# PUM1 is a biphasic negative regulator of innate immunity genes by suppressing LGP2

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Contributed by Bernard Roizman, July 12, 2017 (sent for review May 25, 2017; reviewed by Adolfo Garcia-Sastre and Richard Longnecker)

**PUM1 is an RNA binding protein shown to regulate the stability and function of mRNAs bearing a specific sequence. We report the following: (*i*) A key function of PUM1 is that of a repressor of key innate immunity genes by repressing the expression of LGP2. Thus, between 12 and 48 hours after transfection of human cells with siPUM1 RNA there was an initial (phase 1) upsurge of transcripts encoding LGP2, CXCL10, IL6, and PKR. This was followed 24 hours later (phase 2) by a significant accumulation of mRNAs encoding RIG-I, SP100, MDA5, IFIT1, PML, STING, and IFNβ. The genes that were not activated encoded HDAC4 and NF-κB1. (*ii*) Simultaneous depletion of PUM1 and LGP2, CXCL10, or IL6 revealed that up-regulation of phase 1 and phase 2 genes was the consequence of up-regulation of LGP2. (*iii*) IFNβ produced 48–72 hours after transfection of siPUM1 was effective in up-regulating LGP2 and phase 2 genes and reducing the replication of HSV-1 in untreated cells. (*iv*) Because only half of genes up-regulated in phase 1 and 2 encode mRNAs containing PUM1 binding sites, the upsurge in gene expression could not be attributed solely to stabilization of mRNAs in the absence of PUM1. (*v*) Lastly, depletion of PUM2 does not result in up-regulation of phase 1 or phase 2 genes. The results of the studies presented here indicate that PUM1 is a negative regulator of LGP2, a master regulator of innate immunity genes expressed in a cascade fashion.**

PUM1 | LGP2 | RIG-I | MDA5 | interferon

In this article we report that depletion of human Pumilio protein 1 (PUM1) also known as PUF or RBF results in a cascade of expression of innate immunity genes that ultimately lead to the synthesis of IFN. To monitor the sequential order of gene expression we depleted PUM1 by transfecting cells with siRNA specifically directed against PUM1 and monitored the changes in gene expression as a function of time after transfection. We report that the entire gene expression cascade evolves within a 48- to 72-h time frame. The first phase extrapolates to an interval between 12 and 48 h after transfection of siPUM1 RNA and is characterized by a significant increase in the accumulation of transcripts encoding LGP2, CXCL10, IL6, and PKR. The second phase, which extrapolates roughly to an interval between 48 and 72 h after siPUM1 transfection, is characterized by an upsurge in the accumulation of mRNAs encoding RIG-I, MDA5, SP100, PML, STING, and IFNβ and to a lesser extent that of IFNγ. The results also show that the increase in the expression of genes in both phase 1 and phase 2 were dependent on the increase in the expression of LGP2 following depletion of PUM1. Lastly, the results show that the production of IFNβ was effective in up-regulating LGP2 and phase 2 genes. Thus, PUM1 emerged as a negative biphasic regulator of innate immune responses.

Human cells express two Pumilio proteins, PUM1 and PUM2 (1–3). The primary function of PUM proteins described to date is that of mRNA posttranscriptional regulators (4–9). Specifically both PUM1 and PUM2 contain a RNA sequence-specific binding domain (10–15). In studies that detailed the consequences of the interaction, the results suggest that PUM proteins alter the function of the RNAs by altering their conformation (7, 16). Other studies reported that PUM proteins mediate translational silencing via a number of mechanisms. These include mRNA deadenylation (17), decapping (18), by blocking translation initiation (19), or by facilitating their interaction with microRNAs (7, 16). PUM proteins have also been reported to be markers of stress granules in cells infected with Newcastle disease virus (20). The findings reported here vastly expand our knowledge of the functions of PUM1.

The PUM1 and PUM2 RNA binding domains have been reported to be 91% identical (2). The remaining amino acid sequences of the two proteins are 75% identical (2). Notwithstanding the close relationship of the two proteins, depletion of PUM2 did not match the consequences of depletion of PUM1.

## Results

**PUM1 Mediates the Suppression of Selected Genes Known to Play a Significant Role in Innate Immune Responses to Infection.** In this series of experiments we measured the accumulation of proteins and mRNAs of selected cellular genes associated with innate immune responses in cells depleted of PUM1 by transfection of targeted (siPUM1) or nontargeted (siNT) siRNAs. We have chosen to deplete cells of selected proteins using siRNAs to trace the depletion of gene products as a function of time after transfection. The sequence of siRNAs used in these studies, the sequences of the probes for quantification of mRNAs, and the sources of antibodies are described in *Materials and Methods* and Table 1.

In preliminary studies we tested three PUM1 siRNAs and a NT siRNAs. The sequences of the siRNAs are listed in *Materials and Methods*. Of the four siRNAs tested, two PUM1 siRNAs (nos. 2652 and 1777) significantly decreased the accumulation of PUM1, whereas the NT siRNA and the PUM1 siRNA no. 412 had no effect.

The NT siRNA and the PUM1 siRNA (412) had no effect on the synthesis and accumulation of PUM1, whereas the remaining two, 1777 and 2652, significantly reduced the accumulation of PUM1 (Fig. 1*A*). The results shown in Fig. 1*A* indicate that the

## Significance

We report that PUM1, a protein linked to control of translation of mRNAs carrying a cognate sequence, is a negative regulator of LGP2. In turn LGP2 emerged as a biphasic master activator of numerous innate immunity genes leading to production of interferon. The studies traced in real-time the changes in innate immune responses following transfection of siPUM1 RNA. This approach enabled the discovery that innate immune genes analyzed in this report were activated sequentially in a cascade fashion. The studies were done in uninfected cells in the absence of viral gene products that could affect the expression of cellular genes. This report seeds the possibility of systemic engagement of innate immune responses following life-threatening viral infections by suppressing PUM1 function.

Author contributions: Yonghong Liu, B.R., and G.G.Z. designed research; Yonghong Liu, L.Q., and Yuanyuan Liu performed research; Yonghong Liu, B.R., and G.G.Z. analyzed data; and B.R. and G.G.Z. wrote the paper.

Reviewers: A.G.-S., Icahn School of Medicine at Mount Sinai; and R.L., Northwestern University Feinberg School of Medicine.

The authors declare no conflict of interest.

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**Table 1. Sequences of primers used to measure gene expression**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *LGP2* | 5′-GAGCCAGTACCTAGAACTTAAAC-3′ | 5′-ATGGCCCCATCGAGTTTG-3′ |
| *CXCL10* | 5′-ACGTGTTGAGATCATTGCTAC-3′ | 5′-ATCTTTTAGACCTTTCCTTGC-3′ |
| *IL6* | 5′-GCCACTCACCTCTTCAGAACG-3′ | 5′-CAGTGCCTCTTTGCTGCTTTC-3′ |
| *PKR* | 5′-GGAAAGCGAACAAGGAGTAAG-3′ | 5′-ATCCCGTAGGTCTGTGAAAAAC-3′ |
| *RIG-I* | 5′-AGAAGAGTACCACTTAAACCCAG-3′ | 5′-TTGCCACGTCCAGTCAATATG-3′ |
| *SP100* | 5′-TTTTCGCCTCAGAACCGTATT-3′ | 5′-CTGCACAAACCCTTCTACTCG-3′ |
| *MDA5* | 5′-AGGAGTCAAAGCCCACCATC-3′ | 5′-GTGACGAGACCATAACGGATAAC-3 |
| *IFIT1* | 5′-CAACCAAGCAAATGTGAG-3′ | 5′-AGGGGAAGCAAAGAAAATGG-3′ |
| *PML* | 5′-CACCCGCAAGACCAACAACATC-3′ | 5′-GCTTGGAACATCCTCGGCAGTAG-3′ |
| *STING* | 5′-TCAGCATTACAACAACCTGCTAC-3′ | 5′-TTATCCAGGAAGCGAATGTTG-3′ |
| *HDAC4* | 5′-TCAAGAACAAGGAGAAGGGCAAAG-3′ | 5′-TCCCGTACCAGTAGCGAGGGTC-3′ |
| *NFκB1* | 5′-TAAAGCCCCCAATGCATCCAAC-3′ | 5′-CCAAATCCTTCCCAGACTCCAC-3′ |
| *IFN-α* | 5′-AGAGTCACCCATCTCAGCAAG-3′ | 5′-CACCAGGACCATCAGTAAAGC-3′ |
| *IFN-β* | 5′-TTGTGCTTCTCCACTACAGC-3′ | 5′-CTGTAAGTCTGTTAATGAAG-3′ |
| *IFN-γ* | 5′-ATGTCCAACGCAAAGCAATAC-3′ | 5′-GCTCTTCGACCTCGAAACAG-3′ |
| *ICP27* | 5′-TCATGCACGACCCCTTTGG-3′ | 5′-CTTGGCCCGCCAACAC-3′ |
| *ICP8* | 5′-GCCTGAAACACACGGTCGTT-3′ | 5′-ATGGTCGTGTTGGGGTTGAG-3′ |
| *UL42* | 5′-TTTCTCCTGAAACCCCAGAAGATTTG-3′ | 5′-AGTCCTGGCTGTCTGTTGGCTC-3′ |
| *VP16* | 5′-CTCGAAGTCGGCCATATCCA-3′ | 5′-CCGGGTCCGGGATTTACC-3′ |
| *18S* | 5′-CTCAACACGGGAAACCTCAC-3′ | 5′-CGCTCCACCAACTAAGAACG-3′ |

NT siRNA and the siPUM1 1777 and 2652 at 100 nM are suitable for the studies described below.

In preliminary studies we noted that depletion of PUM1 by siRNA induces the synthesis LGP2. To verify the link between depletion of PUM1 and activation of transcription of LGP2, HEp-2 cells were transfected with NT siRNA or the PUM1 siRNAs, 412, 2652, and 1777. As shown in Fig. 1*B* the PUM1 siRNAs 1777 and 2652 activated the transcription of LGP2, whereas the PUM1 siRNA 412 and the NT siRNA had no effect. These studies support the conclusion that depletion of PUM1 correlates with activation of transcription of LGP2. We selected PUM1 siRNA 1777 for further studies.

We next examined the effect of depletion of PUM1 on the accumulation of IFIT1, PKR, PKR-p-Thr446, and STING. The amounts of protein were normalized with respect to amounts of loading controls (GAPDH) and the amounts of proteins detected in mock transfected level cells. The results (Fig. 1*C*, lanes 7–9) show that at 66 h after transfection, the amounts of the proteins analyzed in this study increased at least twofold. The amounts of PKR-p-Thr446 increased 4.8-fold. Smaller increases were noted at 48 h after transfection.

We next examined the accumulation of mRNAs of a selected set of 12 cellular genes. In this instance, the amounts of mRNAs were normalized with respect to 18S RNAs. The results are shown in Fig. 2. In brief, few changes in mRNA levels were noted at 12 h after transfection of siRNAs. At later times after transfection, we noted three different responses. Specifically the mRNA levels of LGP2, CXCL10, IL6, and PKR began to increase sometime between 12 and 48 h of transfection and leveled off between 72 and 90 h after transfection. The levels of RIG-I, SP100, MDA5, IFIT1, and to a lesser extent those of PML and STING, increased after 48 h after transfection, consistent with the accumulation of the respective proteins shown in Fig. 1. Lastly, we did not detect a significant increase in the levels of mRNAs encoding HDAC4 and NF-κB1 within the time interval tested.

In summary the results show that the expression of at least 10 genes associated with innate immune responses are up-regulated in uninfected HEp-2 cells depleted of PUM1. The increases in expression of some genes extrapolate to ≈12 h after transfection. In the case of others, notably RIG-I, SP100, IFIT1, MDA5, PML, and STING, the increases in the accumulation of mRNAs began between 48 and 72 h after transfection. One hypothesis that could explain the results is that the genes whose increase in expression began after 48 h after transfection were activated by the products of genes transactivated between 12 and 48 h after transfection.

**Up-Regulation of both Phase 1 and Phase 2 Genes Requires the Expression of LGP2.** The primary function of PUM proteins described to date is that of translational repressors that act by binding to a conserved sequence motif (UGUANAUA) in the 3′ UTR of target mRNAs (14, 21). Analyses of the sequences of the mRNAs of the genes up-regulated in phases 1 and 2 revealed that only half of the 12 tested mRNAs contained the conserved sequence motif UGUANAUA. As indicated by the red circle in Fig. 2, these were the mRNAs encoding CXCL10, PKR, RIG-I, SP100, IFIT1, HDAC4, and NF-κB1. In essence these analyses do not support the hypothesis that up-regulation of innate immune responses is the consequence of derepression of their mRNAs.

We next tested the hypothesis that one of the activated genes in phase 1 plays a key role in activation of both phase 1 and phase 2 genes. In two series of experiments we established that LGP2 plays this role. Specifically, in the first series of experiments we measured the role of LGP2 in the up-regulation of phase 1 genes IL16 and CXCL10. The results summarized in Fig. 3 shows that in cells transfected with siPUM1 and siLGP2 RNAs there was no enhancement of accumulation of LGP2 mRNA (Fig. 3*C*) or of IL6 (Fig. 3*A*) or CXCL10 mRNAs (Fig. 3*B*). Thus, activation of phase 1 genes by depletion of PUM1 required the enhanced expression of LGP2.

In the second series of experiments we examined the role of enhanced expression of LGP2, CXCL10, or IL6 in the up-regulation of phase 2 genes RIG-I, SP100, MDA5, or IFIT1 by measuring the accumulation of the mRNAs of these genes in cells depleted of PUM1 and LGP2 (Fig. 4*A*), PUM1 and CXCL10 (Fig. 4*B*), or PUM1 and IL6 (Fig. 4*C*). Depletion efficiency of LGP2 (Fig. 4 *A*, *a*), CXCL10 (Fig. 4 *B*, *f*), and IL6 (Fig. 4 *C*, *k*) by siRNAs were verified by mRNA accumulation. Next we examined the doubly depleted cells for the accumulation of RIG-I, SP100, MDA5, and IFIT1. The results show that the enhancement of the accumulation of the mRNAs encoding RIG-I, SP100, MDA5, and IFIT1 required the activation of LGP2 (Fig. 4 *A*, *b–e*) but not the activation of CXL10 (Fig. 4 *B*, *g–j*) or IL6 (Fig. 4 *C*, *l–o*).

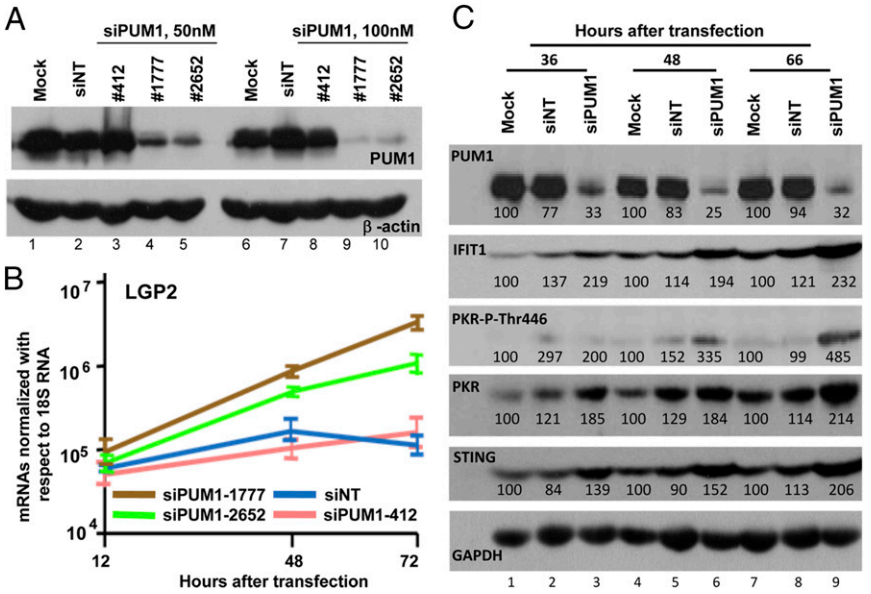


**Fig. 1.** Depletion of PUM1 in HEp-2 cells resulted in up-regulation of selected cellular proteins. (*A*) Depletion of PUM1 by transfection of siRNA. The $3 \times 10^5$ HEp-2 cells seeded on six-well plates were either mock treated or transfected with 50 or 100 nM PUM1 siRNA or nontarget siRNA (siNT). The cells were harvested at 66 h after transfection. Cell lysates containing 70 μg of total proteins were separated on denaturing 10% polyacrylamide gels, electrically transferred to nitrocellulose sheets, and immunoblotted for PUM1 protein. β-Actin was used as a loading control. (*B*) Accumulation of mRNA of LGP2 in HEp-2 cells at times after transfection of NT and PUM1 siRNAs. HEp-2 cells seeded on six-well plates were transfected with 100 nM of PUM1 siRNAs: siPUM1-1777, siPUM1-2652, siPUM1-412 or siNT. The cells were harvested at 12, 48, or 72 h after transfection. Total RNAs were extracted and reverse transcribed to cDNA as described in *Materials and Methods*. mRNAs encoding LGP2 were normalized with respect to 18S RNA and plotted as a function of time after transfection. (*C*) Accumulation of selected cellular proteins in HEp-2 cells depleted of PUM1. The $3 \times 10^5$ HEp-2 cells seeded on six-well plates were mock treated or transfected with 100 nM PUM1 siRNA or siNT. The cells were harvested at 36, 48, or 66 h after transfection. Lysates containing 70 μg of total proteins were electrophoretically separated on denaturing 10% polyacrylamide gels, electrically transferred to nitrocellulose sheets, and immunoblotted for PUM1, IFIT1, PKR, PKR-P-Thr446, and STING. GAPDH was used as loading control. The images of bands were scanned with the aid of ImageJ, and the band density was normalized with respect to GAPDH and shown as a percentage of the values obtained for mock-transfected cells.

In summary, up-regulation of both phase 1 and phase 2 genes required the activation of LGP2 that was induced by depletion of PUM1.

**HSV-1 Gene Expression Is Inhibited in Cells Depleted of PUM1.** The objective of this series of experiments was to determine whether PUM1 depletion affected the replication of HSV-1. We report three series of experiments. In the first replicate, HEp-2 cell cultures were exposed to 0.1 HSV-1(F) per cell 48 h after transfection with siNT or siPUM1. The results (Fig. 5*A*) indicate that the replication of HSV-1 was impaired and resulted in reduced virus yields. In the second series of experiments, the cells were exposed to 0.5 pfu of HSV-1(F) per cell 66 h after transfection with siNT or siPUM1. The cells were harvested at times indicated in Fig. 5*B*, solubilized, subjected to electrophoresis in denatured gels, and probed with antibodies to proteins associated with different kinetic classes of HSV-1 replication. These included ICP0 and ICP27, representative of the α or immediate early genes, ICP8 representative of the β or early genes, and VP16 and US11 representative of γ or late genes (Fig. 5*B*). In the third series of experiments, the cells were exposed to 0.5 pfu of virus per cell. The cells were harvested at times shown in Fig. 5*C* and the amounts of mRNAs of ICP27, ICP8, UL42, and VP16, representative of the various kinetic classes of HSV-1 replication, were measured. The results of three series of experiments indicate diminished viral replication in cells transfected with siPUM1 RNA.

**The Decrease in the Yields of Viral Gene Products in Cells Transfected with siPUM1 RNA Is Associated with Production of IFN.** The activation of genes associated with innate immunity and reductions in viral yields and viral gene products raised the question of whether depletion of PUM1 results in production of IFN. The results presented below suggest that this is the case. We report three series of experiments. In the first series of experiments we tested transfected cells for the synthesis of IFNα, IFNβ, or IFNγ mRNAs. Cells mock treated or transfected with 100 nM of siNT or siPUM1 RNAs were harvested at 12, 48, 72, or 90 h after transfection and analyzed for the accumulation of mRNAs encoding IFNα, IFNβ, or IFNγ using procedures and probes described in *Materials and Methods* and Table 1. The results shown in Fig. 6*A* suggest that IFNβ mRNA begins to accumulate between 48 and 72 h after transfection of siPUM1, that is, during phase 2. The assays also detected the accumulation of small amounts of IFNγ mRNAs.

In the second series of experiments, we verified the accumulation of IFNβ and determined its pattern of accumulation. Specifically the cells were mock treated or transfected with 100 nM of PUM1, siLGP2, or both siPUM1 and siLGP2 RNAs. The cell culture media of transfected cells were replaced daily and the spent media harvested on days 1–5 after transfection were analyzed by ELISA for the accumulation of IFNβ protein using procedures described in *Materials and Methods*. The results shown in Fig. 6*B* suggest that in the cells, depleted PUM1 IFNβ was produced between day 1 and day 2 and reached peak levels by day 4. It then declined to its basal level as detected on day 1 after transfection. IFNβ production was not detected in cells transfected with siLGP2 or both siPUM1 and siLGP2 RNAs. We conclude from these results that IFNβ production requires depletion of PUM1 and activation of LGP2. Both sets of experiments exclude the alternative hypothesis that IFNβ is induced by an alternative pathway as a consequence of transfection of siRNAs.

In the third series of experiments, we ascertained that the antiviral activity produced in siPUM1-transfected cells was due to IFNβ. First we tested for antiviral inhibitory activity in the spent medium harvested 48 h after mock transfection or transfection

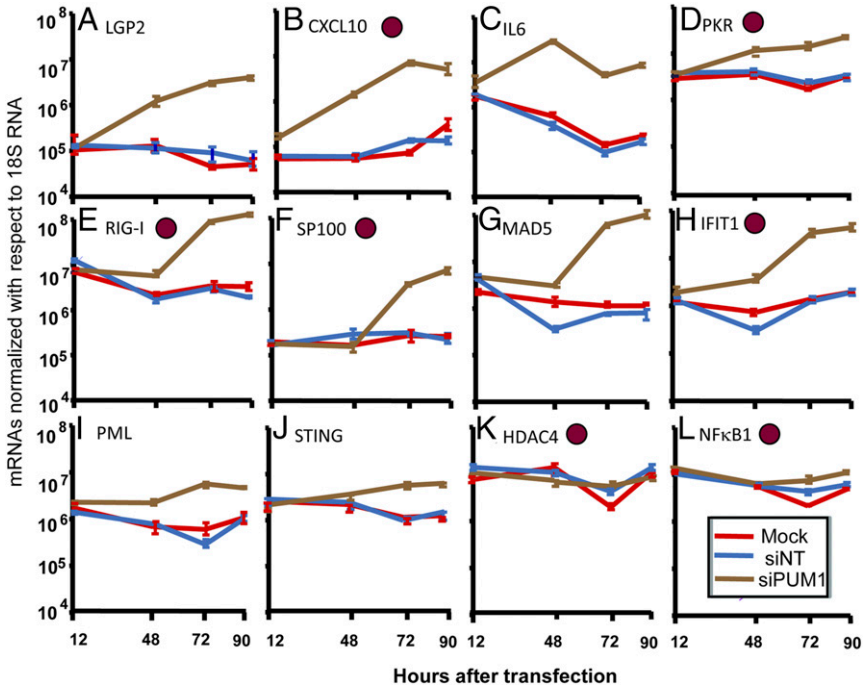


**Fig. 2.** Accumulation of mRNAs of selected cellular genes in HEp-2 cells at times after mock transfection or transfection of NT and PUM1 siRNAs. HEp-2 cells seeded on six-well plates were either mock treated or transfected with 100 nM of PUM1 siRNA or nontarget siRNA (siNT). The cells were harvested at 12, 48, 72, or 90 h after transfection. Total RNAs were extracted and reverse transcribed to cDNA as described in *Materials and Methods*. mRNAs encoding LGP2 (*A*), CXCL10 (*B*), IL6 (*C*), PKR (*D*), RIG-I (*E*), SP100 (*F*), MDA5 (*G*), IFIT1 (*H*), PML (*I*), STING (*J*), HDAC4 (*K*), and NF-κB1 (*L*) were normalized with respect to 18S RNA and plotted as a function of time after transfection. The red circle indicates that the mRNA contains the affinity sequence required from binding to PUM1 or PUM2.

of 100 nM of siNT or siPUM1. In these experiments, replicate cultures of HEp-2 cells in six-well plates were exposed to amounts of spent medium ranging from 0.125 to 2 mL. After 24 h of incubation, the cells were exposed to 0.1 pfu of HSV-1(F) per cell. The cells were harvested 24 h after infection and analyzed for the accumulation of viral proteins representative of different kinetic classes. The results (Fig. 6*C*) show that the yields of viral proteins were not affected by the exposure to 2 mL of spent medium from cultures of mock-transfected or siNT-transfected cells. Infected cells exposed to spent medium from culture transfected with siPUM1 yielded fewer viral proteins in spent medium dose-dependent fashion.

We next tested whether the inhibitor present in spent medium of cells transfected with siPUM1 was IFNα, IFNβ, or IFNγ. In these experiments, 2-mL aliquots of the spent medium from siPUM1-transfected cells (siPUM1-S) were incubated for 2 h at 37 °C with antibody to IFNα, IFNβ, or IFNγ as detailed in *Materials and Methods*. Replicate HEp-2 cell cultures were then exposed to 2-mL amounts of Mock-S, siNT-S, or siPUM1-S or the siPUM1-S antibody mixtures. After 24 h the cells were exposed to 0.1 pfu of HSV-1(F). Fig. 6*D* shows the amounts of various viral proteins present in cells harvested 24 h after infection. The results suggest that the spent medium harvested from cells transfected with siPUM1 contained IFNβ, inasmuch as cells exposed to spent medium neutralized with anti-IFNβ accumulated two- to fourfold more proteins than the controls. We did not detect evidence of antiviral effects due to IFNγ (Fig. 6*D*, lane 7).

Additional data supporting the hypothesis that cells depleted of PUM1 secrete IFN into extracellular fluid emerged from two

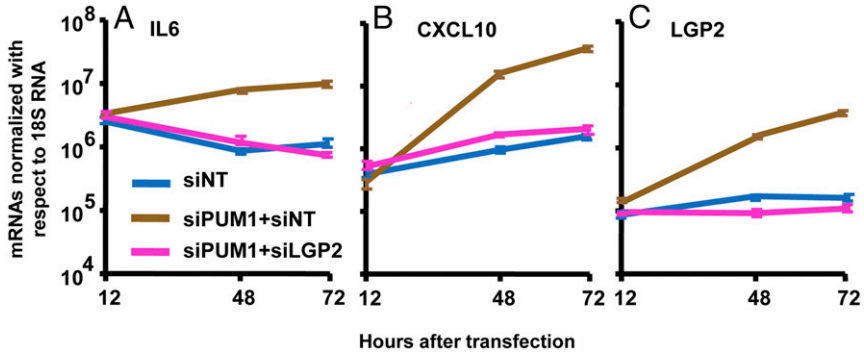


**Fig. 3.** Accumulation of mRNAs encoding IL6 and CXCL10 in HEp-2 cells depleted of PUM1 and LGP2 RNAs. HEp-2 cells seeded on 12-well plates were transfected with nontarget siRNA (siNT) or cotransfected siPUM1 and NT siRNAs or siPUM1 and siLGP2 RNAs. The cells were harvested at 12, 48, or 72 h after transfection. Total RNAs were extracted and reverse transcribed to cDNA as described in *Materials and Methods*. mRNA levels of IL6 (*A*), CXCL10 (*B*), and LGP2 (*C*), were normalized with respect to 18S RNA.

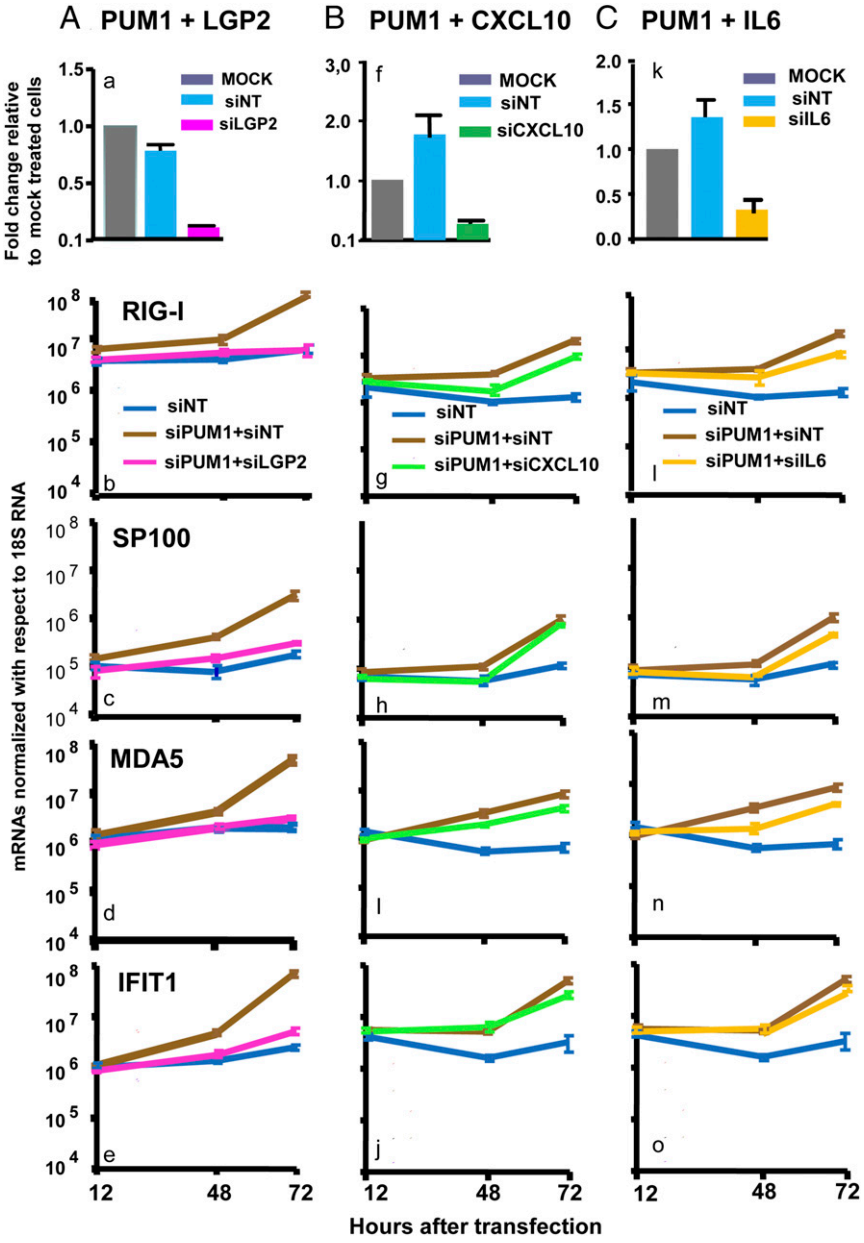


**Fig. 4.** Accumulation of mRNAs of selected cellular genes in HEp-2 cells depleted of PUM1 and LGP2 (*A*), PUM1 and CXCL10 (*B*), or PUM1 and IL6 (*C*) by transfection of siRNAs. HEp-2 cells seeded on 12-well plates were either transfected with nontarget siRNA (siNT) or cotransfected with siPUM1 and siNT RNAs or siPUM1 and siLGP2 RNAs (*A*), siPUM1 and siCXCL10 RNAs (*B*), or siPUM1 and siIL6 RNAs (*C*).The final concentration of total siRNA was 80 nM. The cells were harvested at 12, 48, or 72 h after transfection. Total RNAs were extracted and reverse transcribed to cDNA as described in *Materials and Methods*. mRNA levels of RIG-I, SP100, MDA5, and IFIT1 were normalized with respect to 18S RNA. Depletion efficiency of mRNA levels LGP2 (*A*, *a*), CXCL10 (*B*, *f*), and IL6 (*C*, *k*) by siRNAs were verified and normalized with mock-treated cells.

series of experiments. Thus, cells exposed to Mock-S, siNT-S, or siPUM1-S as above were harvested 48 h after transfection and analyzed with respect to the level of mRNAs encoding known IFN-stimulated genes (ISG). The procedures were the same as those used in preceding experiments (Fig. 2). The results (Fig. 7*A*) show that the spent medium from cells incubated with siPUM significantly up-regulated the mRNA encoding LGP2, RIG-I, STING, IFIT1, and SP100. Although the up-regulation of PKR was significant, it was lower than that of other genes tested in this assay.

In the second experiment, we examined the accumulation of several ISG proteins in lysates of cells maintained in Mock-S, siNT-S, or siPUM1-S for 24 h. The procedures were as described earlier in the text. The results (Fig. 7*B*) show that the cells exposed to siPUM1-S expressed levels of PML, IFIT1, and STING more than twofold higher than those exposed to Mock-S. Again in this assay the up-regulation of PKR was lower than that of other genes tested in this assay.

**Depletion of PUM2 Does Not Mimic the Effects of Depletion of PUM1.** In view of the close relationship between PUM1 and PUM2, the question arises whether PUM2 is also involved in the regulation of innate immune responses described in this report. The following experiments were done to resolve this question.

First, we selected a siRNA that depleted PUM2 in HEp-2 cells. As shown in Fig. 8*A* transfection of HEp-2 cells with

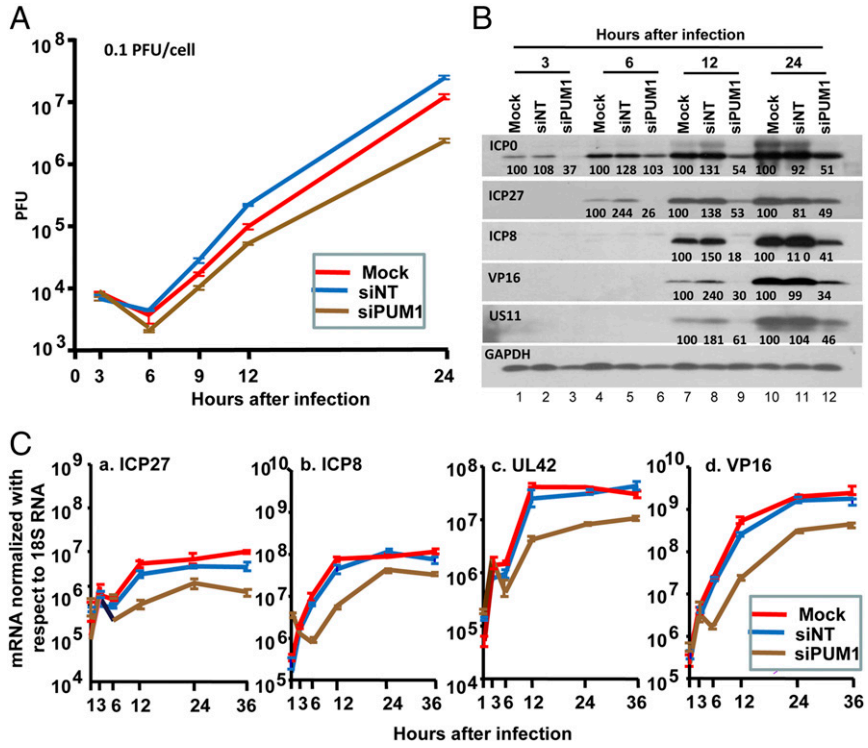


**Fig. 5.** Accumulation of infectious virus, representative viral proteins, and viral mRNAs in cells mock transfected or transfected with NT or PUM1 siRNA. (*A*) Virus yields in PUM1-depleted cells. Replicate cultures of HEp-2 cells in six-well plates were mock treated or transfected with 100 nM PUM1 siRNA or irrelevant nontarget siRNA (siNT). After 48 h of incubation, the cells were exposed to 0.1 pfu of HSV-1(F) per cell for 2 h. The inoculum then was replaced with fresh medium. Virus progeny were harvested at the times shown and titered in Vero cells. (*B*) Accumulation of selected viral proteins in PUM1-depleted cells. HEp-2 cells in six-well plates were either mock treated or transfected with 100 nM siPUM1 or nontarget siNT RNAs for 66 h. The cells were then exposed to 0.5 pfu of HSV-1(F) per cell and harvested at 3, 6, 12, or 24 h after infection. The proteins were electrophoretically separated in a 10% denaturing gel and reacted with indicated antibodies. The band density at 3, 6, 12, and 24 h after infection was normalized with respect to GAPDH and the values were obtained from mock-transfected cells. (*C*) Accumulation of selected viral mRNAs in PUM1-depleted cells. HEp-2 cells were mock transfected or transfected with NT siRNA or PUM1 siRNA and then infected with 0.5 pfu of HSV-1(F) per cell as described in *B*. The cells were harvested at 1, 3, 6, 12, 24, or 36 h after infection. Total RNAs were extracted, reverse transcribed to cDNA as described in *Materials and Methods*. The mRNAs encoding viral proteins ICP27, ICP8, UL42, or VP16 were quantified and normalized with respect to 18S RNA.

100 nM of siRNAs 437, 961, or 1814 effectively depleted PUM2. The sequence of the siRNA selected for the next series of experiments is listed in *Materials and Methods*.

Next, six-well cultures of HEp-2 cells were mock treated or transfected with siNT or PUM2 siRNAs. The cells were harvested at 36, 48, or 60 h after transfection, solubilized, electrophoretically separated on denaturing polyacrylamide gels, and reacted with antibodies against PUM2, PUM1, PKR, or STING. The results in Fig. 8*B* show that, whereas siPUM2 RNA was effective in depleting PUM2, the data do not support the hypothesis that depletion of PUM2 affects the accumulation of PUM1, PKR, or STING.

Lastly, six-well cultures of HEp-2 cells were transfected as above. At 60 h after transfection the cells were exposed to 0.5 pfu of HSV-1(F) per cell. The cells were harvested at 3, 6, 12, or 24 h after infection, processed as above but reacted with viral proteins differing with respect to time of synthesis after infection. The results shown in Fig. 8*C* indicate that depletion of PUM2 had no impact on the accumulation of viral proteins.

## Discussion

PUM1 is known primarily as a sequence-specific mRNA binding protein (1, 10–13, 22). In that role its function is to regulate the stability and function of the mRNA (4, 6–9, 23). PUM1 has also been reported to interact with LGP2 and in that context to affect innate immune response in the course of a Newcastle virus infection (20). The key finding described in this report is that depletion of PUM1 but not of PUM2 induces a biphasic activation of innate immune responses that culminate in the production of IFN. The genes activated in phase 1 between 12 and 48 h after transfection of siPUM1 RNA included LGP2, CXCL10, IL6, and PKR. The genes activated in phase 2 between 48 and 72 h after transfection of siPUM1 RNA included RIG-I, SP100, MDA5, IFIT1, PML, STING, and IFNβ. We could not demonstrate up-regulation of HDAC4 or NF-κB1. We noted a slight up-regulation of IFNγ RNA but not the actual accumulation of the IFNγ. Finally the spent medium containing IFNβ was able to induce in fresh untransfected cells both the genes linked to activation of IFNβ in the first place (e.g., LGP2) as well as genes previously shown to be up-regulated by IFNβ (e.g., PML, SP100, etc.) The list included RIG-I, PKR, STING, and IFIT1.

Relevant to the results reported here are that up-regulation of phase 1 genes required the expression of LGP2. Thus, CXCL10 and IL6 were not up-regulated in cells depleted of both PUM1 and LGP2 and that up-regulation of phase 2 genes also required the expression of LGP2. Thus, representative phase 2 genes (RIG-I, SP100, MDA5, and IFIT1) were up-regulated in cells depleted of PUM1 and CXCL10 or PUM1 and IL6 but not in cells depleted of PUM1 and LGP2.

The results obtained to date do not support the hypothesis that PUM1 represses phase 1 and 2 genes by binding to the mRNAs and that the increase in accumulation of mRNAs is the consequence of increased longevity in the absence of PUM1. Of the phase 1 and 2 up-regulated genes LGP2, IL6, MDA5,

STING, IFNβ, and PML mRNAs do not have a PUM1 binding site. Moreover, whereas PUM2 binds a similar mRNA sequence, cells depleted of PUM2 do not overexpress the genes overexpressed in PUM1-depleted cells. An alternative hypothesis that takes note of the nearly 100-fold increase in the accumulation of LGP2 mRNA in the course of 48 h after transfection of siPUM1 RNA is that PUM1 in association with other factors acts as a transcriptional repressor.

A key question that emerges from the studies reported here is that if LGP2 is a master activator of innate immunity genes why are some genes activated early after depletion of PUM1 (phase 1), whereas others are activated after a significant delay (phase 2). One hypothesis that could explain our results is that the activation of LGP2 is required but is not sufficient for the activation of phase 2 genes. A hypothesis consistent with the results presented here is that LGP2 activates a component of the innate

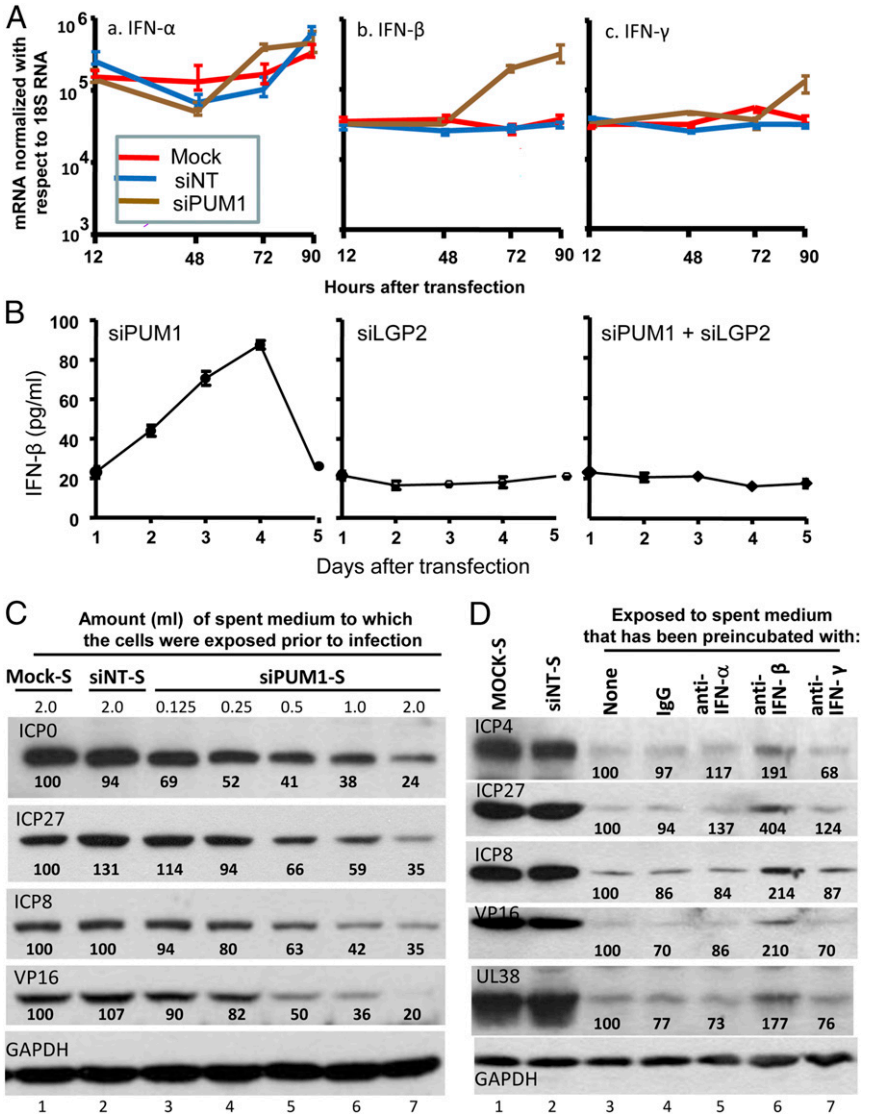


**Fig. 6.** Depletion of PUM1 resulted in the accumulation of IFNβ mRNA and diminished accumulation of selected HSV-1 proteins. (*A*) Depletion of PUM1 resulted in the accumulation of IFNβ mRNA. The $3 \times 10^5$ HEp-2 cells seeded in six-well plates were either mock treated or transfected with 100 nM siPUM1 or siNT RNAs. The cells were harvested at 12, 48, 72, or 90 h after transfection. Total RNAs were extracted and reverse transcribed to cDNA as described in *Materials and Methods*. mRNA expression of IFNα, IFNβ, and IFNγ were normalized with respect to 18S RNA. (*B*) Dynamic state of accumulation of IFNβ protein from cells depleted of PUM1. HEp-2 cells seeded on six-well plates were transfected with siPUM1, siLGP2, or cotransfected with siPUM1 and siLGP2 siRNAs. The 1.5-mL amounts of spent culture medium were harvested from each plate at days 1–5 after transfection. IFNβ levels in culture medium were measured using ELISA kits for IFNβ. (*C*) Spent medium harvested from cultures of PUM1-depleted cells blocked HSV-1 replication in a dose-dependent manner. Spent medium was collected from HEp-2 cells either mock treated or transfected with 100 nM PUM1 siRNA or nontarget siRNA (siNT) for 48 h. Fresh HEp-2 cells were incubated with the spent medium with indicated amount for 24 h, then infected with 0.1 pfu of HSV-1(F) per cell. The cell lysates were collected 24 h postinfection, denatured, electrophoretically separated in a 10% denaturing gel, and reacted with indicated antibodies. The band densities were normalized with respect to GAPDH and shown as percentages of the values obtained for Mock-S–treated cells. (*D*) Anti-IFNβ but not anti-IFNα or anti-IFNγ attenuated the antiviral effects of spent medium collected from cultures of PUM1-depleted cells. The 2-mL amounts of spent medium collected from mock treated, transfected with siNT RNA, or siPUM1 RNAs were incubated with antibodies against IgG (25 μg), IFNα (12.5 μg), IFNβ (25 μg), or IFNγ (25 μg) at 37 °C for 2 h. Fresh cultures of HEp-2 cells in 12-well plate were incubated with the spent medium preincubated with IFN antibodies described above for 24 h, then infected with 0.1 pfu of HSV-1(F) per cell. The cell lysates were collected at 24 h after infection, electrophoretically separated in a 10% denaturing gel, and reacted with indicated antibodies. The band density was normalized with respect to GAPDH and shown as percentages of the values obtained for mock-treated cells.

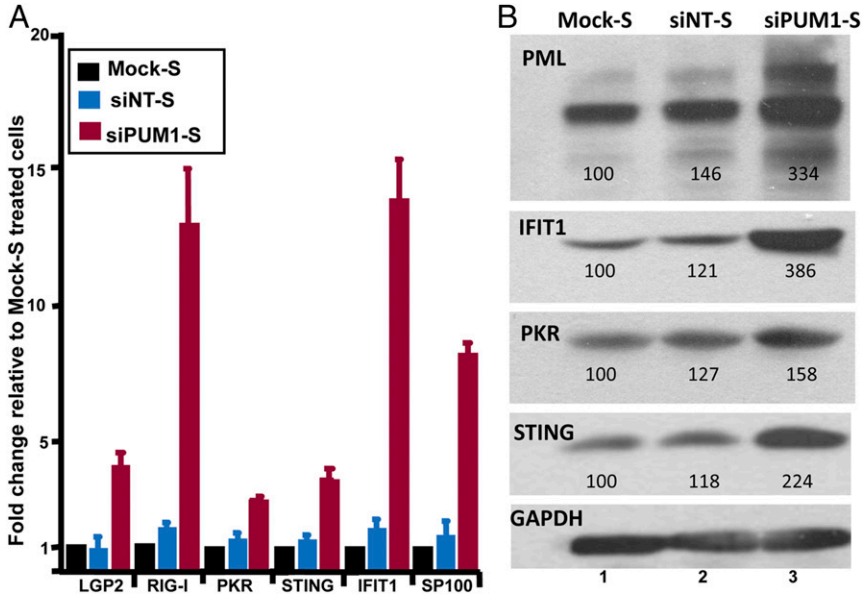


**Fig. 7.** Depletion of PUM1 resulted in accumulation of IFN-stimulated gene (ISG) products. (*A*) mRNA accumulation of ISG mRNAs in cells exposed to spent medium collected from mock treated, transfected with NT, or PUM1 siRNAs. HEp-2 cell cultures are mock transfected or transfected with 100 nM siPUM1 or siNT RNAs. After 48 h, the spent medium was collected and overlayed on fresh cultures of HEp-2 cells. After 20 h the cells were processed as described in Fig. 6*A*. A shows the amounts of mRNAs for several ISGs normalized with Mock-S–treated cells. (*B*) Accumulation of ISG proteins in cells exposed to spent medium collected from mock treated, transfected with NT, or PUM1 siRNAs. The experimental design is identical to that shown in Fig. 6*C*. The lysates of cells, collected 24 h after exposure to spent media, were electrophoretically separated in a 10% denaturing gel and reacted with indicated antibodies. The band density was normalized with respect to GAPDH and is shown as percentages of the values obtained for Mock-S–treated cells.

immune system, which in turn activates phase 2 genes. The identity of this component is not known. One component of the innate immune system, which we cannot exclude, is IFN itself. One example of the potential complexity of innate immune gene regulation relates to regulation of PML and SP100, key components of ND10 nuclear bodies and important innate immune responders to viral infections (24–29). In this report we show that depletion of PUM1 resulted in LGP2-dependent activation of both PML and SP100. Other studies have shown that PML and SP100 are induced on exposure of cells to interferons (30–32). We cannot exclude the possibility that some of the phase 2 genes are in fact up-regulated by IFN.

The significant finding to emerge from the studies reported here is that PUM1 is a negative regulator of LGP2—a master activator of what appears to be a cascade of innate immunity genes. At the same time its precise mode of action of both LGP2 and PUM1 remains obscure. The reports published to date depict LGP2 as a multifunctional protein whose function at any one time may be dependent on interactions with as yet undefined factors. LGP2 was reported to play an essential role in innate immune responses to viral infections that are mediated by RIG-I and MDA5 (33, 34). At the same time overexpression of LGP2 was reported to inhibit RIG-I–mediated antiviral signaling both in the presence or absence of viral ligands (33, 35–37).

LGP2 is a member of the RIG-I–like receptor (RLR) proteins that include RIG-1, MDA5, and LGP2. Most of the studies on the functions of LGP2 were done in the context of viral infections or in cells expressing viral genes. The function of LGP2 as defined in these studies yielded contradictory results and remains to be fully elucidated. Thus, LGP2 expressed from plasmid vectors inhibited IFN-stimulated response element-dependent reporter gene transcription upon Newcastle disease virus or Sendai virus infection (33, 35, 38). LGP2 was also induced during antiviral signaling and functioned as a feedback inhibitor of antiviral responses (36). Other studies reported LGP2 as a positive regulator of antiviral responses. Thus, $LGP2^{-/-}$ mice are more susceptible to infection and have defects in generating antiviral responses, notably in response to the encephalomyocarditis virus and poliovirus infection (36, 38, 39). Moreover, LGP2's positive contributions to antiviral signaling were highlighted by the actions of IFN antagonists, the paramyxovirus V proteins, which can bind directly to the LGP2 helicase domain and disrupt its ATP hydrolysis activity (40, 41).

The studies reported here were done in the absence of infection or the presence of viral proteins that could affect the function of LGP2. In these studies LGP2 functioned as master regulator of numerous genes that included the genes encoding the related RLR proteins. In turn, LGP2 was subject to negative control by PUM1.

Lastly, the findings reported here were based on knockdown rather than knockout of PUM1. By using siRNA to knockdown PUM1, we were able to monitor the status of key innate immunity gene products as a function of time over a period of at least 72 h after transfection of siRNA.

## Materials and Methods

**Cells and Viruses.** HSV-1(F) is a limited-passage prototype strain used in this laboratory (42). HEp-2 cells were obtained from the American Type Culture Collection. All cells were grown in DMEM containing 10% FBS.

**Depletion of PUM1, PUM2, LGP2, CXCL10, or IL6 Levels in HEp-2 Cells by siRNA.** All transfections were carried out using Lipofectamine 2000 (Invitrogen) by its manufacturer's instructions. The sequences for the siRNA were as follows: siPUM1-1777, 5′-GCUGCUUACUAUGACCAAATT-3′; siPUM1-2652, 5′-GGAGAUUGCUGGACAUAUATT-3′; siPUM1-412, 5′-GGCGGCUAUAAUAAUAGCATT-3′; siLGP2, 5′-CCGGAAAUUUGGGACGCAATT-3′; siCXCL10, 5′-CCUUAUCUUUCUGACUCUATT-3′; and siIL6, 5′-CCCAGGAGAAGAUUCCAAATT-3′. The siNT (5′-UUCUCCGAACGUGUCACGUTT-3′) was used as negative control.

**RNA Extraction and Measurements by Real-Time Quantitative PCR.** Total RNAs were extracted with the aid of TRI Reagent solution (Thermo Scientific) according to the manufacturer's instructions, standardized by optical density measurements, and then treated with DNase I (Takara). Viral and cellular cDNA were synthesized from 0.5 μg of total RNA with the aid of the

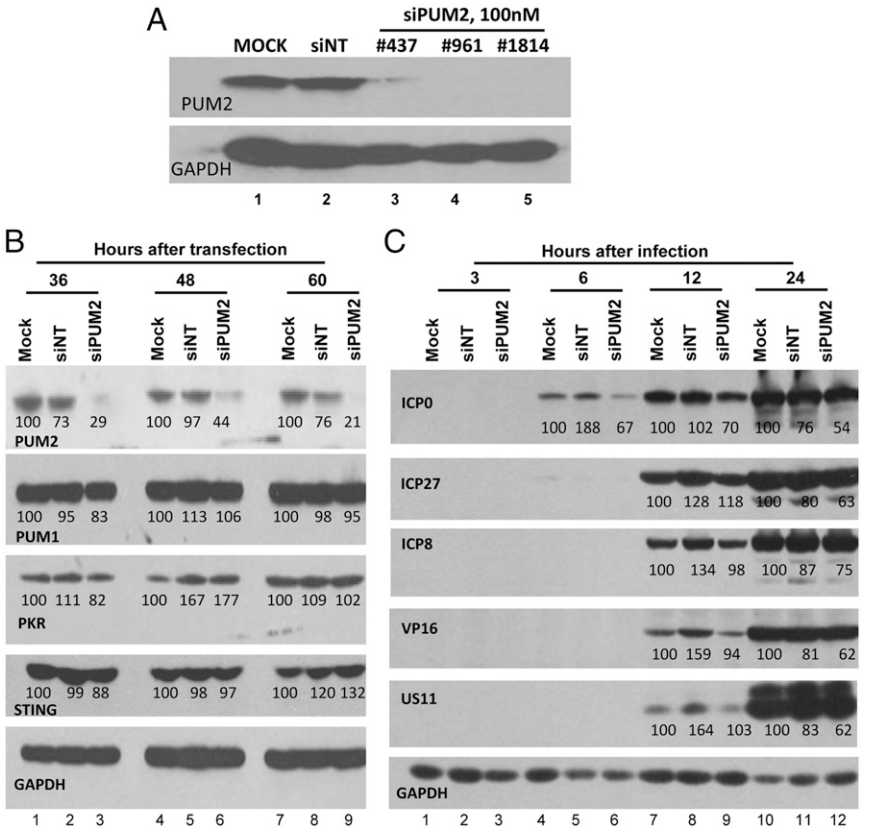


**Fig. 8.** Unlike PUM1, depletion of PUM2 in HEp-2 cells has no effect on the accumulation of host or viral proteins. (*A*) Efficiency of PUM2 depletion by siRNA. The $3 \times 10^5$ HEp-2 cells seeded in six-well plates were either mock treated or transfected with 100 nM siPUM2 or siNT RNA. The cells were harvested at 70 h after transfection. Lysates containing 70 μg of total proteins were separated on denaturing 10% polyacrylamide gels, electrically transferred to nitrocellulose sheets, and immunoblotted for PUM2 protein. GAPDH was used as loading control. (*B*) Depletion of PUM2 protein has no effect on accumulation of host proteins. The $3 \times 10^5$ HEp-2 cells seeded on six-well plates were either mock treated or transfected with 100 nM siPUM2 or siNT RNAs. The cells were harvested at 36, 48, and 60 h after transfection. Lysates containing 70μg of total proteins were separated on denaturing 10% polyacrylamide gels, electrically transferred to nitrocellulose sheets, and immunoblotted for indicated proteins. The band density was normalized with respect to GAPDH and shown as percentages of the values obtained from mock-transfected cells. (*C*) Depletion of PUM2 has no effect on HSV-1(F) replication. HEp-2 cells in six-well plates were mock treated or transfected with 100 nM siPUM2 or siNT RNAs. At 60 h after transfection, the cells were exposed to 0.5 pfu of HSV-1(F) per cell and harvested at 3, 6, 12, or 24 h after infection. The cells were solubilized and the denatured proteins were electrophoretically separated in a 10% denaturing gel and reacted with indicated antibodies. The band densities at 6, 12, and 24 h after infection were normalized with respect to GAPDH and shown as percentages of the values obtained fom mock-transfected cells.

ReverTra Ace q-PCR RT kit (Toyobo) in accordance with instructions provided by the suppliers. Viral and cellular DNA copy numbers were then quantified by SYBR green real-time PCR technology (Toyobo) using primers listed in Table 1, normalized with respect to 18S RNA.

**Immunoblot Assays.** Cells were collected at the indicated times after infection. The procedures for harvesting, solubilization, protein quantification, SDS/PAGE, and transfer to nitrocellulose membranes were as described previously (43). Images were quantified with ImageJ by integration of pixel values across the area of specific bands.

**Antibodies.** Antibodies against to HSV-1 ICP4 (44), ICP27 (44), ICP0 (44), ICP8 (Rumbaugh Goodwin Institute for Cancer Research), VP16 (45), UL38 (46), and US11 (47) have been reported elsewhere. These studies used rabbit monoclonal antibodies against PUM1 (92545, Abcam), PUM2 (92390, Abcam), PKR (32506, Abcam), PKR-p-T446 (32036, Abcam), PML (179466, Abcam), IFIT1 (D2 × 9Z, CST), GAPDH (2118, CST), β-actin (60008–1-Ig, Proteintech), and rabbit polyclonal against STING (19851–1-AP, Proteintech).

**Virus Titration.** HEp-2 cells were transfected with 100 nM of PUM1 siRNA. After 48 h, the cells were exposed to 0.1 pfu of HSV-1(F) per cell. The cells were harvested at indicated times after infection. Viral progeny was titrated on Vero cells.

**Assays of Spent Medium.** Spent medium collected 48 h after transfection of HEp-2 cells with 100 nM of PUM1 siRNA was overlayed on fresh cultures of HEp-2 cells as described in *Results*. The treated cell cultures were harvested for quantitative PCR assay of indicated host cell mRNAs or exposed to 0.1 pfu of HSV-1(F) per cell and harvested at 24 h after infection for quantification of viral proteins.

**IFN Neutralization Assay.** Two-milliliter amounts of spent culture medium harvested from HEp-2 cells 48 h after transfection of PUM1 siRNA (100 nM) were incubated with either anti-IgG (B900610, Proteintech), anti-IFNα (21118–1, PBL), anti-IFNβ (MAB814-100, R&D Systems), or anti-IFNγ (MAB285-100, R&D Systems) antibodies at 37 °C for 2 h. Fresh HEp-2 cells grown in 12-well plates were then exposed to the mixtures of spent medium and antibodies as described above. After 24 h of incubation the cultures were exposed to 0.1 pfu of HSV-1(F) per cell and harvested at 24 h after infection.

**ELISA Analysis.** The 1.5-mL amounts of spent culture medium were harvested from HEp-2 cells at days 1–5 after transfection of 100 nM of either PUM1 siRNA, LGP2 siRNA alone, or PUM1 siRNA plus LGP2 siRNA. Culture supernatants were collected and the IFN concentrations were measured using ELISA kits for IFNβ (Thermo Scientific) according to the manufacturer's instructions.

**ACKNOWLEDGMENTS.** These studies were supported by a Grant from Shenzhen Overseas High-Caliber Peacock Foundation (KQTD2015071414385495) to Shenzhen International Institute for Biomedical Research; National Nature Science Foundation of China Grant NSFC 81472826, Guangdong Nature Science Foundation Grant 2016A030308007, and Guangzhou Science Technology and Innovation Commission Project Grant 201504010016 to Guangzhou Medical University.

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