6. SUMMARY

TLR3-mediated endosomal signaling induces a wide range of cellular responses (Matsumoto & Seya, 2008). Activation of the TICAM-1–TBK1–IRF-3 axis is critical for IFN-β gene expression and DC-mediated NK and CTL induction. Interestingly, recent reports have further demonstrated that TLR3 signaling participates in chromatin remodeling and nuclear reprogramming (Lee et al., 2012) and also in the control of endogenous retroviruses in mice (Yu et al., 2012). TLR3 recognizes viral dsRNA and poly(I:C), but it is still unknown what RNA molecules activate TLR3 in viral infection and sterile inflammatory states. Recently, it has been reported that TLR3 recognizes virus-derived ssRNA with mismatched stems, host cell mRNA, and RNA from necrotic cells, indicating that structures other than dsRNA can activate TLR3 (Bernard et al., 2012; Cavassani et al., 2008; Karikó, Ni, Capodici, Lamphier, & Weissman, 2004; Tatematsu, Nishikawa, Seya, & Matsumoto, 2013). Thus, identification of endogenous/exogenous TLR3 ligands and assessment of their signaling are important for a full understanding of the role of the TLR3 pathway in innate and adaptive immunity. Additionally, the development of the TLR3-specific ligands would be useful to analyze human cells and also to discriminate between endosomal and cytoplasmic signaling.

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PLOS | PATHOGENS

A Novel Function of Human Pumilio Proteins in Cytoplasmic Sensing of Viral Infection

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Abstract

RIG-I-like receptor (RLR) plays a pivotal role in the detection of invading pathogens to initiate type I interferon (IFN) gene transcription. Since aberrant IFN production is harmful, RLR signaling is strictly regulated. However, the regulatory mechanisms are not fully understood. By expression cloning, we identified Pumilio proteins, PUM1 and PUM2, as candidate positive regulators of RIG-I signaling. Overexpression of Pumilio proteins and their knockdown augmented and diminished IFN-β promoter activity induced by Newcastle disease virus (NDV), respectively. Both proteins showed a specific association with LGP2, but not with RIG-I or MDA5. Furthermore, all of these components were recruited to NDV-induced antiviral stress granules. Interestingly, biochemical analyses revealed that Pumilio increased double-stranded (ds) RNA binding affinity of LGP2; however, Pumilio was absent in the dsRNA-LGP2 complex, suggesting that Pumilio facilitates viral RNA recognition by LGP2 through its chaperon-like function. Collectively, our results demonstrate an unknown function of Pumilio in viral recognition by LGP2.

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Introduction

The host innate immune system is the first line of defense against invading pathogens. Pattern-recognition receptors (PRRs) detect pathogen molecules, termed pathogen-associated molecular patterns (PAMPs), to initiate innate immune responses [1,2,3,4,5]. Viruses invade host cells to replicate their genome and produce new infectious virions. RIG-I-like receptors (RLRs), including RIG-I, MDA5 and LGP2, sense the invasion and generation of viral RNA PAMPs and trigger antiviral responses [6,7]. In the resting state, RIG-I and MDA5 exist in an autorepressed state, in which N-terminal caspase activation and recruitment domains (CARDs) are masked by the helicase domain; however, upon virus infection, these helicases are activated and oligomerized along with RNAs to form filament-like structures [8,9]. Signals from RLRs are relayed to an adaptor, IPS-1 (also known as MAVS, VISA, Cardif) [10,11,12,13,14,15], which then recruits TRAF adaptors, protein kinases TBK-1, IKK-i and IKK complex to activate transcription factors IRF-3, -7 and NF-κB [16,17].

Knockout mouse studies have shown that RIG-I and MDA5 play a pivotal role in the detection of a series of RNA viruses in vivo [18]. RIG-I detects Sendai virus, NDV and influenza A virus, whereas viruses belong to picornaviridae are sensed by MDA5. Although the mechanism underlying the differential sensing of different viruses by RIG-I and MDA5 is not completely understood, it is proposed that virus specificity comes from the dsRNA length and 5′-end structure of viral RNA [19,20,21,22]. LGP2 was originally thought to be a negative regulator because it lacks CARD, which is crucial for signal transduction. However, knockout and knock-in mouse studies have shown that LGP2 functions as a positive regulator via its ATPase activity [23], consistent with its high affinity binding with dsRNA [7,24].

Recent studies have reported that RLR signaling is subject to numerous regulations [25]. TRIM25 positively regulates signaling through interactions with RIG-I and ubiquitination [26]. Riplet (also termed RNF135 and REUL) positively regulates RIG-I signaling through ubiquitination of RIG-I, independent of TRIM25 [27,28]. On the other hand, ubiquitin ligases, RNF125 [29] and A20 [30], and deubiquitinating enzymes, DUBA [31] and CYLD [32], are reported to function as negative regulators of RIG-I signaling. In addition to the ubiquitination of signaling peptides, involvement of the free ubiquitin chain has been proposed [33]. Furthermore, accumulating reports suggest the importance of the virus-induced stress response in antiviral innate

Author Summary

Mammals utilize innate immune system to counteract viral infections. The host pattern-recognition receptors, such as RIG-I-like receptors (RLRs), sense invading pathogens and initiate innate immune responses. RLRs are composed of three RNA helicases, RIG-I, MDA5 and LGP2, and detect a series of RNA viruses, such as influenza or hepatitis C virus, in the cytoplasm. Upon RNA virus infection, RLRs transmit signals through mitochondrial adaptor protein, IPS-1, to activate transcription factor IRF-3/7, resulting in the production of type I interferon (IFN). Type I IFN plays a crucial role in innate immune system by inducing a hundreds of interferon-stimulated genes and its induction is tightly controlled at transcriptional and translational steps. Pumilio proteins are originally identified as translational repressor through direct binding to specific sequence motifs in the 3′ untranslated regions of specific mRNA, and regulate critical biological processes, such as development and differentiation. In this report, we identified human Pumilio proteins, PUM1 and PUM2, as candidate regulators of IFN signaling. Our results demonstrated an unknown function of Pumilio in viral recognition by LGP2.

immunity. In particular, viral infection induces antiviral stress granules (avSGs), including RIG-I, MDA5, LGP2 and viral RNA [34,35,36,37].

Our expression cloning for antiviral signal regulators identified Pumilio proteins. Pumilio proteins (also termed PUF, Pumilio/FBF) are evolutionary conserved from plants to mammals and were originally identified as translational repressors through direct binding to the specific sequence termed the Nanos response element (NRE) present within the 3′-UTR of target mRNAs, thereby regulating various processes: embryonic development, stem cell differentiation, cell cycle and mitochondrial biogenesis [38,39,40,41,42]. In this report, we describe a novel and non-translational function of Pumilio proteins in viral recognition by LGP2.

Results

Pumilio Proteins Positively Regulate RIG-I Signaling in Response to NDV Infection

We previously identified RIG-I by expression cloning of the human cDNA library using virus-inducible reporter gene activity as the readout [6]. This strategy allowed us to identify other candidate regulators of antiviral signaling, Pumilio proteins. Pumilio proteins share a highly conserved C-terminal Pumilio-homology domain (PUM-HD) [43]. In humans, two genes encoding PUM1 and PUM2 exist. Human PUM1 and PUM2 have similar domain structures and have high homology in their primary structure (Fig. 1A). It was reported that PUM-HD is responsible for sequence-specific RNA binding, whereas the function of the N-terminal portion is unknown. We obtained two independent clones encoding full-length PUM1 and one clone of PUM2 (missing coding amino acids 1-368) by expression cloning. We constructed expression vectors for full-length PUM1 and PUM2 and examined their effect on *IFNB* promoter activity in L929 cells. Overexpression of PUM1 and PUM2 augmented *IFNB* promoter activity induced by NDV infection (Figure 1B). As *IFNB* promoter is regulated by both IRFs and NF-κB, we investigated whether PUM1 and PUM2 affect promoter activity regulated by IRFs (p-55C1B) and NF-κB (p-55A2). PUM1 and PUM2 augmented p-55C1BLuc activity (Figure 1C), as well as p-55A2Luc activity (Figure 1D), suggesting that PUM1 and PUM2 mediate the activation of both IRFs and NF-κB transcription factors. In accord with the increased IFN promoter activity, NDV RNA replication was suppressed by the overexpression of PUM1 and PUM2 24 h after NDV infection, suggesting that PUM1 and PUM2 share antiviral potential (Figure 1E). It is known that translational repression by PUM-HD depends on H850 in PUM2 [44] and this residue was conserved between PUM1 (H972) and PUM2. Therefore, we constructed expression vectors for their alanine mutants and tested their antiviral activity. Interestingly, these mutants markedly enhanced NDV-induced *IFNB* promoter activity (Figure 1F), suggesting that the enhancing function is independent of the translational repression function of Pumilio proteins.

To further investigate the function of PUM1 and PUM2 in IFN induction, we performed siRNA-mediated knockdown. siRNA targeting for human PUM1 and PUM2 suppressed the expression of endogenous PUM1 and PUM2 protein, respectively (Figure 2A). As expected, knockdown of endogenous PUM1 or PUM2 impaired the mRNA expression of *IFNB1* and *CXCL10*, one of the IFN-stimulated genes, in response to NDV infection (Figure. 2B and C). We also examined IRF-3 phosphorylation in the PUM knockdown cells, as well as IRF-3 dimerization. As shown in Figure S1A and B, both phosphorylation and dimerization of IRF-3 were impaired in the Pumilio knockdown cells. Furthermore, the production of IFN-β protein was also reduced by PUM1 or PUM2 knockdown upon NDV infection (Figure 2D). Conversely, NDV RNA copies were increased by knockdown of PUM1 or PUM2 (Figure 2E). To rule out the possibility that Pumilio proteins regulate NDV-induced IFN production through affecting the expression level of RLRs, we examined the basal level of RLRs in the Pumilio knockdown cells. The knockdown of Pumilio proteins did not affect the basal and IFN-induced expression level of RLRs (Figure S2). Finally, we also tested synthetic oligonucleotides, such as poly I:C, *in vitro*-transcribed 5′pppRNA and poly dA:dT. In contrast to NDV infection, the knockdown of Pumilio proteins did not affect the IFN production in response to these stimuli (Figure S1C). These results suggest that PUM1 and PUM2 positively regulate antiviral responses against NDV by controlling IFN production.

Physical Association of PUM1 and PUM2 with LGP2

It has been shown that NDV infection is mainly detected by one of the RLRs, RIG-I [18]. TRIM25 regulates the activation of RIG-I through ubiquitination of RIG-I [26]. In addition, IPS-1 is an adaptor protein essential for RLR signaling [14,15]. To elucidate the regulatory mechanism, the physical association of full-length PUM1 and PUM2 with RLRs, TRIM25 and IPS-1 was examined by co-immunoprecipitation. As shown in Figure 3A, LGP2, but not RIG-I nor MDA5 was precipitated with PUM1 or PUM2. No interaction of PUM1 and PUM2 with TRIM25 and IPS-1 was detectable, indicating that PUM1 and PUM2 selectively interact with LGP2. We also investigated whether PUM1 and PUM2 interacted with each other. As shown in Figure S3, PUM2 associated with PUM1. This result suggested that PUM1 and PUM2 exist as heteromeric complex.

LGP2 was shown to function as a positive regulator of RIG-I- and MDA5-mediated antiviral responses [23]. Specific associations between LGP2 and PUM1 and PUM2 prompted us to investigate the involvement of LGP2 in Pumilio-mediated transactivation. L929 cells were transfected with the expression vector for shRNA either non-targeted or targeted to LGP2, then transactivation by PUM1 or PUM2 was examined (Figure 3B).

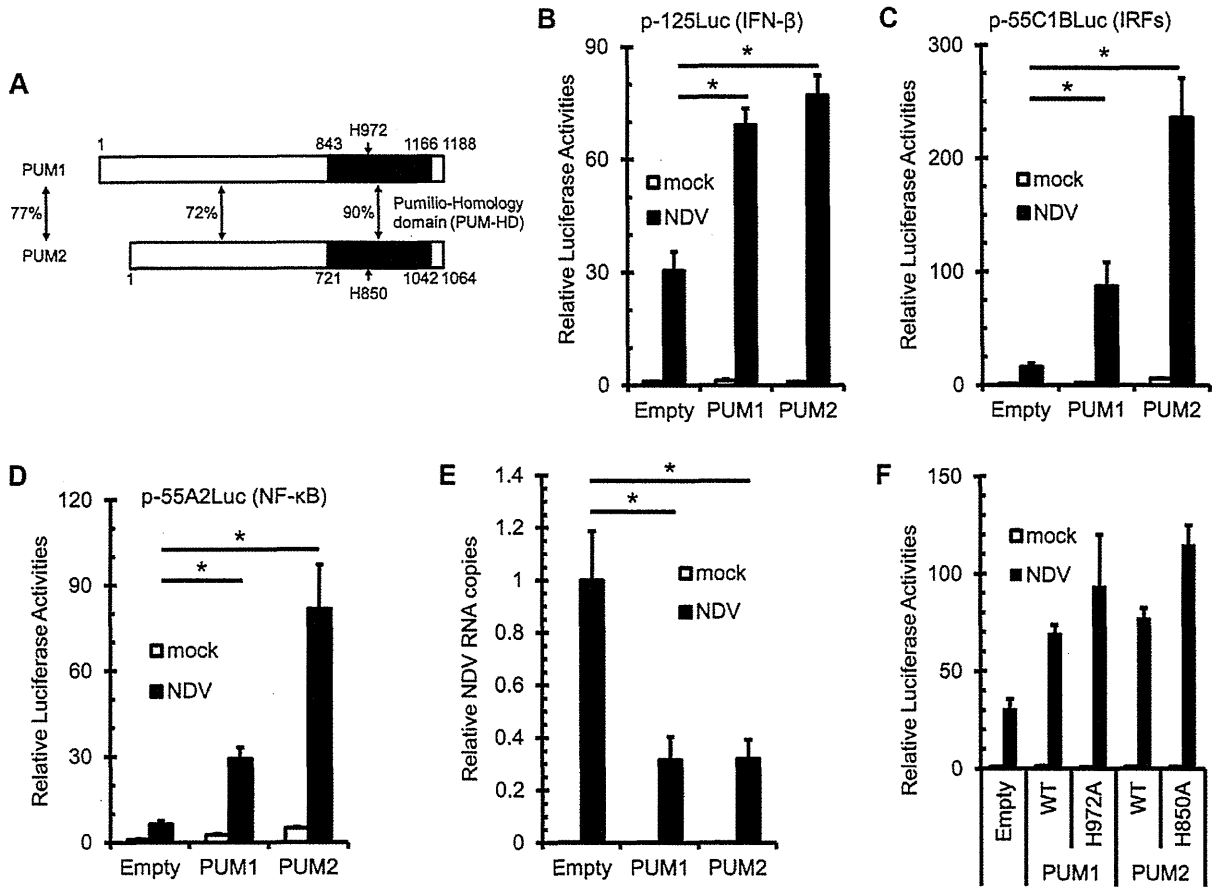

Figure 1. Overexpression of PUM1 and PUM2 results in enhanced NDV-induced *IFNB* promoter activity. (A) Schematic representation of PUM1 and PUM2. PUM-HD shows high sequence similarity between PUM1 and PUM2. Positions of histidine (H) residues critical for NRE recognition are indicated. (B–D) L929 cells were transfected with the indicated reporter gene, p-125Luc (B), p-55C1BLuc (C) or p-55A2Luc (D), and pRL-tk, together with the expression vector for PUM1 or PUM2. The cells were stimulated by NDV infection for 9 h and subjected to a dual-luciferase assay. (E) L929 cells were transfected with an expression vector for PUM1 or PUM2. The cells were infected with NDV for 24 h, and then NDV RNA levels were determined by quantitative RT-PCR. (F) L929 cells were transfected with p-125Luc and pRL-tk, together with the expression vector for wt and histidine mutants of PUM1 or PUM2 as indicated. The cells were stimulated by NDV infection and subjected to a dual-luciferase assay. Data are from one representative of at least two independent experiments; means and S.D. of duplicate experiments are shown (*$p<0.05$).
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Knockdown of LGP2 markedly attenuated transactivation by PUM1 or PUM2, suggesting that the observed physical interaction between LGP2 and PUM1 and PUM2 is relevant to the biological activity of these regulators.

To elucidate the involvement of C-terminal PUM-HD in the association between LGP2 and PUM1 and PUM2, expression vectors for PUM-HD (PUM1dN and PUM2dN) and the rest (PUM1dC and PUM2dC) were constructed (Figure 3C). Co-immunoprecipitation using the mutants revealed that LGP2 interacted with PUM1 and PUM2 lacking PUM-HD as strongly as with the respective full-length proteins, while interaction with PUM-HD (dN constructs) was undetectable. Consistent with the lack of interaction between PUM-HD and LGP2, PUM1H972A and PUM2H850A efficiently co-precipitated with LGP2 (Figure 3D). We also determined the domain of LGP2 responsible for the interaction with Pumilio proteins. As shown in Figure S4, LGP2 helicase domain is important for LGP2 to interact with Pumilio proteins. These results suggest that PUM1 and PUM2 interact with LGP2 through the N-terminal domain.

Involvement of Both N- and C-Terminal Domains of PUM1 and PUM2 in the Activation of Antiviral Response

To further elucidate the mechanism of transactivation by PUM1 and PUM2, full-length and dN and dC mutants were tested for *IFNB* promoter activation (Figure 3E). Full-length PUM1 and PUM2 but neither dC nor dN enhanced NDV-induced IFN-β reporter activity, indicating that both PUM-HD and the N-terminal portion are necessary for transactivation.

Co-localization of PUM1 and PUM2 in avSGs upon NDV Infection

It is reported that PUM1 and PUM2 are recruited to stress granules (SGs) upon stress responses, such as oxidative stress or starvation [45]. Previously, we reported that virus infection induces SG-like aggregates containing SG markers, RLR and several antiviral proteins, and termed the aggregate antiviral SGs (avSGs) [34]. avSGs are thought to function as a platform for detection of viral RNA by RLR and as action sites of antiviral

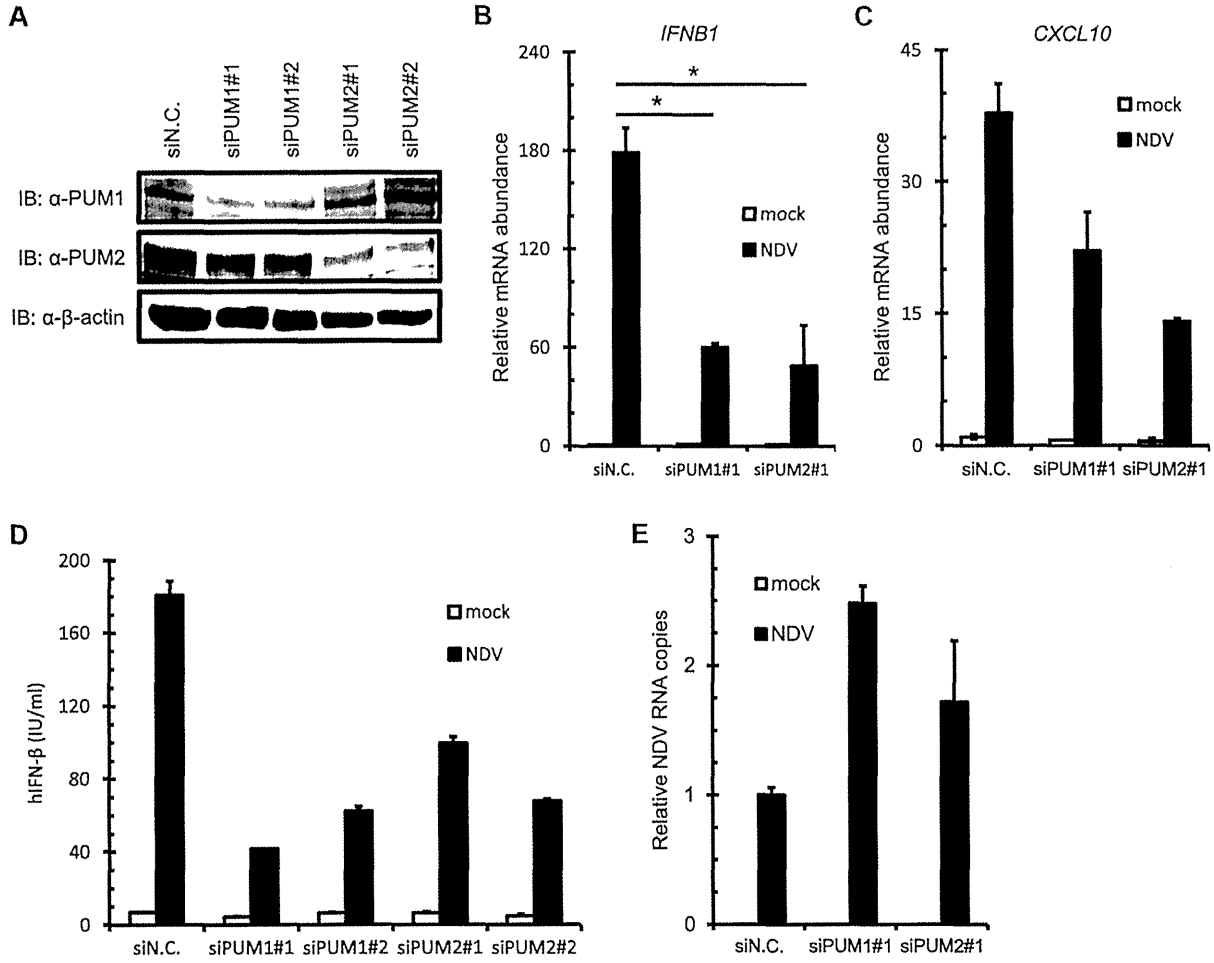

Figure 2. Knockdown of PUM1 and PUM2 downregulates NDV-induced gene activation. (A–C) HEK293T cells were transfected with control siRNA (siN.C.) or siRNA targeting human PUM1 or PUM2 for 48 h. Knockdown efficiency was confirmed by immunoblotting with anti-PUM1, anti-PUM2 and anti-β-actin antibodies (A). The cells were infected with NDV for 9 h, and *IFNB* (B) and *CXCL10* (C) mRNA levels were determined by quantitative RT-PCR. (D and E) HEK293T cells were transfected with control siRNA or siRNA targeting PUM1 or PUM2 for 48 h. The cells were infected with NDV for 24 h. The culture media were collected and subjected to IFN-β ELISA (D). Total cellular RNA was extracted and subjected to qRT-PCR for NDV RNA (E). Data are from one representative of at least two independent experiments; means and S.D. of duplicate experiments are shown (*$p<0.05$, **$p<0.01$).

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proteins. We determined the cellular localization of PUM1 and PUM2 by immunostaining. In uninfected cells, PUM1 and PUM2 localized diffusely in the cytoplasm (Figure 4A). NDV infection induced co-localization of PUM1 and PUM2 into cytoplasmic speckle-like aggregates (Figure 4A). We confirmed that a SG marker, TIAR, localized with the speckles containing PUM1 in NDV-infected cells (Figure 4B); therefore, these aggregates correspond to avSG. We also confirmed that LGP2 localized in the avSG (Figure 4C)

Both N- and C-terminal domains of Pumilio proteins are required for transactivation (Figure 3E) and the N-terminal domain is responsible for interaction with LGP2. We therefore explored the function of C-terminal PUM-HD in terms of cellular localization. Flag-tagged PUM1dN and PUM2dN were expressed in cells and the cells were infected with NDV (Figure 4D). In uninfected cells, these mutants were diffusely accumulated in nuclei and cytoplasm; however, upon viral infection, these proteins localized with avSG, suggesting that PUM-HD is responsible for the localization of PUM proteins in avSG. We also determined the cellular localization of PUM1dC and PUM2dC. As shown in Figure S5D, PUM1dC diffusely localized in the cytoplasm of NDV-infected cells, whereas PUM2dC was recruited to the avSGs in response to NDV infection.

To explore the possibility that PUM1 and PUM2 are required for avSG formation, the effect of knockdown of PUM1 and PUM2 on avSG formation was examined. As shown in Figure S5A, knockdown of PUM1 or PUM2 expression did not alter avSG induction in NDV-infected cells, suggesting that PUM1 and PUM2 do not notably affect avSG assembly. We also examined the recruitment of Pumilio proteins and LGP2 in LGP2 KO cells and Pumilio knockdown cells, respectively. The knockdown of Pumilio proteins did not affect the localization of LGP2 (Figure S5B). Furthermore, Pumilio proteins were recruited to the avSGs in response to NDV in LGP2 KO cells (Figure S5C), indicating that Pumilio proteins were not involved in the recruitment of LGP2 to the avSGs and vice versa.

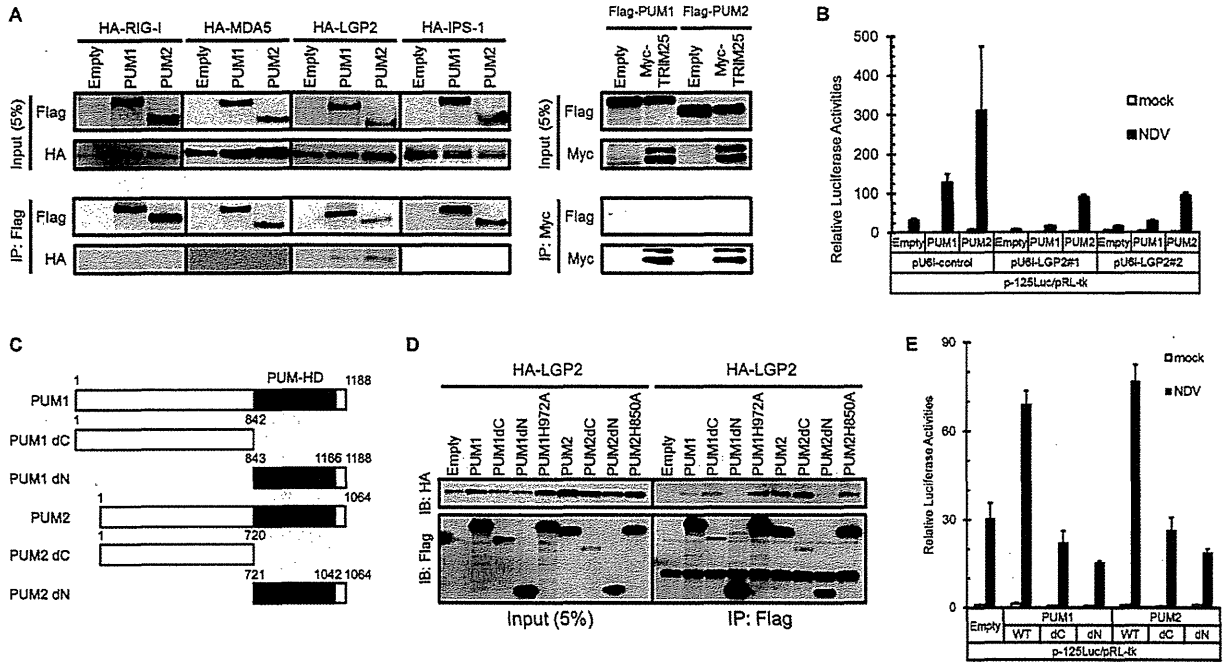

Figure 3. Physical association of PUM1 and PUM2 with LGP2 and involvement of N- and C-terminal domains of PUM1 and PUM2 in IFN induction. (A) HEK293T cells were transfected with expression vector HA-tagged RIG-I, MDA5, LGP2 or IPS-1, together with Flag-tagged PUM1 or PUM2. For TRIM25, HEK293T cells were transfected with Flag-tagged PUM1 or PUM2, together with Myc-tagged TRIM25. The cell lysates were subjected to anti-Flag or anti-c-Myc immunoprecipitation (IP), followed by Western blotting. Western blotting result of total lysate is shown as a reference (Input, 5%). (B) L929 cells were transfected with control shRNA construct (pU6i-control) or shRNA for LGP2 (pU6i-LGP2#1 and #2) and expression vectors for PUM1 or PUM2 and p-125Luc reporter and pRLtk as indicated. The cells were stimulated by infection with NDV for 9 h and subjected to the dual luciferase assay. (C) Schematic representation of PUM1 or PUM2 deletion mutants used for IP experiments. (D) HEK293T cells were transfected with expression vectors for the indicated proteins and for HA-tagged LGP2. The cell lysates were subjected to IP with anti-Flag, followed by immunoblotting with anti-HA. Immunoblotting result of total lysate is shown as a reference (Input, 5%). (E) L929 cells were transfected with expression vectors for the wild type or mutant of PUM1 or PUM2 and p-125Luc reporter and pRL-tk as indicated. Cells were stimulated by infection with NDV for 9 h and subjected to the dual luciferase assay. Data are from one representative of at least two independent experiments (means and s.d. of duplicate experiments.)
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PUM1 and PUM2 Augment dsRNA Binding Activity of LGP2

Finally, to elucidate the mechanism of the enhancement of IFN gene expression by PUM1 and PUM2, we examined dsRNA binding activity of LGP2 in the presence or absence of PUM1 and PUM2. Electrophoresis mobility shift assay (EMSA) was performed using synthetic dsRNA and recombinant proteins. LGP2 bound to the probe, resulting in a slow-migrating complex. We confirmed that this slow-migrating band was a complex of the probe and LGP2 by a supershift experiment (Figure 5A). PUM1 and PUM2 exhibited very weak binding with the probe (Figure 5B). Interestingly, the LGP2-dsRNA complex intensity was increased with the addition of PUM1 and PUM2; however, the mobility of the complex is hardly affected. Dissociation constant for LGP2 in the absence and presence of PUM1 or PUM2 was determined by Scatchard plot analysis (Figure 5C). The Kd value indicates that PUM1 and PUM2 increased the dsRNA-binding activity of LGP2. We also purified recombinant PUM1 and PUM2 lacking PUM-HD (PUM1dC and PUM2dC) and subjected them to a binding assay (Figure S6A and B). Essentially similar results were obtained, indicating that the N-terminal domain of PUM1 and PUM2 is sufficient for increasing the dsRNA binding affinity of LGP2.

Because the complex mobility in EMSA did not change in the presence or absence of Pumilio proteins, we examined whether the association between Pumilio proteins and LGP2 is affected in the presence or absence of dsRNA. Recombinant PUM1 and PUM2 proteins produced as GST fusion were mixed with recombinant Flag-tagged LGP2 in the presence or absence of dsRNA and pulled down with glutathione Sepharose (Figure S7). In the absence of dsRNA, we confirmed the association between PUM1 and PUM2 with LGP2; however, in the presence of dsRNA, this association was undetectable, suggesting that upon binding of LGP2 with dsRNA, PUM1 and PUM2 are released from the complex, consistent with the EMSA results. Taken together, we hypothesized that Pumilio proteins changed the conformation of LGP2 through physical associations to increase its dsRNA binding affinity (Figure 6).

Discussion

We found that Pumilio proteins enhanced NDV-induced activation of the *IFNB* gene (Figure 1). Knockdown of PUM1 and PUM2 respectively attenuated gene activation (Figure 2), suggesting that PUM1 and PUM2 function non-redundantly. Furthermore, PUM1 and PUM2 accelerated the activation of IRFs and NF-κB transcription factors, suggesting their action in signal transduction, rather than post-transcriptional steps. Interestingly, PUM1 and PUM2 selectively interact with LGP2 but not with RIG-I, MDA5, IPS-1 or TRIM25 (Figure 3A). LGP2

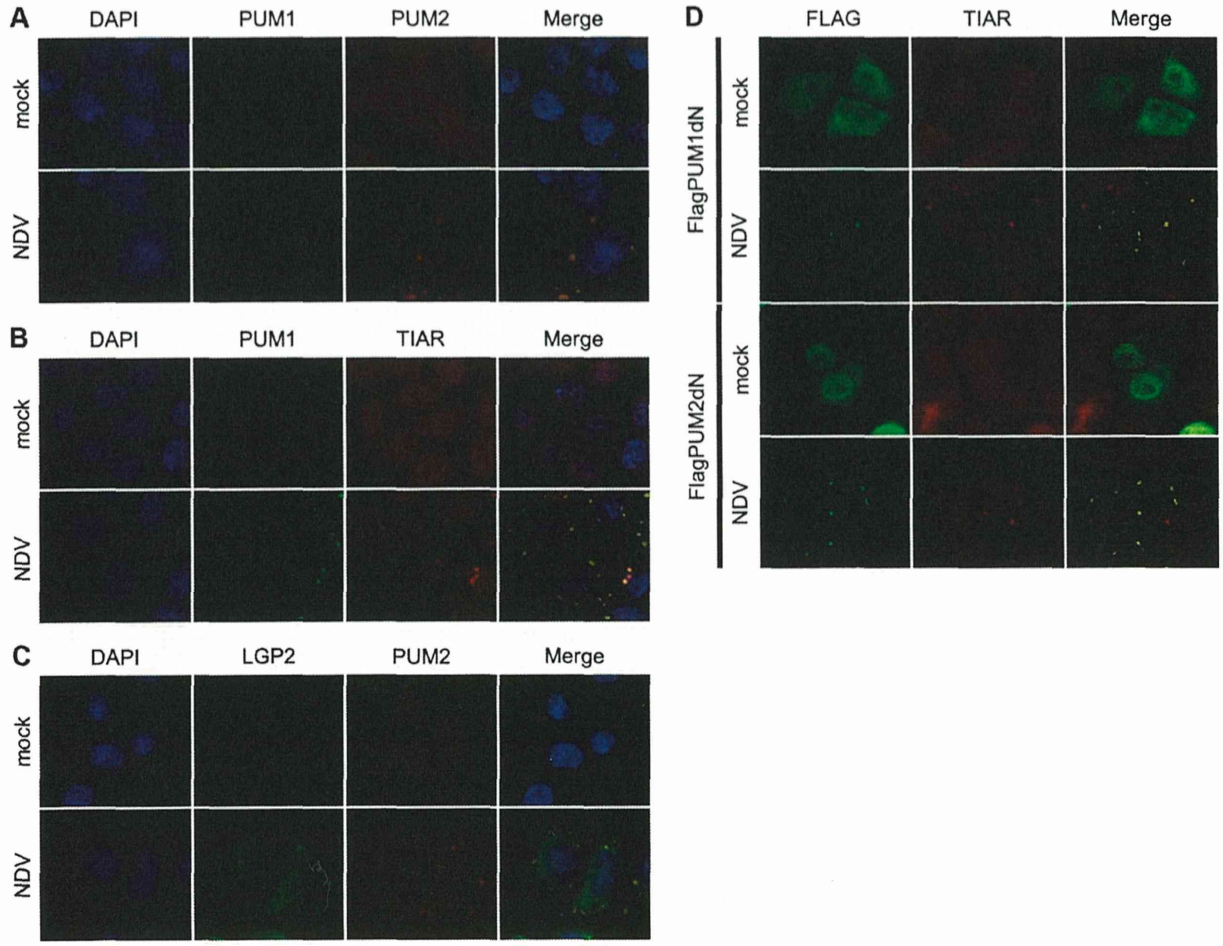

Figure 4. Cellular localization of PUM1, PUM2 and LGP2. (A–C) HeLa cells were mock-treated or infected with NDV for 9 h, fixed and stained with the indicated antibodies. Nuclei were stained with DAPI. (D) HEK293T cells were transfected with the expression vector for Flag-PUM1dN or Flag-PUM2dN for 48 h and mock treated or infected with NDV for 9 h. The cells were stained with anti-Flag or anti-TIAR.
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knockdown diminished *IFNB* gene induction augmented by PUM1 and PUM2 (Figure 3B), suggesting that PUM1 and PUM2 augment viral RNA sensing mediated by LGP2. It was shown that LGP2 does not participate in the detection of synthetic oligonucleotides, such as poly I:C or in vitro-transcribed 5′pppRNA [23]. Consistent with this, the knockdown of PUM1 and PUM2 did not affect the IFN production induced by poly I:C, 5′pppRNA or poly dA:dT (Figure S1C). LGP2 exhibits strong binding activity to dsRNA; however, it lacks CARD, through which the signal is relayed to IPS-1. Therefore, it has been hypothesized that LGP2 cooperates with either RIG-I or MDA5. Our findings uncovered a new mechanism of sensing viral RNA by LGP2, PUM1 and PUM2.

Deletion analyses of PUM1 and PUM2 revealed that both N- and C-terminal regions are required for up-regulation (Figure 3E). The N-terminal region is sufficient for interaction with LGP2 and to increase the binding affinity to dsRNA (Figure 3D and S7). The C-terminal region, also termed PUM-HD, is sufficient for translocation to avSG upon viral infection (Figure 4D), although the underlying mechanism is unknown.

PUM1 and PUM2 have been known to regulate mRNA translation through sequence-specific recognition of NRE within the target mRNA. It was proposed that PUM-HD consists of 8 repeats of the module and each module recognizes a single nucleotide in NRE [43]. It was shown that single amino acid substitution is sufficient to abolish NRE binding and the translational regulation of PUM2 [44]. On the other hand, we found that NRE binding-deficient mutants (PUM1H972A and PUM2H850A) augment virus-induced signaling as strongly as the respective wt protein (Figure 1F), suggesting that Pumilio proteins facilitate two independent biological functions in translational and antiviral signal regulation. It is tempting to speculate that the repeated modules recognize component(s) associated with avSGs.

Concerning the molecular mechanism, we found that PUM1 and PUM2 increased dsRNA binding affinity of LGP2 (Figure 5). However, interestingly, we did not observe the ternary complex of dsRNA, LGP2 and PUM1 or PUM2; furthermore, the interaction between LGP2 and PUM1 or PUM2 was lost when dsRNA was added (Figure S7). We therefore hypothesize that PUM1 and PUM2 have a cooperative function with LGP2 through physical association to increase its binding affinity to dsRNA (Figure 6). It has been shown that LGP2 facilitates RIG-I- and MDA5-mediated signaling [23]. Also it was shown that LGP2 and RIG-I interact