A non-canonical role of the p97 complex in RIG-I antiviral signaling

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Abstract

RIG-I is a well-studied sensor of viral RNA that plays a key role in innate immunity. p97 regulates a variety of cellular events such as protein quality control, membrane reassembly, DNA repair, and the cell cycle. Here, we report a new role for p97 with Npl4-Ufd1 as its cofactor in reducing antiviral innate immune responses by facilitating proteasomal degradation of RIG-I. The p97 complex is able to directly bind both non-ubiquitinated RIG-I and the E3 ligase RNF125, promoting K48-linked ubiquitination of RIG-I at residue K181. Viral infection significantly strengthens the interaction between RIG-I and the p97 complex by a conformational change of RIG-I that exposes the CARDs and through K63-linked ubiquitination of these CARDs. Disruption of the p97 complex enhances RIG-I antiviral signaling. Consistently, administration of compounds targeting p97 ATPase activity was shown to inhibit viral replication and protect mice from vesicular stomatitis virus (VSV) infection. Overall, our study uncovered a previously unrecognized role for the p97 complex in protein ubiquitination and revealed the p97 complex as a potential drug target in antiviral therapy.

Keywords antiviral signaling; mechanism; p97-Npl4; RIG-I; ubiquitination

Subject Categories Immunology; Post-translational Modifications, Proteolysis & Proteomics

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Introduction

Viral infections pose increasing threats to public health throughout the world. Innate immunity serves as the first line of defense in the response against the pathogenic invasion of hosts by viruses. Such responses are usually initiated by the detection of viral nucleic acid via pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) in the endosome and RIG-I-like receptors (RLRs) in the cytoplasm (Kawai & Akira, 2010; Takeuchi & Akira, 2010; Barbalat et al., 2011). TLR family members TLR3, 7, 8, and 9 detect endosomal DNA and RNA viruses, while RIG-I, MDA5, and LGP2 of the RLR family are the sensors specific for cytoplasmic RNA viruses (Nakhaei et al., 2009; O’Neill & Bowie, 2010; Kato et al., 2011). RIG-I and MDA5 are made up of two tandem caspase activation and recruitment domains (CARDs) at the N-terminus, a central RNA helicase domain containing a conserved DExD/H box, and a carboxyl-terminal domain (CTD) (Kolakofsky et al., 2012). The CARDs are required for the interaction of RIG-I (or of MDA5) with its adaptor protein MAVS (also known as IPS-1/CARDIF/VISA), which resides in the mitochondrial outer membrane (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). The association of MAVS with RIG-I as mediated by CARDs results in the activation of the downstream signaling cascades and the eventual production of pro-inflammatory cytokines and type I interferons (Kawai & Akira, 2008). Unlike RIG-I and MDA5, LGP2 lacks N-terminal CARDs and thus fails to induce an antiviral immune response by itself (Onoguchi et al., 2011). Instead, LGP2 positively regulates RIG-I- and MDA5-mediated antiviral responses (Satoh et al., 2010).

Accurate regulation of innate antiviral immunity ensures that the spread of virus in the host is restricted, and at the same time that the development of autoimmune and/or inflammatory diseases caused by excessive interferon production is prevented. The ubiquitin system has been demonstrated to play a crucial role in precisely controlling RLR-mediated signal transduction (Maelfait & Beyaert, 2012; Oshiumi et al., 2012). Activation of RIG-I-mediated antiviral signaling starts with the ubiquitination of RIG-I. Infection by an RNA virus results in a conformational rearrangement of RIG-I that exposes its N-terminal CARDs to solvent (Jiang et al., 2011; Kowalsinski et al., 2011; Luo et al., 2011); this exposure leads to the recruitment of the E3 ligases TRIM25 (Gack et al., 2007) and Riplet (Gao et al., 2009; Oshiumi et al., 2009), and these two ligases contribute to the conjugation of K63-linked polyubiquitin chains onto RIG-I. Additionally, unanchored K63-linked polyubiquitin chains also bind to the CARDs (Zeng et al., 2010). Both covalently linked and non-covalently bound K63-polyubiquitin chains appear to promote RIG-I antiviral activity. MAVS then recruits a series of E3 ligases essential for the activation of the downstream signaling events. Specifically,
auto-ubiquitination of TRAF6 with K63-linked polyubiquitin chains leads to the recruitment of the TAK1 complex and the subsequent activation of the IKK complex (Xu et al., 2005; Yoshida et al., 2008; Konno et al., 2009), which eventually cause nuclear translocation of the transcription factor NF-kB to activate pro-inflammatory genes (Konno et al., 2009; Liu & Chen, 2011; Maelfait & Beyaert, 2012). In parallel, K63 auto-ubiquitination of TRAF3 causes the activation of TBK1 and IKKε, which in turn promote the phosphorylation, dimerization, and nuclear translocation of another transcription factor, IRF3, resulting in the expression of type I interferons (Oganesyan et al., 2006; Saha et al., 2006; Paz et al., 2011).

Proteasomal degradation mediated by K48-linked ubiquitination is involved in the attenuation of RLR signal transduction to terminate sustained immune responses. RNF125, which is characterized as a RING-type E3, suppresses antiviral immune response by inducing the degradation of RIG-I, MDA5, and MAVS (Arimoto et al., 2007). Other E3s, those that do not directly associate with their substrates, depend on adaptor proteins to be recruited to their specific substrates. Siglec-G, which is upregulated upon RNA virus infection, interacts with E3 ligase c-Cbl and RIG-I via SHP2, facilitating c-Cbl-mediated K48-linked ubiquitination of RIG-I prior to proteasomal degradation (Chen et al., 2013). PCBP2 acts as a negative regulator of RLR signaling, sequestering the HECT domain-containing E3 ligase AIP4 to MAVS and catalyzing the conjugation of K48-linked polyubiquitin to MAVS for degradation (You et al., 2009). In addition, Ndfip1 has been reported to enhance MAVS degradation mediated by Smurf-1, which is another member of the HECT E3 ligase family (Wang et al., 2012).

p97 (also known as VCP/CDC48) is a member of the type II AAA (ATPase associated with various activities) ATPase family and has been found to participate in a wide range of independent cellular processes including ER- and mitochondria-associated degradation (Ye et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002; Heo et al., 2010; Tanaka et al., 2010; Xu et al., 2011), autophagy (Ju et al., 2009; Vesa et al., 2009; Krick et al., 2010; Tresse et al., 2010), membrane reassembly (Latterich et al., 1995; Kondo et al., 1997; Hetzer et al., 2001; Ramirez et al., 2007), protein aggregation (Higashiyama et al., 2002; Yamanaka et al., 2004; Kobayashi et al., 2007; Song et al., 2007; Nishikori et al., 2008), DNA repair (Partridge et al., 2003; Indig et al., 2004), cell cycle progression (Cao et al., 2003; Fu et al., 2003; Ramirez et al., 2007; Mouysset et al., 2008), sex determination (Sasagawa et al., 2009), and neutralization of virus (Hauler et al., 2012). Many studies have shown that p97 is recruited, with the help of cofactors including Npl4, Ufd1, p47, UBXD7, and FAF1, to ubiquitinated substrates (Kondo et al., 1997; Ye et al., 2001; Song et al., 2005; Alexandru et al., 2008; Meyer et al., 2012; Yamanaka et al., 2012)—and through its segregase activity, p97 extracts its target proteins from their cellular environments mostly for proteasomal degradation. However, Alexandru and colleagues suggested that p97 may be also involved in the ubiquitination of its substrates (Alexandru et al., 2008), indicating that the mechanisms by which p97 targets its substrates for degradation are probably more complicated than previously thought. So far, the exact mechanism by which the p97 complex facilitates ubiquitination of its substrates remains unclear, and it is also unknown whether the p97 complex could bind directly to the non-ubiquitinated substrates.

Previously, we studied the mechanism by which RIG-I signaling is activated (Feng et al., 2013). In the current work, we identified a previously unknown role of the p97-Npl4 complex in antiviral response, in which the complex was shown to recruit recruits RNF125 to promote K48-polyubiquitination and degradation of RIG-I. Notably, knockdown of p97/Npl4 resulted in concurrent decrease of RNF125-mediated ubiquitination of RIG-I, instead of accumulation of ubiquitinated substrates as was previously expected. In addition to its classic function of binding ubiquitinated substrates, p97-Npl4 was shown also to recognize non-ubiquitinated substrates and simultaneously recruit E3 ligase in the process of substrate ubiquitination. Upon viral infection, the interaction between RIG-I and p97-Npl4 was substantially enhanced by the following: (i) a conformational change that exposes the CARDs of RIG-I; and (ii) K63-linked ubiquitination of the CARDs. Meanwhile, administering p97 inhibitors to mice protected them from VSV infection, which further suggests that p97-Npl4 should be considered as a promising target for antiviral therapy. Considering the potential for inhibitors of p97 in treating human cancer and the significance of antiviral treatments in cancer patients (Wang et al., 2010; Magnaghi et al., 2013), our findings may also provide a mechanistic basis for using p97 inhibitors as both anticancer and antiviral agents.

### Results

**p97 negatively regulates type I interferon signaling in an ATPase-dependent manner**

How the viral RNA sensor RIG-I is activated has been extensively studied. The mechanism by which RIG-I antiviral signaling is turned off remains, however, only partially understood. In this work, we set out to investigate such mechanism by performing mass spectrometry (MS) assays using RIG-I as bait. Our MS analysis indicated p97 to be a potential interaction partner of RIG-I (data not shown). Since p97 is important for the degradation of various proteins, we hypothesized that p97 may also play a role in the control of RIG-I protein turnover and thus antiviral signaling.

To determine whether p97 indeed plays a role in type I interferon response and antiviral immunity, we transfected human embryonic kidney (HEK293T) cells with interferon beta (IFNβ)-containing, IFNβ-positive regulation domain PRDII and PRDII (PRDII-I)-containing, and interferon-stimulated response element (ISRE)-containing promoters, as well as p97-specific siRNA targeting its off remains, however, only partially understood. In this work, we set out to investigate such mechanism by performing mass spectrometry (MS) assays using RIG-I as bait. Our MS analysis indicated p97 to be a potential interaction partner of RIG-I (data not shown). Since p97 is important for the degradation of various proteins, we hypothesized that p97 may also play a role in the control of RIG-I protein turnover and thus antiviral signaling.

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To further demonstrate a functional correlation between p97 and antiviral immunity, we knocked down p97 in MEF cells and then infected the cells with VSV-GFP at a multiplicity of infection of 2.
Knockdown of p97 rendered the cells resistant to viral infection, resulting in obviously fewer GFP-positive (virus-infected) cells than those treated with control siRNA (n.c.) (Fig 1E). Consistent with these results, flow cytometry showed that relatively few (i.e. only 9%) MEF cells that were transfected with p97-specific siRNA became infected with VSV-GFP, while the vast majority (i.e. 93%) of the MEF cells transfected with control siRNA were observed to be positive for GFP (Fig 1F). Together, these observations indicate that p97 is a negative regulator of antiviral immunity.

Next, we performed a rescue assay to verify the regulatory function of p97 in type I interferon signaling, as well as to address whether such a function depends on its ATPase activity. In cells transfected with p97-specific siRNA targeting its UTR sequence, expression of wild-type (WT) p97 restored the inhibitory effect of p97 on SeV-induced IFNβ luciferase activities and IFNB transcription (Fig 1G and H; Appendix Fig S1B). In contrast, the mutant p97QQ (Ye et al, 2003), whose ATPase activity is defective, failed to rescue such inhibitory effects. Together, these results confirmed the inhibitory effect of p97 on type I interferon signaling, and demonstrated that such inhibition is dependent on its ATPase activity.

Npl4-Ufd1 is the cofactor of p97 in antiviral immune response

Since multiple cofactors of p97 have been identified to control its differential functions in various cellular processes, we then asked which specific cofactors are required for its negative regulation of antiviral signaling. For this purpose, we measured the luciferase activity of IFNβ and the transcription of IFNB in SeV-infected HEK293T cells after knocking down various p97 cofactors including Npl4, Ufd1, p47, and FAF1 (Fig 2A and B; Appendix Fig S2A–D). Depletion of Npl4, but not p47 or FAF1, markedly enhanced IFNβ transactivity and IFNB transcription, as did p97 knockdown.
addition, knockdown of Ufd1, which constitutively interacts with Npl4 to form a stable complex, also significantly increased type I interferon response to SeV infection. Together, these results suggest that Npl4-Ufd1 is the cofactor of p97 during its regulation of anti-viral signaling.

To verify the above observations and further examine the regulatory effect of Npl4 in antiviral signaling, we then transfected the SeV-infected cells with increasing amounts of Npl4. As shown in Fig 2C, Npl4 potently inhibited SeV-induced activation of IFNβ- and ISRE-luciferase reporters in a dose-dependent manner. Consistent with these results, cells overexpressing Npl4 also showed substantially decreased mRNA levels of IFNB and ISG56 in response to SeV infection (Fig 2D). Similar results were obtained when the cells were transfected with poly(I:C) (Appendix Fig S2E and F), indicating that Npl4 inhibits RNA/virus-induced type I interferon response. To further confirm these observations, we silenced endogenous Npl4

Figure 2. Npl4 cooperates with p97 to suppress IFNβ antiviral response. A, B Transactivity of IFNβ promoter (A) and transcription of IFNB (B) in SeV-infected cells after transfection with indicated siRNA. C, D Luciferase activities of IFNβ (left) and ISRE (right) promoters (C) and mRNA levels of IFNB (left) and ISG56 (right) (D) in SeV-infected cells transfected with various doses of Npl4. E, F Luciferase (E) and real-time PCR (F) analyses in Npl4-depleted cells upon SeV treatment. G Rescue assay in Npl4-knockdown MEFs after infection with VSV-GFP. H IFNB transactivation and IFNB transcription in sip97 cells transfected with the indicated plasmids.

Data information: Error bars represent SD of data obtained in three independent experiments. One-way analysis of variance (ANOVA) and Student’s t-test were used. n.s., no significant difference; *P < 0.05; **P < 0.01; ***P < 0.001 in comparison with control group. e.v., empty vector. Other abbreviations as in Fig 1. Wedge represents increasing amounts of plasmids.

See also Appendix Fig S2.
using a mixture of three individual siRNAs and then examined the activation of the IFNB and ISRE promoters. Knockdown of Npl4 led to a greater activation of IFNB-, PRDIIB1-N, and ISRE-luciferase reporters when compared with the control group (Fig 2E). Accordingly, the transcription of IFNB, RANTES, and ISG56 was promoted in Npl4-knockdown cells infected with SeV for different amounts of time (Fig 2F). Such inhibitory effects of Npl4 were also observed in cells transfected with poly(I:C) (Appendix Fig S2G and H). A subsequent rescue study showed that depletion of endogenous Npl4 by siRNA targeting its UTR sequence rendered the cells resistant to VSV infection, while back-transfection of Npl4 induced the cells to be more susceptible to VSV infection (Fig 2G). Together, these data indicate that Npl4, similar to p97, is a negative regulator of type I interferon signaling and antiviral immunity.

We next investigated whether Npl4 indeed acts as the cofactor of p97 during its negative regulation of antiviral signaling. For this purpose, we first knocked down p97 and then transfected cells with Npl4 in combination with wild-type p97 or its ATPase-defective QQ mutant. Overexpression of Npl4 alone in p97-knockdown cells failed to inhibit SeV-induced IFNB reporter activation and IFNB transcription, whereas co-transfection of Npl4 with wild-type p97 but not its QQ mutant efficiently suppressed IFNB transactivation and ISRE transcription (Fig 2H). Together with the above observations, these results indicate that the inhibitory role of Npl4 on type I interferon signaling is dependent on p97; that is, Npl4 per se acts as the cofactor of p97 during this process. Hereinafter, we mainly focused our efforts on p97 and Npl4.

Structure determination and mutational studies of the p97-Npl4 complex

To assess the atomic interactions between p97 and Npl4 in antiviral signaling, we performed structural and structure-directed mutational studies. Npl4 comprises four domains: the N-terminal UBD (amino acids 1–80) essential for interaction with p97, the central zf-Npl4 domain (amino acids 104–246) and Npl4 domain (amino acids 248–557), and the C-terminal NSF domain (amino acids 580–608) responsible for binding ubiquitin (Ye et al, 2003; Alam et al, 2004; Isaacscon et al, 2007; Lass et al, 2008). In order to evaluate the functional importance of individual domains of Npl4, we generated a series of Npl4 truncation constructs: ΔNZF (amino acids 1–579), ΔUBD (amino acids 81–608), and the UBD domain alone (Figs 3A and EV1A). Compared with the wild-type Npl4, deletion of the UBD domain abolished its inhibitory effect on SeV-induced IFNB activation and IFNB transcription (Fig EV1B), indicating that association of Npl4 with p97 is essential for the regulation of antiviral response.

Subsequently, the crystal structure of the drosophila Ter94 (p97) N-terminal domain in complex with the drosophila Npl4 (dNpl4) UBD was determined by the single-wavelength anomalous diffraction method to 2.0 Å resolution with a crystallographic R factor of 20.82% and a free R factor of 25.13% (Figs 3B and EV1C; Appendix Table S1). Similar to other adaptors such as p47 and FAF1, the dNpl4 UBD binds to the hydrophobic groove formed by the two N-terminal subdomains (Nn and Nc) of Ter94 (Fig 3B). The overall structures of Ter94 and dNpl4 in the complex are similar to those previously determined individually (Huyton et al, 2003; Dreveny et al, 2004; Isaacscon et al, 2007; Hanzelmann & Schindelin, 2011; Hanzelmann et al, 2011). A total of 1082 Å² of the molecular surface was buried upon complex formation between dNpl4 and Ter94. The heterodimeric interface between Ter94 and dNpl4 is primarily composed of two sites termed Site 1 and Site 2 (Fig 3B). At Site 1, residues L7, R9, and R18, which are located in the β1 and β2 strands of dNpl4, form hydrophobic interactions with residue F49 from the Nn domain of Ter94. Meanwhile, hydrogen bonding and electrostatic interactions were formed between residues R9, R18, and H69 of dNpl4 and R50, D52, and Q47 of Ter94. For Site 2, residues A13 and R49 of dNpl4 were observed to interact with residues Y107 and Y140 from the Nc domain of Ter94 through hydrophobic packing, while residues A13, Q11, and R49 of dNpl4 formed hydrogen bonding and electrostatic interactions with Y107 of Ter94. In addition, R49 of dNpl4 was observed to pair with Y140 of Ter94 via a cation–π interaction. It is worth noting that all these interface residues are highly conserved between drosophila and human (Fig EV1C and D), supporting our rationale of using Ter94-dNpl4 as a substitute for p97-Npl4 in the structural analysis of this complex.

Several structures of p97 in complex with adaptors including p47, FAF1, and OTU1, were previously reported (Dreveny et al, 2004; Hanzelmann et al, 2011). Comparing these structures revealed an overall conserved fashion through which all adaptors bind to the same hydrophobic groove of p97 (Fig EV1E). However, the detailed relative positioning between p97 and its adaptors varies. It is helpful in this regard to refer to the structure of the Npl4 UBD, which was determined by nuclear magnetic resonance (NMR), and to refer to its interaction with p97, which was modeled with experimental probes (Isacscon et al, 2007). Compared with this NMR structure of Npl4, the crystal structure of dNpl4 determined here revealed a similar overall fold yet with significant conformational differences, especially in loops α1/β3 and β3/α4 (Fig EV1F and G). More importantly, the relative orientations between dNpl4/Npl4 and Ter94/p97 were observed to differ considerably when comparing the Ter94-dNpl4 complex structure with the previously modeled structure of p97-Npl4 (Fig EV1G). It is also noteworthy that residues Val15, Leu74, and Phe76, which were predicted to be critical for complex formation by the previous modeling, are neither evolutionarily conserved nor present in the interface of the current structure of the Ter94-dNpl4 complex. On the other hand, interface residues critical for Ter94 interaction with dNpl4 are strictly conserved across diverse species (see below). Together, these observations indicate the robust reliability of our structural analysis of the p97-Npl4 complex.

Next, we performed structure-directed mutational studies in a context of full-length human p97 and Npl4 to further evaluate the importance of individual interface residues for complex formation. Pulldown assays using purified recombinant proteins of wild-type and mutant (substitution of wild-type residue with alanine) p97 and Npl4 showed that residues of p97 engaging in either hydrophobic interactions (F52, Y110) or hydrogen bonding and electrostatic interactions (D55, Y110) are essential for Npl4 binding (Fig 3C and D). Meanwhile, mutation of the single residue R50 or multiple sites at I6, R8, R17, and R50 of Npl4 abolished its binding to p97 (Fig 3C and E). These observations were further confirmed by biolayer interferometry (Octet Red 96) using purified proteins in vitro (Fig EV1H and I), as well as a co-immunoprecipitation (co-IP) assay in cells (Fig 3F and G). Therefore, we chose hereinafter F52A/D55A/Y110A of p97 (termed 3A) and I6A/R8A/R17A/R50A of Npl4 (termed 4A) as representative mutants that entirely disrupt the p97-Npl4 complex.
Finally, we transfected HEK293T cells with wild-type or mutant p97 and Npl4. Upon SeV infection, overexpression of wild-type p97 but not its 3A mutant in cells depleted of endogenous p97 inhibited IFNβ transactivation and IFNB transcription (Fig 3H). Meanwhile, transfection of wild-type Npl4 but not its 4A mutant suppressed IFNβ promoter activation and IFNB transcription in cells after SeV infection for different durations of time (Fig 3I). Taken together, these results further validate our structural analysis and indicate that direct interaction between p97 and Npl4 to form a complex is essential for their suppressive role in antiviral signaling.

The p97-Npl4 complex promotes degradation of RIG-I

Given that p97 is an AAA ATPase relevant to the control of protein stability, we surmised that p97-Npl4 might function by targeting components of RLR-mediated antiviral signaling such as the RIG-I pathway for proteasome degradation. To test this possibility, we first determined the protein levels of RIG-I, MAVS, and IRF3 in cells transfected with short hairpin RNA (shRNA) interfering the transcription of p97 (Fig 4A). Knockdown of p97 markedly increased poly(I:C)-induced RIG-I expression when compared with scramble.
The protein level of MAVS was also increased, while the total level of IRF3 protein remained unchanged. Consistent with the increased level of RIG-I and MAVS, the phosphorylation of IRF3 was obviously enhanced (Fig 4A). In addition, overexpression of Npl4 significantly reduced poly(I:C)-induced expression of RIG-I and MAVS when compared with the control, leading to decreased phosphorylation of IRF3 (Fig 4B). Moreover, depletion of Npl4 significantly increased the poly(I:C)-induced expression of the proteins RIG-I and MAVS with enhanced IRF3 phosphorylation, as was found for the effect of knocking down p97, but did not affect the protein levels of TBK1, IKKε, and IRF3, suggesting that p97-Npl4 specifically targets the proteins RIG-I and MAVS (Fig 4C). Meanwhile, a similar effect was observed for depletion of Ufd1, while knockdown of p47, another p97 cofactor, did not cause such an effect, again indicating that Npl4-Ufd1, and specifically Npl4-Ufd1, is required for p97 regulation of RIG-I antiviral signaling (Fig EV2A and B).

Next, we examined whether regulation of RIG-I antiviral signaling by p97-Npl4 is dependent on proteasomal degradation. A real-time PCR assay showed that overexpression of Npl4 in cells transfected with poly(I:C) did not affect the mRNA levels of RIG-I (Fig EV2C). Furthermore, RIG-I protein level reduction mediated by p97 together with Npl4 was largely blocked when cells were treated with MG132, a proteasome inhibitor (Fig 4D and E), suggesting that p97-Npl4 regulates the RIG-I protein level through proteasome degradation. Subsequently, we analyzed the potential regulatory role of p97-Npl4 on K48-linked polyubiquitination of RIG-I.

The p97-Npl4 complex is required for K48 ubiquitination of RIG-I

As mentioned before, a mechanism commonly proposed to explain how p97 functions is that, with the help of various cofactors, p97 is recruited to the ubiquitinated substrates and targets them for proteasome degradation. Thus, depletion of p97 and its cofactors would lead to an accumulation of ubiquitinated substrates (Ballaré et al., 2006; Alexandru et al., 2008; Meerang et al., 2011; Raman et al., 2011; Kim et al., 2013; Chan et al., 2014; Li et al., 2014). To examine the potential effect of p97-Npl4 on K48-linked polyubiquitination of RIG-I, we transfected Flag-tagged RIG-I into HEK293T cells with wild-type p97/Npl4 or their mutants that disrupt complex formation. Unexpectedly, co-transfection of Flag-RIG-I with wild-type p97, but not of its 3A mutant defective in binding Npl4, in p97-depleted cells led to accumulation of K48-linked RIG-I polyubiquitination in these cells (Figs 4F and EV2D). Similarly, overexpression of wild-type Npl4 enhanced K48-linked polyubiquitination of RIG-I compared to overexpression of its 4A mutant, which is unable to bind p97 (Figs 4G and EV2E). Consistent with these results, knockdown of either p97 or Npl4 substantially decreased K48-linked ubiquitination of RIG-I (Fig 4H and I). Taken together, these results indicate that p97-Npl4 suppresses antiviral response by promoting K48-linked polyubiquitination and proteasomal degradation of RIG-I, and this process depends on the direct interaction between p97 and Npl4.

**RNA25 is the E3 ligase required for p97-Npl4-mediated RIG-I turnover**

To explore the underlying mechanism of p97-Npl4-related degradation of RIG-I, we set out to identify the potential E3 ligase responsible for RIG-I turnover during this process. So far, two E3 ligases, RNF125 and c-Cbl, have been identified to mediate K48-linked ubiquitination of RIG-I (Arimoto et al., 2007; Chen et al., 2013). To assess these two ligases, we first designed two groups of shRNAs targeting RNF125 and c-Cbl respectively. The knockdown efficiency of each of these shRNAs was validated by real-time PCR analysis (Fig EV3A). According to the results of a dual-luciferase reporter assay, depletion of endogenous RNF125 abrogated the ability of Npl4 to downregulate IFNβ promoter activity induced by SeV infection, whereas knockdown of c-Cbl had no such effect (Fig 5A), suggesting that the inhibition of RIG-I antiviral signaling by Npl4 is dependent on RNF125. Similarly, Npl4 failed to suppress IFNβ transactivation triggered by overexpression of RIG-I CARDs in cells depleted of RNF125, but not c-Cbl (Fig 5B). Consistent with these observations, overexpression of Npl4 significantly decreased poly(I:C)-induced accumulation of RIG-I protein, while depletion of endogenous RNF125 impaired such an effect of Npl4 (Fig 5C).

Together, these findings suggest that RNF125 is specifically required for p97-Npl4-mediated inhibition of RIG-I antiviral signaling.

We next examined the ubiquitination levels of RIG-I in cells transfected with Npl4 and/or RNF125. Consistent with the aforementioned results, overexpression of Npl4 significantly increased K48-linked ubiquitination of RIG-I, while co-expression of Npl4 with RNF125 further enhanced this effect (Fig 5D), indicating that Npl4 acts synergistically with RNF125 to facilitate K48-linked ubiquitination of RIG-I. Consistently, Npl4 could no longer increase K48-linked ubiquitination of RIG-I when endogenous RNF125 was depleted (Fig EV3B). These observations were further confirmed by a QPCR assay showing that co-transfection of Npl4 and RNF125 suppressed IFNβ transcription to a larger extent than did Npl4 or RNF125 alone (Fig 5E). Taken together, these results indicate that p97-Npl4 inhibits RIG-I-related type I interferon response by promoting RNF125-mediated K48-linked ubiquitination of RIG-I.
Figure 4.

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The EMBO Journal

p97 regulates K48 ubiquitination of RIG-I Qian Hao et al

2910
Figure 5. RNF125 is essential for p97-Npl4 inhibition of RIG-I signaling.

A Luciferase activity detected in HEK293T cells transfected with an IFNβ luciferase reporter, plus scramble, shRNF125, or shc-Cbl, and increasing doses of Npl4 after SeV infection.
B Luciferase analysis of IFNβ promoter activity in cells transfected with the tandem CARDs of RIG-I, plus scramble, shRNF125, or shc-Cbl, and increasing doses of Npl4.
C Protein levels of RIG-I in shRNF125 cells with or without transfection of Npl4.
D Ubiquitination of RIG-I in cells transfected with Npl4 in combination with RNF125 after being challenged with SeV.
E mRNA levels of IFNB in SeV-infected cells after transfection with Npl4 or RNF125 or both.
F Mass spectrometric analysis of ubiquitination sites of RIG-I after co-transfection with RNF125 and HA-K48Ub.
G Immunoblotting analysis of RIG-I in cells transfected with wild-type or mutant RIG-I and Npl4 or RNF125.
H Ubiquitination of wild-type or mutant RIG-I in cells transfected with Npl4 and RNF125.
I IFNβ transactivity in SeV-infected cells transfected with wild-type or mutant RIG-I in combination with Npl4 and RNF125.
J Fluorescence microscopy (GFP) of VSV-GFP-infected cells after transfection with shNpl4 and shRNF125, as well as wild-type RIG-I and its mutants.

Data information: Error bars represent SD of data obtained in three independent experiments. One-way analysis of variance (ANOVA) and Student's t-test were used. 

* P < 0.05; ** P < 0.01; *** P < 0.001 in comparison with control group. For abbreviations, see Figs 1 and 2.

See also Fig EV3.

Source data are available online for this figure.
K181 is the key site for RNF125-p97-Npl4-mediated ubiquitination of RIG-I

While a previous study identified RNF125 as an E3 ligase that contributes to K48-linked ubiquitination of RIG-I (Arimoto et al., 2007), its specific target site remains unknown. To determine the RNF125-mediated ubiquitination site(s) on RIG-I, we carried out a mass spectrometry analysis with and without overexpression of RNF125. Previously, c-Rel was reported to catalyze K48-linked ubiquitination of RIG-I on lysine 812 (Chen et al., 2013). We thus used K812 of RIG-I as a control in our study. MS analysis revealed K181, but not K812, as a candidate site for K48-linked ubiquitination of RIG-I catalyzed by RNF125 (Fig 6F). To verify this result, wild-type and mutant RIG-I were each co-transfected with Npl4 or RNF125 in SeV-infected cells. An immunoblotting assay showed that overexpression of Npl4 or RNF125 in SeV-infected cells significantly downregulated the protein levels of wild-type RIG-I as well as of its K812R mutant, but not that of the RIG-I K181R mutant (Fig 5G), indicating that K181 of RIG-I is a major site targeted by RNF125 and p97-Npl4 for regulation of RIG-I-related antiviral signaling. Moreover, Npl4 could not enhance K48-linked ubiquitination of the RIG-I K181R mutant as it does on wild-type RIG-I (Figs 5H and EV3C). Consistent with these observations, inclusion of RNF125 and Npl4 together significantly inhibited IFNβ promoter activation induced by wild-type RIG-I or its K812R mutant, but failed to inhibit that induced by the K181R mutant of RIG-I (Fig 5I).

Next, the antiviral effects of wild-type RIG-I and its K181R mutant were each monitored upon depletion of Npl4 and RNF125. When compared with scramble, knockdown of Npl4 and RNF125 further inhibited VSV infection (MOI, 20) in cells transfected with wild-type RIG-I, but failed to do so in cells transfected with the K181R mutant of RIG-I, again indicating that K181 per se is the targeted site (Fig 5J). We then examined the local structural environment of K181 in RIG-I and found this site to be in the vicinity of K172, a major site for K63-linked ubiquitination critical for RIG-I activation; it was also observed to be near T170, a phosphorylation site implicated in negative regulation of RIG-I signaling (Fig EV3D). Taken together, these results strongly indicate that K181 of RIG-I is the key site for RNF125/p97-Npl4-mediated RIG-I ubiquitination, and suggest that K48-linked ubiquitination at K181 could be regulated by posttranslational modifications of K172 and K170 (see more below).

The p97-Npl4 complex directly interacts with both RIG-I and RNF125

To further dissect the mechanism by which p97-Npl4 mediates inhibition of RIG-I antiviral signaling, we sought to determine whether the p97-Npl4 complex physically associates with RIG-I and with RNF125. A co-IP assay in HEK293T cells showed that endogenous RIG-I interacted with endogenous Npl4, and such interaction was substantially increased upon being infected with SeV (Fig 6A). Meanwhile, the previously reported association of RIG-I with RNF125 (Arimoto et al., 2007) was also detected. Consistent with the above results, confocal microscopy showed enhanced colocalization of endogenous Npl4 and RIG-I upon SeV infection (Fig 6B). Furthermore, an in vitro pulldown assay using purified recombinant proteins of Npl4 and RIG-I indicates that the interaction between Npl4 and RIG-I is a direct one (Fig 6C).

Next, we mapped the specific domains of RIG-I and Npl4 mediating their association. Three truncated forms of RIG-I were generated and co-expressed with full-length Npl4 in HEK293T cells. A co-IP assay showed that the portion of RIG-I containing the CARDs (amino acids 1-238) interacts with Npl4 as indicated by the band next to the red asterisk in Fig 6D, whereas the helicase and carboxyl-terminal domains of RIG-I were not involved in this interaction (Fig 6D). In addition, two deletion mutants of Npl4 were constructed and co-expressed with full-length RIG-I. The resulting co-IP assay showed that deletion of the NZF domain completely disrupted the interaction of Npl4 with RIG-I, whereas deletion of the UBD did not affect this interaction (Figs 6E and EV1A), suggesting that the NZF domain is essential for association between Npl4 and RIG-I. These observations were further confirmed by an in vitro pulldown assay showing a direct interaction between Npl4 and the CARDs of RIG-I (Fig EV4A).

Finally, we sought to determine whether RNF125 interacts with p97. An endogenous IP assay showed that RNF125 could not only associate with RIG-I, but also interact with p97 (Fig 6F). Subsequently, we used purified recombinant proteins to further assess the interactions among p97-Npl4, RIG-I, and RNF125. p97 was readily pulled down by RNF125 but not by an MBP control, indicating a direct interaction between p97 and RNF125 (Fig 6G). Furthermore, RNF125 could directly pull down RIG-I; and the p97-Npl4 complex further promoted the interaction between RNF125 and RIG-I (Fig 6H). Together, these observations suggest that the p97-Npl4 complex physically associates with both RIG-I and RNF125 and that Npl4 directly interacts with the CARDs even without ubiquitination of RIG-I.

RIG-I interaction with Npl4 is enhanced by exposure and K63 ubiquitination of the CARDs

Since Npl4 could directly bind to non-ubiquitinated RIG-I CARDs, while SeV greatly enhanced the binding of Npl4 to RIG-I in cells, we then investigated how such an interaction is regulated upon viral infection. Given that binding of dsRNA triggers a conformational change of RIG-I that results in its oligomerization and the exposure of the CARDs, we first evaluated whether dsRNA could enhance Npl4 interaction with non-ubiquitinated RIG-I. To this end, we performed an in vitro pulldown assay using purified recombinant proteins of Npl4 and RIG-I and synthesized dsRNA that binds to RIG-I and induces its dimerization (Feng et al., 2013). The interaction between Npl4 and RIG-I was obviously increased when dsRNA and ATP were added (Fig 6I). This result suggests that a viral infection-triggered conformational change of RIG-I could facilitate its interaction with Npl4 by exposing the CARDs.

Considering the key role of K63-linked polyubiquitin in RIG-I antiviral signaling, we next studied the potential function of K63-linked ubiquitination in regulating the interaction of Npl4 with RIG-I CARDs. It is well established that the E3 ubiquitin ligase TRIM25 activates RIG-I by generation of K63-linked polyubiquitin chains covalently attached to K172 of RIG-I (Gack et al., 2007). Meanwhile, separated K63-linked polyubiquitin chains can bind to RIG-I non-covalently for synergistic activation (Zeng et al., 2010). Furthermore, T170 was found constitutively phosphorylated under physiological conditions, preventing RIG-I from binding to TRIM25 and MAVS (Gack et al., 2010). Moreover, phosphorylation of T170 not only...
Figure 6. p97-Npl4 directly interacts with both RIG-I and RNF125.

A  Endogenous co-IP assays for Npl4 and RIG-I with or without SeV infection.
B  Colocalization of Npl4 and RIG-I with or without SeV infection.
C  In vitro pulldown assay to assess the direct interaction between Npl4 and RIG-I.
D, E Domain mapping of Npl4 and RIG-I interaction by co-IP experiment. Red asterisk in panel D marks the position of RIG-I (1-238).
F  Endogenous co-IP assay for p97 and RNF125 with or without SeV infection.
G  In vitro pulldown assay to assess the direct interaction between p97 and RNF125.
H  In vitro pulldown assay to assess the interaction between RIG-I and RNF125 with or without the p97-Npl4 complex.
I  Pulldown assay to assess the effect of dsRNA/ATP on direct interaction between RIG-I and Npl4.
J  Co-IP assay for Npl4 with RIG-I-CARDs and its mutants.

See also Fig EV4.

Source data are available online for this figure.
impaired ubiquitination of RIG-I at K172, but also abolished the non-covalent binding between RIG-I and K63-linked polyubiquitin chains. Based on these studies, we constructed two RIG-I-CARDs mutants (K172R and T170E/K172R) to evaluate the possible effect of K63-linked ubiquitination on the interaction between RIG-I and Npl4.

We then transfected cells with wild-type or mutant RIG-I-CARDs, together with Npl4. When compared with wild-type RIG-I-CARDs, the K172R mutant that blocks K63-linked ubiquitination of RIG-I showed significantly decreased binding to Npl4 (Fig 6J). Similarly, the double mutation T170E/K172R that blocks both covalent ubiquitination of RIG-I and non-covalent binding of K63-linked polyubiquitin chains to RIG-I impaired the interaction between RIG-I and Npl4. These results indicate that K63-linked ubiquitination of RIG-I facilitates the binding of Npl4 to RIG-I. Next, we co-transfected cells with wild-type or mutant RIG-I-CARDs, Npl4, TRIM25, and ubiquitin. Consistent with previous report, TRIM25 promoted ubiquitination of wild-type RIG-I-CARDs, but not the K172R and T170E/K172R mutants (Fig EV4B). More importantly, the altered ubiquitination levels of RIG-I-CARDs appear to be correlated with Npl4 binding to RIG-I. These observations were further confirmed by an in vitro pulldown assay showing that Npl4 binds stronger to the wild-type RIG-I-CARDs when compared with the T170E/K172R mutant RIG-I-CARDs (Fig EV4C). Taken together, these results indicate that K63-linked ubiquitination of RIG-I may greatly enhance its interaction with the p97 complex.

Targeting p97-Npl4 promotes RIG-I antiviral signaling and protects mice from VSV infection

To further corroborate our mechanistic study of the regulation of the RIG-I antiviral signaling by p97-Npl4 and to explore the therapeutic potential of this complex, we took advantage of the recently developed p97 inhibitors DBeQ and NMS-873 (Fig 7A), which bind distinct sites of p97 to specifically turn off its ATPase activity (Chou & Deshaies, 2011; Chou et al., 2011; Magnaghi et al., 2013). As expected, both DBeQ and NMS-873 increased, in a dose-dependent manner, IFNβ promoter transactivation in cells infected with SeV (Fig 7B), confirming that the ATPase activity of p97 is essential for its regulatory function in RIG-I antiviral signaling. Consistent with these results, DBeQ and NMS-873 inhibited K48-linked ubiquitination of endogenous RIG-I upon virus challenge (Fig 7C), again indicating that p97 promotes ubiquitination of RIG-I in an ATPase-dependent manner. Moreover, cells treated with either DBeQ or NMS-873 were endowed with enhanced resistance to VSV infection (Fig 7D). Together, these results further support the notion that p97-Npl4 inhibits RIG-I antiviral signaling by promoting K48-linked ubiquitination and proteasomal degradation of RIG-I and that p97-Npl4 could be targeted for therapeutic purposes.

We next assessed the potential therapeutic effects of p97 inhibitors by treating mice intravenously. Strikingly, the average survival rates of VSV-infected mice treated with DBeQ and with NMS-873 were each significantly enhanced in a dose-dependent manner when compared with that of mice treated with saline (Fig 7E). Consistent with this observation, VSV titers in serum of mice treated with DBeQ and in serum of mice treated with NMS-873 were much lower than those of control mice (Fig 7F). Consistently, IFNβ production was significantly increased in VSV-infected mice that were treated with DBeQ or NMS-873 compared with that of control mice (Fig EV5A).

Moreover, DBeQ and NMS-873 did not exhibit a detectable toxicity (no significant weight change of mice treated with these compounds) at the indicated dosage that displayed clear evidence of an antiviral effect (Fig EV5B). Together, these observations clearly indicate that therapeutic targeting of the p97-Npl4 complex can enhance antiviral response and protect mice from viral infection.

Discussion

As a sensor of RNA viruses, RIG-I needs to have its activation accurately regulated. Upon infection, the conformation of RIG-I is converted from an auto-suppressed state to an active configuration, enclosing viral RNA within a central cavity formed by the helicase and C-terminal domains; this conformational change also exposes the N-terminal CARDs to solvent and thus allows them to bind MAVS and affect downstream signaling (Kolakofsky et al., 2012). Sustained RIG-I-MAVS signaling is then attenuated by different types of negative regulators after clearance of invasive virus. For example, the deubiquitinases CYLD (Friedman et al., 2008; Zhang et al., 2008) and USP21 (Fan et al., 2014) can reverse K63-linked ubiquitination of RIG-I and thus inhibit its downstream signaling. Meanwhile, RIG-I is also regulated at the level of protein degradation as shown by studies on RNF125 and c-Cbl (Arimoto et al., 2007; Chen et al., 2013).

The ATP-driven chaperone p97 has been found to participate in a wide range of independent cellular processes (Hauler et al., 2012; Yamanaka et al., 2012). Previously, p97 was implicated in the regulation of both classical and alternative NF-kB signaling pathways (Dai et al., 1998; Asai et al., 2002), indicating the functional relevance of p97 in inflammatory responses. In this work, we have identified the p97-Npl4-Ufd1 complex as a novel negative regulator of RIG-I-mediated antiviral signaling and uncovered a non-canonical role for p97 in the control of protein stability. Mechanistically, the p97 complex directly interacts with both RIG-I and the E3 ligase RNF125 to promote K48-linked ubiquitination of RIG-I at K181 and therefore facilitate its proteasomal degradation. During this process, the NZF domain of Npl4 and the CARDs of RIG-I are critical for the interaction between Npl4 and RIG-I, but this interaction does not depend on the UBD of Npl4, which is instead essential for association with p97. Meanwhile, RNF125 was shown to interact with both RIG-I and p97. Compared with the well-studied canonical function of p97—that is, “remodeling and transporting ubiquitinated proteins”—our results suggest that the p97 complex may also act as an adaptor to bridge substrate proteins with E3 ligase and thus facilitate the ubiquitination process.

A structure-directed mutational study, as well as analyses using p97 inhibitors, revealed that an intact state of the p97 complex, with its ATPase activity being functional, is required for the regulation of RIG-I antiviral signaling. Moreover, lysine 181 of RIG-I was identified as a primary site for RNF125-p97-mediated downregulation of RIG-I antiviral signaling. In this regard, it is worth noting that K181 (site for K48-linked ubiquitination) is adjacent to other critical regulatory sites in the 3D structure, namely K172 (site for K63-linked ubiquitination) and T170 (site for phosphorylation) in the RIG-I CARDs. Such a conformation may allow for cross talk among different types of PTM to regulate RIG-I signaling, which could involve not only E3 ubiquitin ligase, but also deubiquitinase, or even kinase and phosphatase.
Based on our results, we propose a working model in which the p97 complex helps to recruit RNF125 and facilitate K48-linked ubiquitination and proteasomal degradation of RIG-I, thus dampening the antiviral response (Fig EV5C). The p97 complex is able to bind non-ubiquitinated RIG-I, yet viral infection-induced activation of RIG-I could markedly strengthen such binding through conformational exposure and K63-linked ubiquitination of the CARDs. It is likely that the p97 complex may help to avoid spontaneous RIG-I signaling or maintain a proper threshold at basal level. Upon viral infection, RIG-I undergoes a conformational change that exposes its CARDs. Subsequent K63-linked polyubiquitination of RIG-I rapidly enhances its binding with the p97 complex, which in turn recruits RNF125 for K48-linked polyubiquitination of RIG-I and subsequent degradation to “turn off” the signaling. As such, a prolonged RIG-I signaling would be terminated right after an effective antiviral response is elicited.

Figure 7. Enzymatic inhibition of p97 protects mice from viral infection.
A Hypothetical structural model of p97 hexamer bound with its inhibitors DBeQ and NMS-873.
B IFNβ transactivation in SeV-infected cells after treatment with DBeQ or NMS-873.
C Ubiquitination of RIG-I in HEK293T cells after treatment with DBeQ or NMS-873.
D Viral loads in drug-treated cells infected with VSV-GFP.
E Survival of VSV-infected mice after treatment with different doses of DBeQ or NMS-873 (n = 20). The mice were intranasally challenged with VSV. Two days after infection, tissue fluids were collected from the mice. Equal volumes (10 ml) of serum were added to HEK293 cell culture media and incubated for 24 h, followed by the viral titer being calculated.
F Viral titer in the serum of VSV-infected mice treated with DBeQ or NMS-873. Horizontal lines represent the means.

Data information: Error bars represent SD of data obtained in three independent experiments. Log-rank test and Student’s t-test were used for survival curves and continuous variables, respectively. n.s., no significant difference; *P < 0.05; **P < 0.01; ***P < 0.001 in comparison with control group.
See also Fig EV5.
It has been well established that the ATPase p97 possesses seque-gase activity able to segregate its substrates from their binding partners or cellular surfaces (Meyer et al., 2012). In this regard, we also observed a negative regulatory role of p97-Npl4 on the stability of the protein MAVS. Such a phenomenon is not surprising considering that RNF125 has also been implicated in regulating the turnover of MAVS (Arimoto et al., 2007). Furthermore, we found that RIG-I binding to the p97 cofactor Npl4 could be significantly enhanced by dsRNA-triggered oligomerization (therefore exposing the CARDs) and K63-linked ubiquitination of RIG-I, two key events for subsequent interaction with and activation of MAVS. Thus, it is reasonable to speculate that the p97 complex may also regulate the disassembly of RIG-I-MAVS signaling complex.

Notably, p97 has been emerging as a potential target for cancer therapy on account of its levels being positively correlated with prognosis of multiple types of cancer including gastric carcinoma (Yamamoto et al., 2003a), non-small cell lung carcinoma (Yamamoto et al., 2004a; Valle et al., 2011), hepatocellular carcinoma (Yamamoto et al., 2003b; Liu et al., 2012), follicular thyroid cancer (Tsujimoto et al., 2004), and pancreatic endocrine neoplasm (Yamamoto et al., 2004b). Inhibitors of p97 have been experimentally indicated to induce cancer cell death, making them potent adjuvants for preventing tumor growth and metastasis. For instance, DBEq, a reversible ATP-competitive p97 inhibitor, can selectively induce apoptosis in human cancer cells (Chou & Deshaies, 2011; Chou et al., 2011). NMS-859, a covalent inhibitor of p97 that blocks its ATP-binding site, can impede the proliferation of cancer cells (Magnaghi et al., 2012). NMS-873 showed cell-killing activity in hematological tumors (Chou et al., 2015). The drosophila Ter94 N domain (amino acids 20-186) and the dNpl4 UBD (amino acids 1-77) were cloned into the vector HT-pET28a, which contains a 6x His tag and a TEV protease cleavage site at the N-terminus. Proteins were expressed and purified according to the methods described above.

Plasmids and protein purification

Mammalian expression vectors for HA-/myc-Npl4, V5-p97, and HA-/Flag-Rig-I were described previously (Feng et al., 2013; Zhang et al., 2013). The drosophila Ter94 N domain (amino acids 20-186) and the dNpl4 UBD (amino acids 1-77) were cloned into the vector HT-pET28a, which contains a 6x His tag and a TEV protease cleavage site at the N-terminus. Proteins were expressed in E. coli BL21 (DE3) Codon Plus cells (Stratagene) by induction with 0.5 mM isopropyl β-D-thiogalactopyranoside at OD600 = 1.0 in Terrific Broth medium for 18 h at 16°C. The cells were harvested and lysed by using a high-pressure homogenizer (JNBIO3000 plus) at 1300 bar in lysis buffer containing 20 mM Hepes pH 7.5, 500 mM NaCl, 5% glycerol, 20 mM imidazole, 1 mM DTT, and 1 mM PMSF. Proteins were then purified by nickel affinity chromatography and finally eluted by the lysis buffer plus 500 mM imidazole. After TEV cleavage to remove the His tag, the protein was applied to a HiLoad 16/60 Superdex 200 column (GE Healthcare) pre-equilibrated with a buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, and 1 mM DTT. Purified Ter94 N and dNpl4 UBD, in an equimolar ratio, were mixed and applied to the gel filtration column once more to obtain the binary complex.

Selenomethionine (SeMet)-labeled Ter94 N and dNpl4 UBD were expressed in E. coli BL21 (DE3) Codon Plus cells, which were cultured in M9 medium containing an amino acid supplement (lysine, phenylalanine, and threonine to a final concentration of 100 mg/l, isoleucine, leucine, and valine to a final concentration of 50 mg/l, and L-SeMet to a final concentration of 60 mg/l). SeMet-labeled protein was purified in the same way as the native protein described above.

Human full-length p97 (amino acids 1–806), Npl4 (amino acids 1–608), and RIG-1 (amino acids 1–925) were separately cloned into the vector pET28a, leading to the target protein with a C-terminal His tag. Human full-length p97 (amino acids 1–806) and Npl4 (amino acids 1–608) were also cloned into the vector pET51b, which contains a Strep tag at the N-terminus. All the proteins were expressed and purified according to the methods described above.

Materials and Methods

Cells, reagents, and antibodies

Human HEK293, HEK293T, and HeLa cells were purchased from the Cell Resource Center (Institute of Life Sciences, Chinese Academy of Sciences). Cells were maintained in Dulbecco’s minimum essential medium (DMEM), supplemented with 10% fetal calf serum (FBS) and penicillin/streptomycin. Sendai virus (SeV) was kindly provided by Dr. Hong-Bing Shu (Wuhan University). NMS-873 was purchased from Selleck Chemicals (Houston, TX). Poly(I:C), DBEq, and antibodies specific for Flag, Myc, and β-actin were purchased from Sigma (St. Louis, MO). Antibodies specific for p97, RIG-1, MAVS, p-TBK, TBK, IKKe, β-IRF3, IRF3, and K48Ub were purchased from Cell Signaling (Danvers, MA); those for HA, ubiquitin, and RNF125 were from Santa Cruz (Santa Cruz, CA) and those for Npl4 (IF) were from Novus Biologicals (Littleton, CO). An antibody specific for Npl4 (IP and WB) was bought from Abcam (Cambridge, UK).

Structure determination and refinement

The Ter94 N–Npl4 UBD complex was concentrated to 25 mg/ml for the crystallization screen. Crystals were obtained by sitting-drop vapor diffusion at 16°C using a reservoir solution consisting of 0.1 M Tris pH 8.5, 23% w/v polyethylene glycol 3350, and 0.2 M...
ammonium sulfate. Crystals were cryoprotected by soaking them in a solution composed of 0.1 M Tris pH 8.5, 23% w/v polyethylene glycol 3350, 0.2 M ammonium sulfate, and 10% glycerol and then flash-frozen in liquid nitrogen.

Diffraction data were collected on beamlines BL17U at Shanghai Synchrotron Radiation Facility (SSRF) and 1W2B at Beijing Synchrotron Radiation Facility (BSRF) and processed using HKL2000. The structure was solved by the single-wavelength anomalous diffraction method from a SeMet derivative with the program AutoSol in Phenix (Adams et al, 2010). The structure was refined using phenix.refine, and model building was performed in Coot (McCoy et al, 2007; Emsley et al, 2010). The coordinate files and structure factors for the p97-Npl4 complex crystal structure were deposited in the Protein Data Bank under accession code 4RV0.

**Viral infection of mice**

For infection, 7- to 8-week-old mice were narcotized and then intranasally inoculated with VSV. Negative controls included saline and PBS. Clinical symptoms were observed 2–8 days post-inoculation, and the analyses described below were performed. All procedures for animal experimentation were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the Animal Core Facility of the Institutes of Biochemistry and Cell Biology (SIBCB). The approval ID for using the animals was No. 081 by the Animal Core Facility of SIBCB.

**Viral titer assay**

After VSV infection, peripheral blood and tissue were collected to obtain serum and tissue fluid, respectively. For the VSV titer assay, sera or tissue fluids were added into culture media of HEK293 cells, and the viral titer was determined by a standard plaque assay.

**Confocal imaging**

HeLa cells were plated on coverslips in 6-well plates and transfected with the indicated plasmids. Twenty-four hours later, the cells were treated with or without SeV (MOI = 1) for 24 h. Coverslips with the cells were washed once with PBS and fixed in 3.7% formaldehyde in PBS for 15 min. After permeabilization with Triton X-100 (0.25%) in PBS for 15 min, the cells were blocked with PBS containing BSA (5%) for 1 h and then incubated with primary antibodies for 1 h. After three separate washes, the cells were incubated with secondary antibody for another hour and then stained with DAPI for 2 min. The coverslips were washed extensively and fixed on slides. Images of these cells were captured using a Leica laser scanning confocal microscope (Leica TCS SP2 AOBS).

**Statistical analysis**

Statistical analysis was performed using the SAS statistical software package (9.1.3). Data are presented as mean ± SD. One-way analysis of variance (ANOVA) and Student’s t-test were used for continuous variables. Survival curves were calculated according to the Kaplan–Meier method; survival analysis was performed using the log-rank test. $P < 0.05$ was considered significantly different.

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