-I, GFP-TBK1, GFP-IRF-3(5D), ISRE-luciferase, pRLTK, FLAG-NEMO, FLAG-TRAF3, HA-Ub, MYC-HOIP and HA-HOIL-1 were previously described (Paz et al., 2009; Paz et al., 2011; Tokunaga et al., 2009; Zhao et al., 2007). The Mda5 expression plasmid was a gift from I. Julkunen (Siren et al., 2006). To generate GFP-Mda5 expression plasmid, Mda5 cDNA was amplified by PCR from Mda5 expression plasmid and cloned into pEGFPc1. Flag NEMO was inserted into pMSCV puro vector (Clontech). FLAG-NEMO-Ub1, Ub2 and Ub4 were constructed by adding one ubiquitin or tandem linkage of two or four ubiquitin moieties at the C-terminus of Flag NEMO. To generate S-NEMO, S-NEMO-Ub1 and S-NEMO-Ub2 expression plasmids, the cDNA encoding S-NEMO, S-NEMO-Ub1 and S-NEMO-Ub2 were cloned into pTriEX-4 Neo vector (Novagen).

*Cell culture, transfection and luciferase assay.* Wt MEFs and cpdm MEFs from mice deficient in SHARPIN have been described (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). HEK 293 and MEFs were grown in DMEM media (Wisent) supplemented with 10% (vol/vol) FBS, L-glutamine and antibiotics (Wisent). For luciferase assays, MEF cells grown to subconfluency in DMEM (Wisent) supplemented with 10% (vol/vol) FBS, L-glutamine and antibiotics were transfected with 200 ng of pRLTK reporter (renilla luciferase; internal control), 200 ng of ISRE-Luc, NF-κB-Luc, IFNB-pGL3 or IFNA4-pGL3 luciferase reporter (firefly luciferase; experimental reporter) and 200 ng of MAVS, ΔRIG-I, TRIF, TBK1 or IRF-3(5D) expression plasmid using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). HEK 293 cells were transfected with 50 ng of PRLTK reporter, 100 ng of ISRE-Luc or NF-κB-Luc reporter, expression plasmid encoding Mda5 (200 ng), ΔRIG-I (200 ng), MAVS (50 ng), TRIF (50 ng), TBK1 (50 ng), or IRF-3 (5D) (50 ng), together with 400 ng of empty vector, LUBAC (HOIL-1 and HOIP), NEMO-Ub1, NEMO-Ub2 or NEMO-Ub4 expression plasmid by the calcium phosphate transfection method (Zhao et al., 2007). At 24 h
after transfection, luciferase activity was measured with a Dual-Luciferase Reporter Assay System according to the manufacturer’s instructions (Promega). Some cells were treated with SeV (40 hemagglutination units per ml, Charles River).

**Generation of Flag-NEMO, Flag-NEMO-Ub1 and Flag-NEMO-Ub2 expressing cell lines.** Plasmids Flag pMSCV puro, Flag-NEMO pMSCV puro, Flag-NEMO-Ub1 pMSCV puro and Flag-NEMO-Ub2 pMSCV puro were introduced into HEK 293 cells by the calcium phosphate method. Cells were selected beginning at 48h for approximately 2 weeks in DMEM containing 10% FBS, L-glutamine, antibiotics, and 2 µg/ml puromycin (Sigma).

**Short interfering RNA.** For siRNA experiments, siRNA targeting human HOIL-1L (RBCK1) (Smartpool, Dharmacon) and control non-targeting siRNA pool (Dharmacon) were transfected in A549 or HEK 293 cells using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). 48h after transfection, A549 cells were infected with SeV and harvested 4, 8 and 12h later. Luciferase reporter gene assays were performed on siRNA control and siRNA HOIL-1L HEK 293 cells, as described above.

**Immunoblot analysis.** Whole-cell lysates (40 µg) were separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred for 1 h at 4 °C to nitrocellulose membranes (0.45 µ, Bio-Rad) in a buffer containing 30 mM Tris, 200 mM glycine and 20% (vol/vol) methanol. Membranes were blocked for 1 h at 25 °C in 5% (wt/vol) dried milk in PBS and 0.1% (vol/vol) Tween-20 (PBST) and then were probed with antibody to the Flag-tag (anti Flag (M2); Sigma Aldrich), VSV whole virus antisera, SeV antisera, anti NEMO (DA10-12; Cell Signaling), anti HOIL-1L (RBCK1, a gift from Dr. Shu, Wuhan University (Zhang et al., 2008a) antibody to IRF-3 phosphorylated at Ser396 (EMD-Millipore), anti MYC (Sigma), anti HA (Sigma), anti SHARPIN (Ikeda et al., 2011), anti RIG-I (EMD-Millipore), anti TRAF3(Santa Cruz Biotechnology), anti actin (EMD-Millipore) at a dilution of 1 µg/ml in 5% (wt/vol) milk in PBS. After three 10-minute washes with 0.1% (vol/vol) Tween-20 in PBS, membranes were incubated for 1 h with horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse (Amersham) at a dilution of 1:3,000 in blocking solution. The reaction was then visualized with an enhanced
chemiluminescence detection system as recommended by the manufacturer (PerkinElmer). Densitometry analysis were performed using ImageJ software (NIH Windows version).

**Protein-protein interaction analysis.** For S-tag precipitation, 500 µg of whole cell lysate was incubated with 90 µl bed volume of S-protein agarose (Novagen), rotating at 4°C for 6h, then washed 3 times with 1 ml lysis buffer (20mM Tris [pH 7.5], 150mM NaCl, 10% glycerol, 1% triton, 1mM DTT, 1mM PMSF, 40mM beta-glycerol phosphate, 1mM sodium orthovanadate, Protease inhibitor, 5mM sodium fluoride, 10 mM N-ethylmaleimide). Washed beads were eluted by resuspension in 45 µl Laemmli Sample Buffer with β-mercaptoethanol, followed by boiling for 10 minutes. Eluates were electrophoresed in a 10% SDS polyacrylamide gel and transferred onto nitrocellulose blot. The transferred blots were autoclaved for 30 minutes in water then 15 minutes dry. Blots were blocked for one hour at room temperature in 20% heat inactivated bovine calf serum-TBS-0.45% Tween then processed for immunoblot analysis with the appropriate antibodies, as described above.

For endogenous immunoprecipitation experiments in MEFs, cells were harvested and lysed in immunoprecipitation lysis buffer (20mM Tris [pH 7.0], 250mM NaCl, 3mM EDTA, 3mM EGTA, 0.5% NP-40, 1mM DTT, 1mM PMSF, 40mM beta-glycerol phosphate, 1mM sodium orthovanadate, Protease inhibitor, 5mM sodium fluoride, 10 mM N-ethylmaleimide). To detect the ubiquitinated form of NEMO, lysed cells were incubated at 95 °C for 30 min in 1% SDS in immunoprecipitation lysis buffer, and then diluted to 0.1% SDS with immunoprecipitation lysis buffer. NEMO was immunoprecipitated using an antibody against NEMO (BD pharmingen) at 4°C for 2 h, followed by incubation with protein A/G-PLUS agarose beads overnight. MAVS was immunoprecipitated using an antibody against MAVS (EMD Millipore 06-1043), TRAF3 was immunoprecipitated using an antibody against TRAF3 (Santa-Cruz, sc-6933). Immunoprecipitates were resolved on 10% SDS–PAGE and processed for immunoblot analysis with the appropriate antibodies, as described above.

**Virus production, quantification and infection.** Recombinant VSV-GFP, which harbors the methionine 51 deletion in the matrix protein-coding sequence (Stojdl et al., 2003), was kindly
provided by J. Bell (Ottawa Health Research Institute). Virus stocks were grown in Vero cells, concentrated from cell-free supernatants by centrifugation, and titrated by standard plaque assay. Cells were infected with VSV in a small volume of medium without FBS for 1h at 37°C; cells were then incubated in complete medium for the indicated period of time prior to analysis. To evaluate MEF infectivity, cells were infected at different MOI with VSV-GFP. GFP fluorescence intensity was measured by flow cytometry using a FACSCalibur (BD Biosciences) and data were analysed with FCS Express 3 (De Novo Software). IFNα, IFNβ and IL6 in cell supernatants were quantified by ELISA (PBL Interferon Source).

**Quantitative real-time PCR.** DNase-treated total RNA from MEFs was prepared using the RNeasy kit (Qiagen). RNA concentration was determined by absorption at 260 nm, and RNA quality was ensured by a 260/280 ratio ≥ 2.0. Total RNA was reverse transcribed with 100 U of Superscript II Plus RNAse H reverse transcriptase using oligo AnCT primers (Gibco BRL Life Technologies). qPCR assays were performed using the SYBR Green I on a Light Cycler apparatus (Roche Diagnostics). Murine primers sequences used in this study are as follows: IRF-7 Forward: 5’ – AAG CAT TTC GGT CGT AGG G – 3’; IRF-7 Reverse: 5’– GAG CCC AGC ATT TTC TCT TG – 3’; DDX58 (RIG-I) Forward: 5’ – AAG CAA GGC TGA TGA GGA TG–3’; DDX58 (RIG-I) Reverse: 5’– CTC GCA ATG TTG TAC CCA AG – 3’; OAS1 Forward: 5’ – GGC TGA AGA GGC TGA TGT GT – 3’; OAS1 Reverse: 5’– ACC AAG CGT GTG TTC TTT CC – 3’; IL6 Forward: 5’ – CCA CGG CCT TCC CTA CTT C – 3’; IL6 Reverse: 5’– TTG GGA GTG GTA TCC TCT GTG A – 3’; GAPDH Forward: 5’ – GGG AAG CCC ATC ACC ATC T – 3’; GAPDH Reverse: 5’– CGG CCT CAC CCC ATT TG – 3’. PCR efficiency results were obtained from duplicate measurements of two individual cDNA samples. Experiments were performed at least twice. All data are presented as a relative quantification, based on the relative expression of target genes versus GAPDH as reference gene and analyzed using Prism 5 software.
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FIGURE LEGENDS

Fig 1. **LUBAC inhibits the IFN response downstream of MAVS.** (a) HEK 293 cells were transfected with either the NF-κB-Luc or ISRE-Luc promoter along with empty vector or LUBAC and were left untreated or challenged with SeV for 16h. Luciferase activity was analyzed at 24h post-transfection and fold activation was determined compared to empty vector; values represent the average +/- S.D. Results are representative of at least three experiments run in triplicate. (b) HEK 293 were challenged with either SeV or the following activators encoding, Mda5 (200 ng), ΔRIG-I (200 ng), MAVS (50 ng), TRIF (50 ng), TBK1 (50 ng), or IRF-3 (5D) (50 ng), along with either empty vector (-) or LUBAC (400 ng) and the ISRE–Luc reporter plasmids. Luciferase activity and analysis was performed as in (a). (c) HEK 293 were transfected with empty vector (-), GFP-Mda5, GFP-ΔRIG-I, GFP-MAVS, GFP-TBK1 or GFP-IRF-3(5D), with or without LUBAC. Whole cell lysates were subjected to immunoblot analysis and blotted with anti ISG15 (top panel), anti GFP (second panel), anti MYC (third panel), anti HA (fourth panel), or anti actin (last panel) antibodies. (d) A549 cells transfected with either siRNA control (si control) or siRNA HOIL-1L (si HOIL-1L) then left untreated or treated with SeV for 4, 8 or 12h. Immunoblot analysis was performed using either anti p-s396-IRF-3 antibody (top panel), anti IRF3 antibody (second panel), anti HOIL-1L antibody (third panel) or anti actin antibody (bottom panel). (e) HEK 293 cells were transfected with either siRNA control or siRNA against HOIL-1L. At 48h post-transfection, cells were again transfected with the ISRE-Luc promoter along with empty vector or Mda5, ΔRIG-I, MAVS, TBK1, IRF-3(5D). Luciferase activity was monitored as in (a) and (b).

Fig 2. **Linear ubiquitination of NEMO inhibits IFN induction.** (a) HEK 293 cells were transfected with empty vector (-), NEMO, NEMO-Ub1, NEMO-Ub2, NEMO-Ub4, HOIL-1L, HOIP or LUBAC along with the NF-κB-Luc reporter plasmid. Luciferase activity was analyzed as described in Fig 1a. (b,c) HEK 293 cells were challenged with either SeV or the following
activators, Mda5 (200 ng), ΔRIG-I (200 ng), MAVS (50 ng), TRIF (50 ng), TBK1 (50 ng), or IRF-3 (5D) (50 ng), along with either empty vector (-), NEMO-Ub1, NEMO-Ub2 or NEMO-Ub4 (400 ng each) and the ISRE–Luc reporter plasmids. Luciferase activity was analyzed as in (a). (d) HEK 293 cells were either treated or not with IFNα (16h); infected or not with SeV (12h); and transfected with either empty vector (-), NEMO, NEMO-Ub1 or NEMO-Ub2. Whole cell lysates were subjected to immunoblot analysis using anti ISG56 (top panel), anti ISG15 (second panel), anti RIG-I (third panel), anti FLAG (fourth panel), anti SeV (fifth panel) or anti actin (last panel) antibodies.

**Fig 3. Linear ubiquitinated NEMO interacts with TRAF3.** (a) HEK 293 cells were transfected with FLAG-tagged TRAF3 and an empty vector or S-tagged wt-NEMO (S-NEMO), S-NEMO-Ub1 or S-NEMO-Ub2. S-tagged proteins were then precipitated using the S-tag purification technique and immunoblotted using anti FLAG antibody (top panel) to reveal TRAF3 interaction. Equal pull down of NEMO was verified using anti NEMO antibody (second panel). Whole-cell lysates were subjected to immunoblot analysis using anti FLAG (third panel, TRAF3), anti NEMO (fourth panel) or anti actin (last panel) antibodies. (b) HEK 293 cells were transfected with S-NEMO, FLAG-TRAF3, S-NEMO and FLAG-TRAF3 or S-NEMO and FLAG-TRAF3 in combination with HOIL-1L and HOIP (LUBAC). S-NEMO was pulled down as in (a) and immunoblotted using anti NEMO (first panel, pull down control) or anti FLAG (second panel, TRAF3 interaction). Whole-cell lysates were subjected to immunoblot analysis using anti FLAG (third panel, TRAF3), anti NEMO (fourth panel), anti MYC (fourth panel, HOIL-1L), anti HA (fifth panel, HOIP) or anti actin (last panel) antibodies. (c) HEK 293 cells were transfected with S-NEMO, FLAG-TRAF3, S-NEMO and FLAG-TRAF3 or S-NEMO and FLAG-TRAF3 in combination with HOIL-1L alone, HOIP alone or with both (LUBAC). S-NEMO was pulled down and immunoblotted as in (b). Whole-cell lysates were subjected to immunoblot analysis using the same antibodies as in (b).

**Fig 4. Linear ubiquitinated NEMO competes with MAVS for TRAF3 binding.** (a, b) HEK 293 cells were transfected with MYC-MAVS, S-NEMO, FLAG-TRAF3, HA-HOIL-1L, MYC-HOIP in combination or alone. FLAG-TRAF3 was immunoprecipitated using an anti FLAG antibody
and subjected to immunoblot analysis using an anti MYC antibody (a) to detect MAVS interaction (top panel) or anti NEMO (b) (top panel). (a,b) FLAG antibody was used to probe for immunoprecipitated TRAF3 (second panel). (a,b) Whole-cell lysates were subjected to immunoblot analysis using anti MYC (MAVS and HOIP), anti NEMO, anti HA (HOIL-1L), anti FLAG (TRAF3) and anti actin (eight panel) antibodies. (c) HEK 293 cells were transfected with MYC-MAVS, S-NEMO, FLAG-TRAF3 and LUBAC in combination or alone. S-NEMO was precipitated and immunoblot analysis was performed using FLAG antibody to detect TRAF3 interaction (top panel). Precipitated NEMO was revealed using anti NEMO antibody (second panel). Whole-cell lysates were subjected to immunoblot analysis using anti FLAG (third panel, TRAF3), anti NEMO (fourth panel), and anti MYC (fifth panel, MAVS, and seventh panel, HOIP), anti HA (sixth panel, HOIL-1L) and anti actin (eight panel) antibodies. (d) HEK 293 cells were transfected with either S-NEMO, FLAG-TRAF3 and LUBAC with an increasing amount of MYC-MAVS. S-NEMO was precipitated and immunoblot analysis was performed using FLAG antibody to detect TRAF3 interaction (top panel). Precipitated NEMO was revealed using anti NEMO antibody (second panel). Immunoblots using anti FLAG (third panel, TRAF3), anti MYC (fourth panel, MAVS and HOIP), anti HA (fifth panel, HOIL-1L), anti NEMO (sixth panel), and anti actin (seventh panel) antibodies.

Fig 5. Increased antiviral response and decreased VSV replication in cpdm MEFs. (a) VSV-GFP infection was assessed using flow cytometry in cpdm and wt MEFs 24h following virus infection with different MOIs (0, 0.01, 0.1, 1 and 10). The histogram represents GFP expression in wt and cpdm MEFs at 10 MOI. Two-way ANOVA p<0.0001. (b) Fluorescent (top panel) and phase contrast microscopy (bottom panel) of wt and cpdm MEFs infected with VSV-GFP at 10 MOI for 24 h (magnification X20) or 48h (magnification X10). (c) Wt and cpdm MEFs were infected with VSV (10 MOI) for the indicated times. Whole-cell lysates were subjected to immunoblot analysis using anti RIG-I (top panel), anti VSV (second panel), anti SHARPIN (third panel) and anti actin (bottom panel) antibodies. (d,e) Wt and cpdm MEFs were transfected with the IFNβ, IFNα4 + IRF7, IL6 or NF-κB reporter and infected with VSV at 8h post-transfection. Luciferase activity was analyzed at 24h post-transfection and fold activation was determined compared to empty vector; values represent the average +/- S.D. (f) Wt and cpdm
MEFs infected with VSV-GFP with the indicated MOI for 24 h or 48h. Apoptosis was measured by flow cytometry by Annexin V/PI staining. Wt and cpdm MEFs were infected with VSV (10 MOI) for the indicated times. Whole-cell lysates were subjected to immunoblot analysis using anti cleaved caspase 3 (top panel), anti SHARPIN (second panel) and anti actin (bottom panel) antibodies.

**Fig 6. Linear ubiquitinated NEMO interacts with TRAF3 and dissociates MAVS-TRAF3.** (a) Wt and cpdm MEFs were infected with VSV (10 MOI) for the indicated times. Endogenous NEMO was immunoprecipitated using an anti NEMO antibody and its linear ubiquitination status as well as its ability to interact with TRAF3 was assessed using anti linear ubiquitin (top panel) and anti TRAF3 (second panel) antibodies. Equal amounts of immunoprecipitated NEMO were revealed using an anti NEMO antibody (third panel). Input amounts for NEMO, TRAF3, SHARPIN and actin are shown (last 4 panels). (b) Wt and cpdm MEFs were infected with VSV (10 MOI) for the indicated times. Endogenous TRAF3 was immunoprecipitated using an anti TRAF3 antibody and its ability to interact with NEMO was assessed using anti NEMO antibody (top panel). Equal amounts of immunoprecipitated TRAF3 were revealed using an anti TRAF3 antibody (second panel). Input for NEMO, TRAF3, SHARPIN and actin are shown (last 4 panels). (e) Wt and cpdm MEFs were infected with VSV (10 MOI) for the indicated times. Endogenous MAVS was immunoprecipitated using an anti MAVS antibody and its ability to interact with TRAF3 was assessed using anti TRAF3 antibody (top panel). Equal amounts of immunoprecipitated MAVS were revealed using an anti MAVS antibody (second panel). Input amounts for TRAF3, MAVS, SHARPIN and actin are shown (last 4 panels).

**Fig 7. Increased IFN response following virus infection in cpdm MEFs.** (a,c) qPCR analysis of total RNA isolated from wt and cpdm MEF cells. Relative fold expression levels of IRF7, DDX58 (RIG-I), OAS1 (a) and IL6 (c) versus GAPDH mRNA are shown. Data is representative of at least two experiments run in duplicate. (b,d) ELISA of IFNa, IFNβ (b) and IL6 (d) in the supernatant of wt and cpdm MEFs infected with VSV at 10 MOI for 48h. Data is representative of two experiments run with three individual samples. (e) A schematic of the inhibition of RIG-I signaling by linear ubiquitinated NEMO-mediated dissociation of the MAVS-TRAF3 complex.
Figure 1
Figure 2
Figure 4
Figure 6
Figure 7