CORRESPONDENCE

Virus-inducible IGFALS facilitates innate immune responses by mediating IRAK1 and TRAF6 activation

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Activation of TLR signaling is a first line of the host defense system in the elimination of invading pathogens.[1] Insulin-like growth factor binding protein, acid-labile subunit (IGFALS) is a leucine-rich glycoprotein. By prolonging the half-life of IGF-I in the vascular system,[2] IGFALS regulates the bioavailability of IGF, which is crucial for normal growth and development and metabolic regulation.[3,4] However, the role of IGFALS in antiviral innate immune responses has not been established. In this study, we found that IGFALS is virus inducible, while it in turn inhibits viral replication. Mechanistically, IGFALS directly associates with IRAK1 and TRAF6, facilitating IRAK1/TRAF6 complex formation and enhancing K63-linked polyubiquitination of both proteins for full activation, thereby facilitating antiviral signaling.

First, we assessed the interplay of viruses and IGFALS. IGFALS protein expression was significantly enhanced with IAV or SeV infection (Fig. 1A). In addition, IAV replication was inhibited in A549 cells with IGFALS overexpression (Fig. 1B). Moreover, aberrant expression of IGFALS diminished SeV replication (Fig. 1C). Then, we constructed specific shRNAs targeting IGFALS. Consistent with the above findings, IGFALS knockdown enhanced VP1 mRNA levels in EV71-infected RD cells (Fig. 1D). Next, we generated mouse embryonic fibroblasts (MEFs) to confirm the antiviral activity of IGFALS. We found that both the mRNA and protein levels of EV71 VP1 were elevated in IGFALS-deficient MEFs (Fig. 1E).

Activation of antiviral signaling cascades leads to the production of type I and III interferons (IFNs).[5] The results of qPCR experiments indicated that overexpression of IGFALS potentiated VSV-induced IFN production (Fig. 1F). Furthermore, we generated IGFALS-deficient splenocytes; the qPCR analysis results demonstrated that

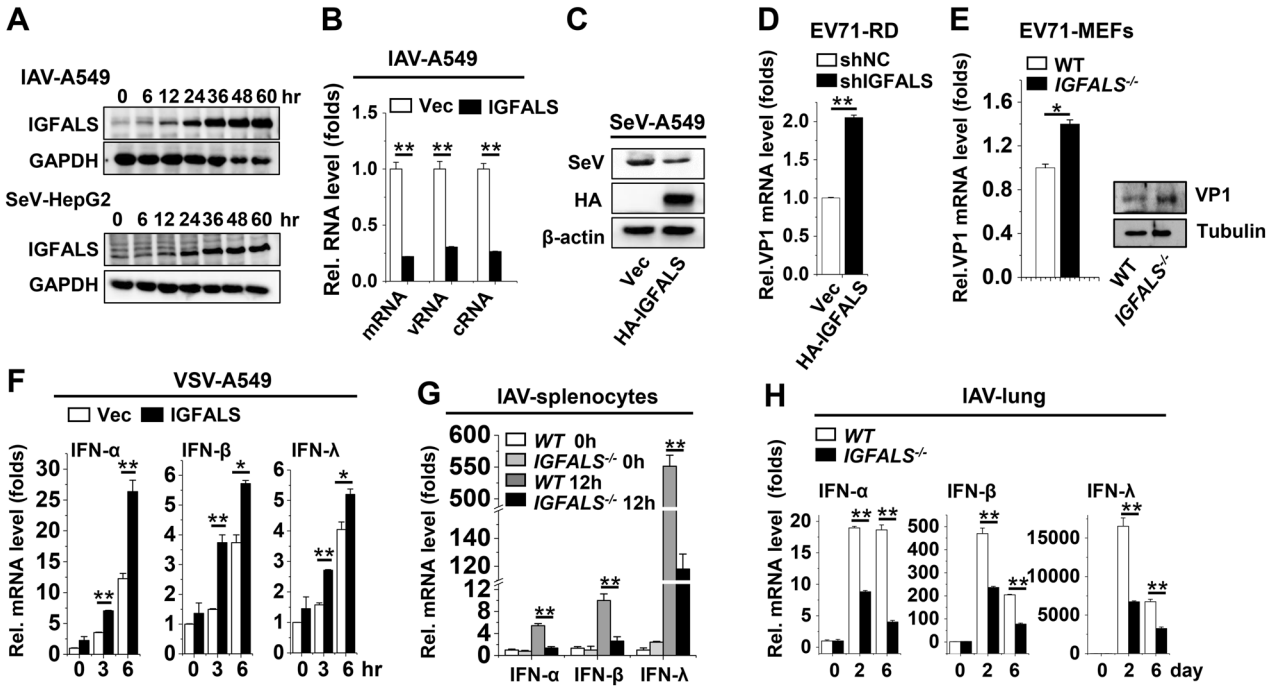

Fig. 1 Continued.

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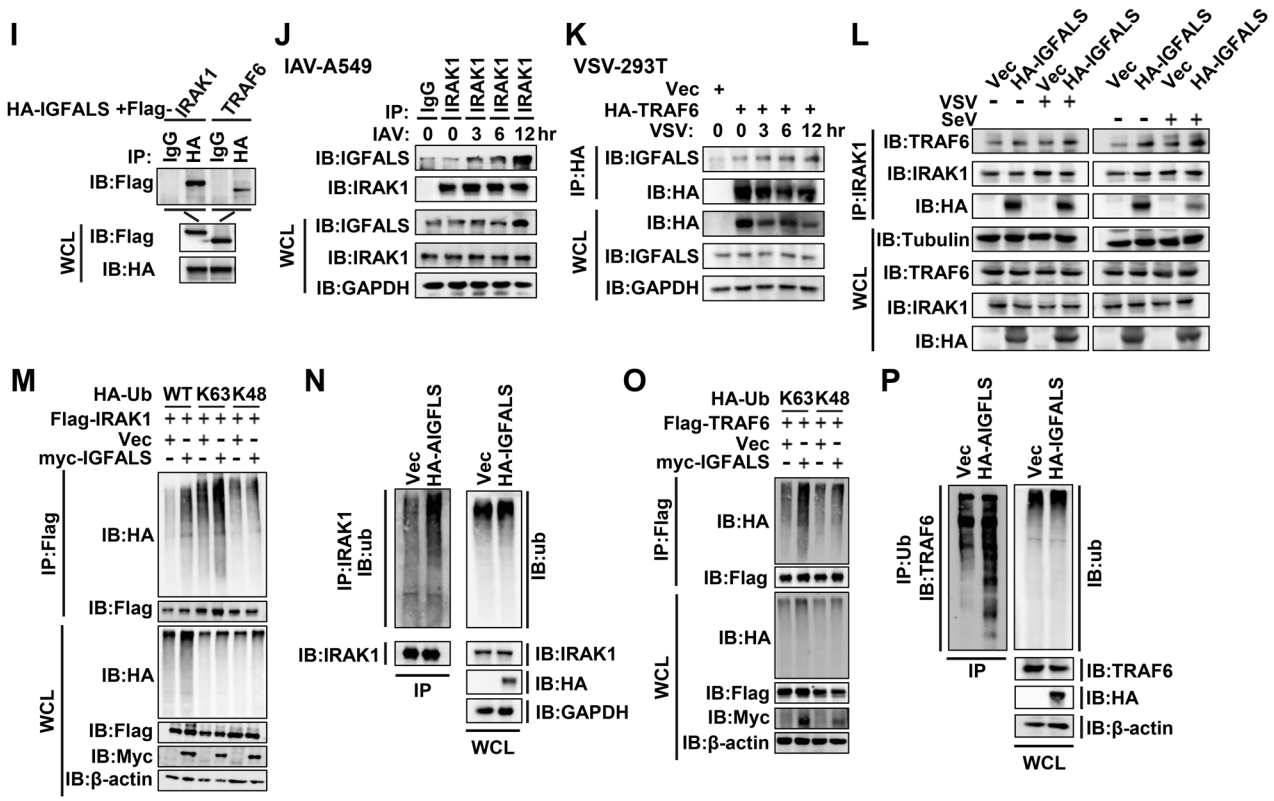

Fig. 1 **A** A549 and HepG2 cells were infected with IAV or SeV at an MOI of 1 for the indicated hours. Cells were then lysed for immunoblot assays. **B** A549 cells were transfected with empty vector (Vec) or HA-tagged IGFALS plasmids for 24 h and were then infected with IAV (MOI = 1) for 24 h. The relative levels of IAV NP-specific mRNA, cRNA, and vRNA were quantified using a real-time RT-PCR assay. **C** A549 cells transfected with the indicated plasmids were infected with SeV (MOI = 1) for 12 h before immunoblot analysis with the indicated antibodies. **D** qRT-PCR analysis of EV71 VP1 mRNA levels in RD cells transfected with shNC or shIGFALS plasmids for 24 h and then infected with EV71 (MOI = 1) for 8 h. **E** Wild-type or IGFALS knockout MEFs were infected with EV71 (MOI = 1), and VP1 mRNA and protein expression was quantified by qRT-PCR (left graphs) and western blot analysis (right panels), respectively. **F** qRT-PCR analysis of IFN-α, IFN-β, and IFN-λ mRNA expression in A549 cells transfected with empty vector or the IGFALS expression plasmid and then infected with VSV (MOI = 1) for 0, 3, or 6 h. **G** WT and IGFALS$^{-/-}$ splenocytes were left uninfected or infected with IAV (MOI = 1) for 0–12 h before qPCR analysis of IFN mRNA levels. **H** WT and IGFALS$^{-/-}$ mice ($n = 3$) were infected with IAV at 10^4 TCID50 for 0, 2, or 6 days. mRNA levels of IFN in mouse lung tissues were quantified by qRT-PCR. **I** Immunoprecipitation and immunoblot analysis of 293T cells transfected with the indicated plasmids for 24 h. **J** A549 cells were infected with IAV (MOI = 1) for the indicated times. Subsequently, cells were lysed and subjected to immunoprecipitation and immunoblot analysis. **K** 293T cells were transfected with empty vector or HA-TRAF6 plasmids as indicated and infected with VSV (MOI = 1) for 0–12 h. The cell lysates were subjected to coimmunoprecipitation and immunoblot analysis, as indicated. **L** 293T cells were transfected with the indicated plasmids for 24 h, infected with VSV or SeV at an MOI of 1 for 6 h, and subjected to immunoprecipitation and immunoblot analysis. **M** 293T cells were transfected with HA-tagged WT, K63 or K48 Ub plasmids together with Flag-IRAK1 and empty vector or myc-IGFALS plasmids for 24 h. Coimmunoprecipitation and immunoblotting were performed. **N** 293T cells were transfected with empty vector or HA-IGFALS for 24 h, infected with VSV (MOI = 1) for 6 h, and subjected to immunoprecipitation and immunoblot analysis. **O** 293T cells were transfected with the indicated plasmids for 24 h and were then subjected to coimmunoprecipitation and immunoblot analysis. **P** The experiments were performed similarly to those in **N**, except an anti-Ub antibody was used for immunoprecipitation and an anti-TRAF6 antibody was used for immunoblot analysis. The data are representative of three independent experiments and are presented as the mean ± SEM values. *$p < 0.05$, **$p < 0.01$

IGFALS deletion inhibited the transcription of IFNs in these cells (Fig. 1G). In addition, we intranasally administered a lethal dose of IAV to IGFALS KO mice and WT mice and found that IFN production in the lungs was inhibited by IGFALS deficiency (Fig. 1H).

To further identify the molecular mechanism of IGFALS in facilitating innate immune responses, we investigated the interaction of IGFALS with several molecules involved in the antiviral signaling pathway and found that both exogenous IRAK1 and TRAF6 were coimmunoprecipitated by overexpressed IGFALS (Fig. 1I). Consistent with this result, the association of endogenous IGFALS with IRAK1 and TRAF6 was increased after IAV and VSV infection (Fig. 1J, K). Recognition of pathogens by TLRs leads to myddosome formation and activation of IRAK1 and its subsequent association with its downstream effector TRAF6.[6] We hypothesized that IGFALS might act as a scaffold protein mediating their interaction. We performed coimmunoprecipitation assays and found that endogenous recruitment of IRAK1 and TRAF6 was potentiated by IGFALS in 293T cells with or without VSV or SeV stimulation (Fig. 1L). TRAF6 catalyzes the addition of K63-linked ubiquitin chains to IRAK1 and itself and triggers transcriptional programs for host immune defense.[7] Therefore, we determined whether IGFALS alters IRAK1 and TRAF6 polyubiquitination. The results of ubiquitination assays revealed that IGFALS enhanced K63-linked but not K48-linked polyubiquitination of IRAK1 and TRAF6 (Fig. 1M, O). Substantially increased levels of endogenous IRAK1 and TRAF6 polyubiquitination were observed in IGFALS-overexpressing 293T cells challenged with VSV (Fig. 1N, P).

In conclusion, our present study reveals evidence for the essential role of IGFALS in antiviral immune responses and provides new insights into the biological function of IGFALS.

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AUTHOR CONTRIBUTIONS

G.X. and Y.Z. conceived and designed the research. G.X. performed the research and analyzed the data. F.D., Q.Z., L.L., K.D., Z.C., W.C., C.L., C.Y., and S.L. contributed to performing the experiments and participated in discussion. Y.Z. contributed critical reagents. G.X. and Y.Z. wrote the manuscript with editorial input from all authors.

ADDITIONAL INFORMATION

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Competing interests: The authors declare no competing interests.

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