

Real-time 2-5A kinetics suggest that interferons β and λ evade global arrest of translation by RNase L

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Cells of all mammals recognize double-stranded RNA (dsRNA) as a foreign material. In response, they release interferons (IFNs) and activate a ubiquitously expressed pseudokinase/endoribonuclease RNase L. RNase L executes regulated RNA decay and halts global translation. Here, we developed a biosensor for 2',5'-oligoadenylate (2-5A), the natural activator of RNase L. Using this biosensor, we found that 2-5A was acutely synthesized by cells in response to dsRNA sensing, which immediately triggered cellular RNA cleavage by RNase L and arrested host protein synthesis. However, translation-arrested cells still transcribed IFN-stimulated genes and secreted IFNs of types I and III (IFN- β and IFN- λ). Our data suggest that IFNs escape from the action of RNase L on translation. We propose that the 2-5A/RNase L pathway serves to rapidly and accurately suppress basal protein synthesis, preserving privileged production of defense proteins of the innate immune system.

interferon | translation reprogramming | 2-5A | RNase L | RNA decay

Interferons (IFNs) of type I (α and β) and type III (λ 1, λ 2, and λ 3) are cytokines secreted by cells after exposure to pathogens or internal damage. Both types of IFNs activate transcriptional programs in surrounding tissues to induce the innate immune response. IFN production requires protein synthesis; however, during the innate immune response to double-stranded RNA (dsRNA), IFN production is universally accompanied by translational arrest. Inhibition of protein synthesis arises in part from activation of the dsRNA-dependent protein kinase R (PKR) and in part from signaling by conserved small RNAs that contain 2',5'-linked oligoadenylates (2-5A) (1–4). The action of 2-5A is sufficient for the arrest of translation independent of PKR, and at least in some cell lines, 2-5A is the main cause of translational arrest (5).

In human cells, 2-5A is synthesized by three enzymes: oligoadenylate synthetase 1 (OAS1), OAS2, and OAS3 (OASs), which function as cytosolic dsRNA sensors using dsRNA binding for activation (6, 7). The activity of the OASs is normally low to allow for housekeeping protein synthesis, but it increases in the presence of viral or host dsRNA molecules (8). 2-5A has an antiviral effect, against which some viruses have evolved 2-5A antagonist genes that are essential for infection (9–16).

The 2-5A system is also a surveillance pathway for endogenous double-stranded RNAs (dsRNAs) from mammalian genomes. Cells with adenosine deaminase 1 deficiency accumulate self-dsRNA that promotes 2-5A-driven apoptosis (8). 2-5A synthesis in the presence of low amounts of endogenous dsRNAs does not cause cell death, but rather functions as a suppressor of adhesion, proliferation, migration, and prostate cancer metastasis (17, 18). In addition, activation of the 2-5A system also blocks secretion of milk proteins and stops lactation, presumably as a mechanism to prevent passing infection via breast feeding (19).

All the effects of 2-5A arise from the action of a single mammalian 2-5A receptor, pseudokinase-endoribonuclease L (RNase L) (20). 2-5A binds to the ankyrin-repeat (ANK) domain of RNase L and promotes its oligomerization and the formation

of a dimeric endoribonuclease active site (21–23). This dimer further assembles into high-order oligomers (24) that cleave viral RNAs (16, 18) and all components of the translation apparatus, including mRNAs (25), tRNAs (26), and 28S/18S rRNAs (27, 28). The resulting action of RNase L inhibits global translation, which puts all proteins, including IFNs, at risk for arrest during a cellular response to dsRNA.

The impact of translational shutdown by RNase L on IFN synthesis and paracrine IFN signaling is unknown. Measurements of 2-5A/RNase L activity have been limited by the need for biochemical analyses, which are incompatible with live cells. To address this challenge, we have developed a real-time 2-5A biosensor (patent application WO 2017193051 A1) and used it to elucidate the kinetics of 2-5A-mediated RNase L activation and translational arrest occurring during the cellular response to immunostimulatory dsRNAs. Our biosensor can detect in situ 2-5A synthesis in mammalian cells, and thus it provides a heretofore missing platform for cell-based applications. These applications can range from live cell screens for modulators of innate immune responses to mechanistic analysis of dsRNA sensing, which we describe in this paper.

Results

Real-Time 2-5A Dynamics in Live Cells. In the absence of methods to monitor 2-5A without cell disruption, the cellular dynamics of

Significance

RNase L is a mammalian enzyme that can stop global protein synthesis during interferon (IFN) response. Cells must balance the need to make IFNs (which are proteins) with the risk of losing cell-wide translation due to RNase L. This balance can be achieved most simply if RNase L is activated late in the IFN response. However, by engineering a biosensor for the RNase L pathway, we show that RNase L activation actually precedes IFN synthesis. Furthermore, translation of IFN evades the action of RNase L. Our data suggest that RNase L facilitates a switch of protein synthesis from homeostasis to specific needs of innate immune signaling.

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this second messenger are poorly understood. Here we developed a biosensor for continuous and noninvasive 2-5A monitoring in live cells. The biosensor was designed based on the crystal structures of the cellular 2-5A receptor RNase L (22, 24) (Fig. 1A), indicating that the N-terminal ANK domain of RNase L is sufficient for 2-5A sensing and provides a minimal dimerization module (24). In a single ANK protomer, the N and C termini in *cis* are separated, but on dimerization of two ANK domains, which bind notably head-to-tail, the N and C termini become positioned in *trans* next to each other (Fig. 1B). We used this in *cis* vs in *trans* N–C distance decrease to drive a dual split-luciferase sensor.

This combinatorially optimized sensor has two halves, consisting of the core ANK domain fused to a modified split firefly luciferase (Fluc) (29) (Fig. 1B). We modified Fluc by replacing the published overlapping split junction N416/C415 with a non-overlapping junction (Fig. 2A). Due to the head-to-tail structure, the sensor was encoded as one polypeptide, which simplified its use compared with usual two-protein split systems. We optimized the reporter by engineering the linker regions and selected variant V6 with the highest luminescence response to 2-5A for further work (Fig. 2A). We determined that V6 was suitable for 2-5A detection over a range of 2-5A and V6 concentrations from ~10 nM to at least 1 μ M for V6 and 3 μ M for 2-5A (Fig. 2B and C). This luminescence response was specific and abolished by a control mutation Y312A, which removed the key 2-5A-sensing

tyrosine (24) (Fig. 2B and C). The reporter V6 had sufficient sensitivity to detect low amounts of 2-5A purified from human cells treated with poly-inosine/poly-cytidine (poly-IC) dsRNA (*SI Appendix*, Fig. S1A). These reporter measurements agreed closely with the standard endoribonuclease readout based on rRNA cleavage (*SI Appendix*, Fig. S1B).

To determine whether V6 was sufficiently bright and stable in live cells, we expressed FLAG-tagged V6 in HeLa cells and stimulated these reporter cells with poly-IC. The poly-IC treatment conditions were selected to produce cleavage of 28S rRNA in the conventional endpoint assay using cell disruption (22). 28S rRNA cleavage was noticeable after 1 h of poly-IC treatment and increased further after 3 and 4 h (Fig. 3A). HeLa cells expressing WT FLAG-V6 exhibited a time-dependent increase in luminescence in the presence of cell-permeable D-luciferin ethyl ester (Fig. 3B and *SI Appendix*, Fig. S1C). The luminescence increase began at approximately 30 min of poly-IC treatment and before the appearance of visible 28S rRNA cleavage. HeLa cells expressing control FLAG-V6-Y312A produced no increase in luminescence, confirming that WT V6 detects cellular 2-5A in real time.

Further evidence of specific 2-5A detection was obtained in cells in which 2-5A synthetases were knocked out. It has been shown that knockout of OAS3 is sufficient to inhibit 2-5A synthesis in human cells (11). We found that the biosensor exhibited a robust response to poly-IC in OAS1-KO and OAS2-KO cells. In contrast, the response was lost in OAS3-KO cells (*SI Appendix*, Fig. S2), confirming that 2-5A synthesis gave rise to the reporter activity.

It has been proposed that RNase L may be activated by local production of 2-5A (30) at discrete sites inside cells (the cytosol). However, OASs are present not only in the cytosol, but also in the nucleus (31–33), raising a question about the role of nuclear OASs and suggesting that if 2-5A acts locally, then 2-5A accumulation could be nonuniform between cellular compartments. The availability of live cell 2-5A sensor provides an opportunity to evaluate 2-5A accumulation in individual cellular compartments in situ. To this end, we engineered tagged versions of V6 with a nuclear localization signal (NLS) and nuclear export signal (NES). Both variants localized to the expected sites (Fig. 3C). In the presence of poly-IC, NLS-V6 and NES-V6 produced nearly identical luminescence profiles that were similar to the trace obtained with untagged V6 (Fig. 3D). This similarity is most simply reconciled with a model of rapid 2-5A equilibration between the two compartments due to diffusion. Indeed, 2-5A produced at the center of a HeLa cell may take only several seconds to diffuse across the nucleus and through the nuclear pores and to become evenly distributed between the nucleus and the cytosol (Fig. 3E). Our measurements do not support localized 2-5A action and indicate that 2-5A is poised to establish communication between the OASs and RNase L across the cell.

Translational Arrest by 2-5A Precedes the IFN Response. The pathways of 2-5A and IFNs are closely interconnected. IFNs stimulate 2-5A production by transcriptionally inducing the OASs (3, 34, 35). Conversely, 2-5A can amplify (36) and suppress (37) IFN- β protein production. Considering that RNase L stops translation and ultimately causes apoptosis (38), IFNs may critically require mechanisms to delay RNase L activation and evade RNase L. To test whether such mechanisms exist, we generated stable A549 and HeLa human cell lines carrying FLAG-V6 (*SI Appendix*, Fig. S3) and used these cells to measure 2-5A synthesis throughout dsRNA response.

Time-dependent 2-5A synthesis was readily observed over a range of poly-IC concentrations (Fig. 4A). Accumulation of 2-5A started almost immediately after the addition of dsRNA and showed a discernible lag at low poly-IC doses and no lag at higher doses. In contrast, transcription of IFN-stimulated genes

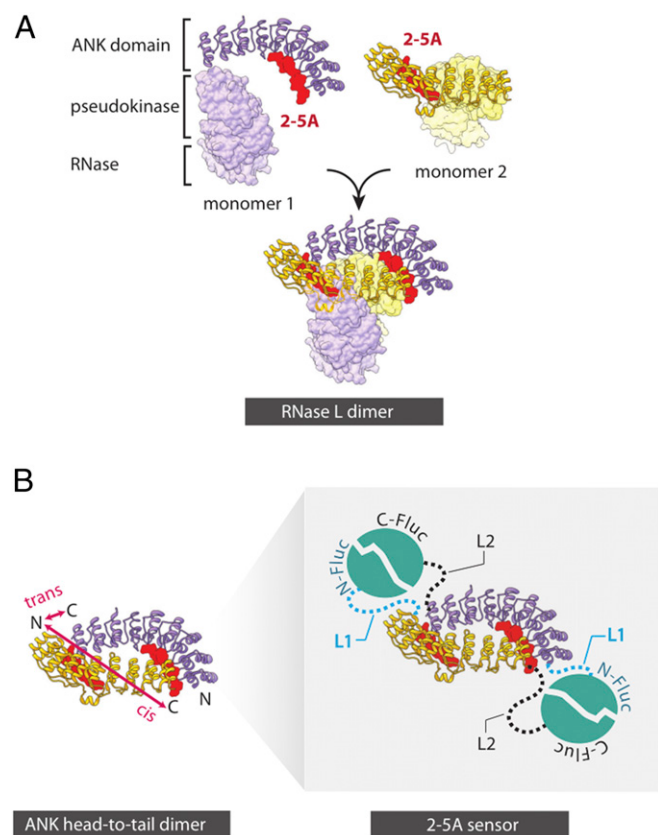


Fig. 1. Structure-guided design of the 2-5A biosensor. (A) The N-terminal ANK domain of RNase L can bind two molecules of 2-5A (red) and form a 2-5A-templated homodimer with a head-to-tail structure. (B) In the 2-5A biosensor, split Fluc halves are engineered on both termini of the ANK domain. On 2-5A binding, the head-to-tail configuration of the ANK domains brings the N and C termini of two different ANK domains in proximity, which reconstitutes two functional luciferase copies per assembled ANK/ANK reporter. Activation of luminescence provides the readout of 2-5A.

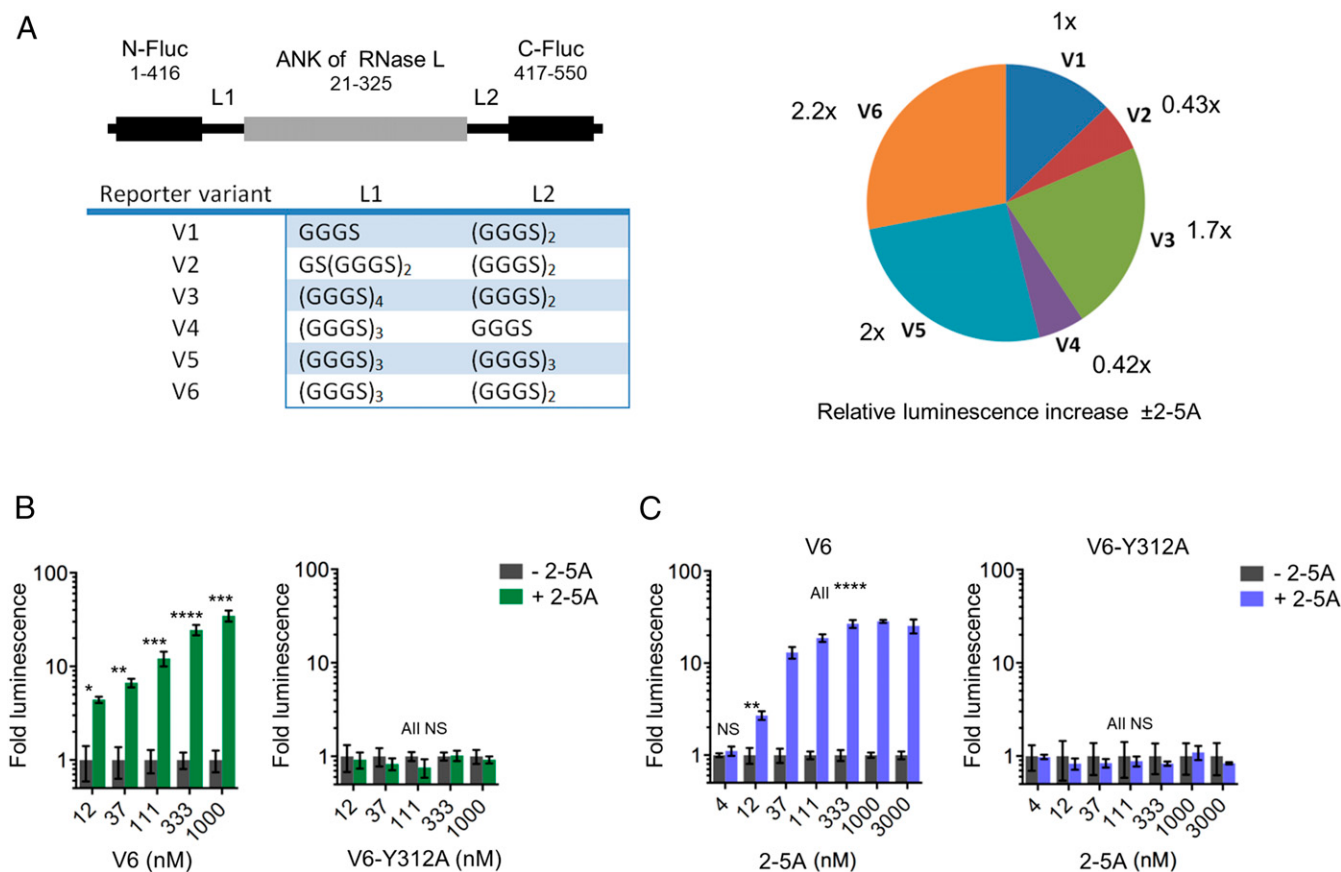


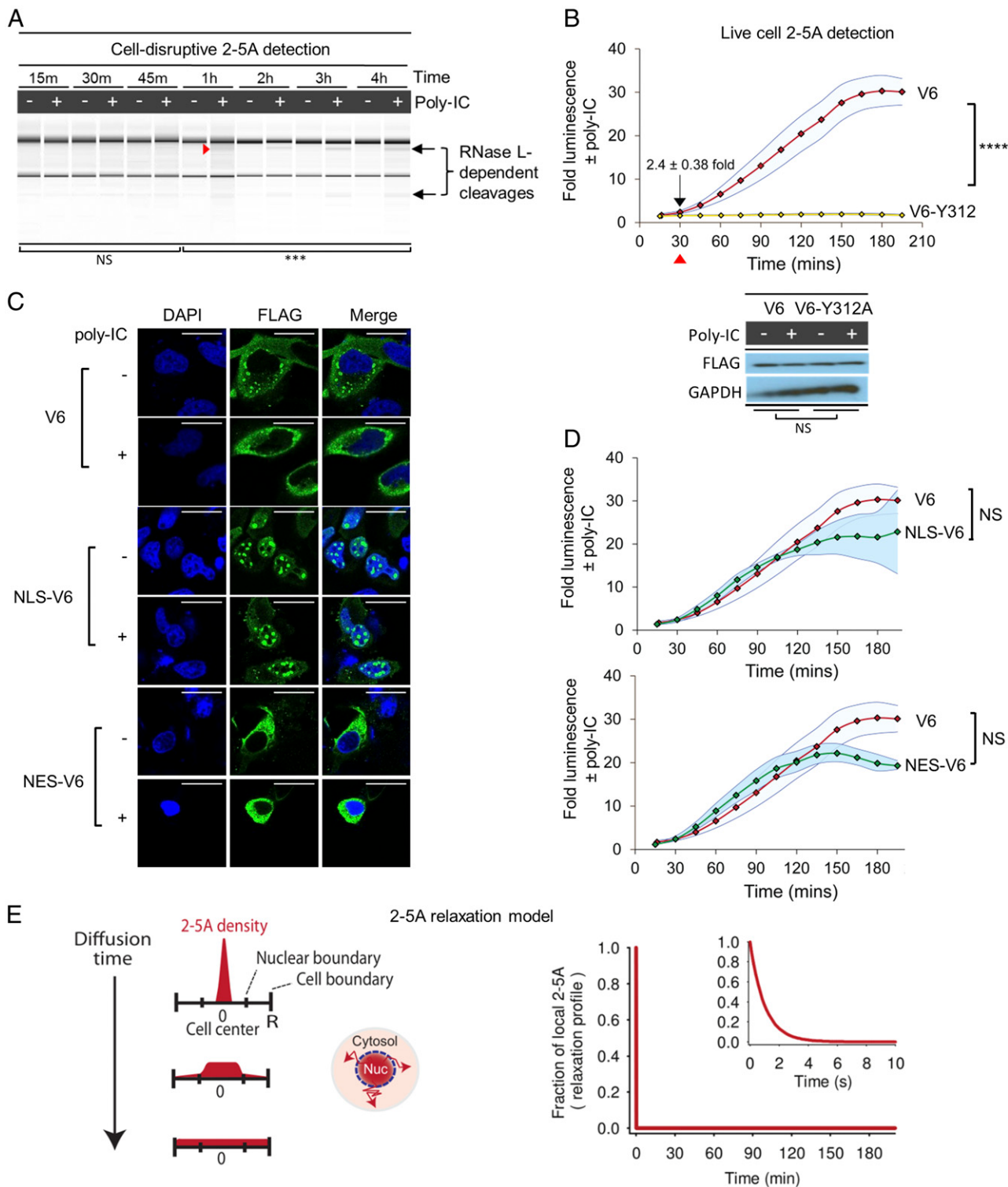
Fig. 2. Light-based detection of nanomolar 2-5A concentrations. (A, Left) Luminescence analysis of 2-5A biosensor variants in A549 cells. (A, Right) Data on the relative activation of these variants by synthetic 2-5A from three independent experiments (two for V1, V3, and V4). (B and C) Luminescence analysis of 2-5A biosensor V6 dose-response in A549 cells at excess 2-5A (B) or in response to varying concentrations of 2-5A (C). The Y312A mutant served as a control. Data are mean $\pm$ SE pooled from at least three independent experiments. NS, nonsignificant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$, Welch's two-tailed unpaired t test (James McCaffrey implementation; <https://msdn.microsoft.com/en-us/magazine/mt620016.aspx>).

(ISGs) measured by qPCR of OAS1/2/3/L and the helicases RIG-I and MDA5 developed with a lag of 2–4 h and became strong after maximal 2-5A production (Fig. 4 A and B). The occurrence of 2-5A synthesis before the IFN response was confirmed by cleavage of 28S rRNA in A549 cells (Fig. 4C) and in HeLa cells using a combination of biosensor and qPCR readouts (SI Appendix, Fig. S4). We found that in HeLa cells, the reporter luminescence was down-regulated at 3–5 h after poly-IC transfection, whereas in A549 cells, the luminescence reached a plateau and began to decrease only after 6 h. Our observations suggest the presence of a negative feedback in HeLa cells that is weaker in A549 cells.

Our observations suggest that 2-5A production precedes the IFN response, and that 2-5A is supplied by basal OASs rather than by IFN-induced OASs. This model is in line with the recent report that basal OASs are sufficient and solely responsible for protecting mouse myeloid cells from murine coronavirus (39). In agreement with a key role of basally expressed OASs, we found that (i) OAS/RNase L activation is not inhibited by actinomycin D, which prevents new mRNA synthesis (SI Appendix, Fig. S5), and (ii) cell pretreatment with IFN- β has a twofold or lesser effect on 2-5A synthesis, while the robust transcriptional response indicates that IFN- β treatment is effective (Fig. 4 D and E and SI Appendix, Figs. S6 and S7). Basally expressed OASs appear to be sufficient for 2-5A production, providing a mechanistic explanation for the 2-5A source ahead of the IFN response.

To determine whether cellular 2-5A dynamics correspond to a rapid arrest of translation by RNase L, we measured nascent protein synthesis by puromycin pulse labeling in WT and RNase L^{-/-} A549 cells (5). Treatment of WT, but not of RNase L^{-/-} cells with poly-IC halted global translation before ISG induction (Fig. 4B and SI Appendix, Fig. S8 A–C). RNase L^{-/-} cells exhibited a delayed and incomplete translational attenuation involving eIF2 α phosphorylation, presumably due to PKR (5). Translational arrest ahead of ISG induction was also present in HeLa cells (SI Appendix, Fig. S4 B and C). Disengagement of basal protein synthesis before the IFN response was further confirmed using metabolic labeling of the nascent proteome with ³⁵S (Fig. 4G).

IFN- β and - λ Escape the Translational Shutoff Caused by 2-5A. 2-5A rapidly stops basal protein synthesis. To examine IFN protein production under these conditions, we treated WT and RNase L^{-/-} cells with poly-IC and assayed the media for IFN activity (SI Appendix, 5A). These tests revealed a time-dependent increase in the media's antiviral activity and ability to induce ISGs, which developed after RNase L-mediated translational arrest (Fig. 5B and SI Appendix, Fig. S8). At time points well beyond translational arrest, we observed an increase in antiviral activity (Fig. 5C) and ISG induction by approximately two orders of magnitude from media of poly-IC-treated WT and RNase L^{-/-} cells (Fig. 5D). In agreement with the ISG induction readout, media from WT and RNase L^{-/-} cells exhibited comparable (within two-fold to threefold) antiviral activity and a similar time-dependent



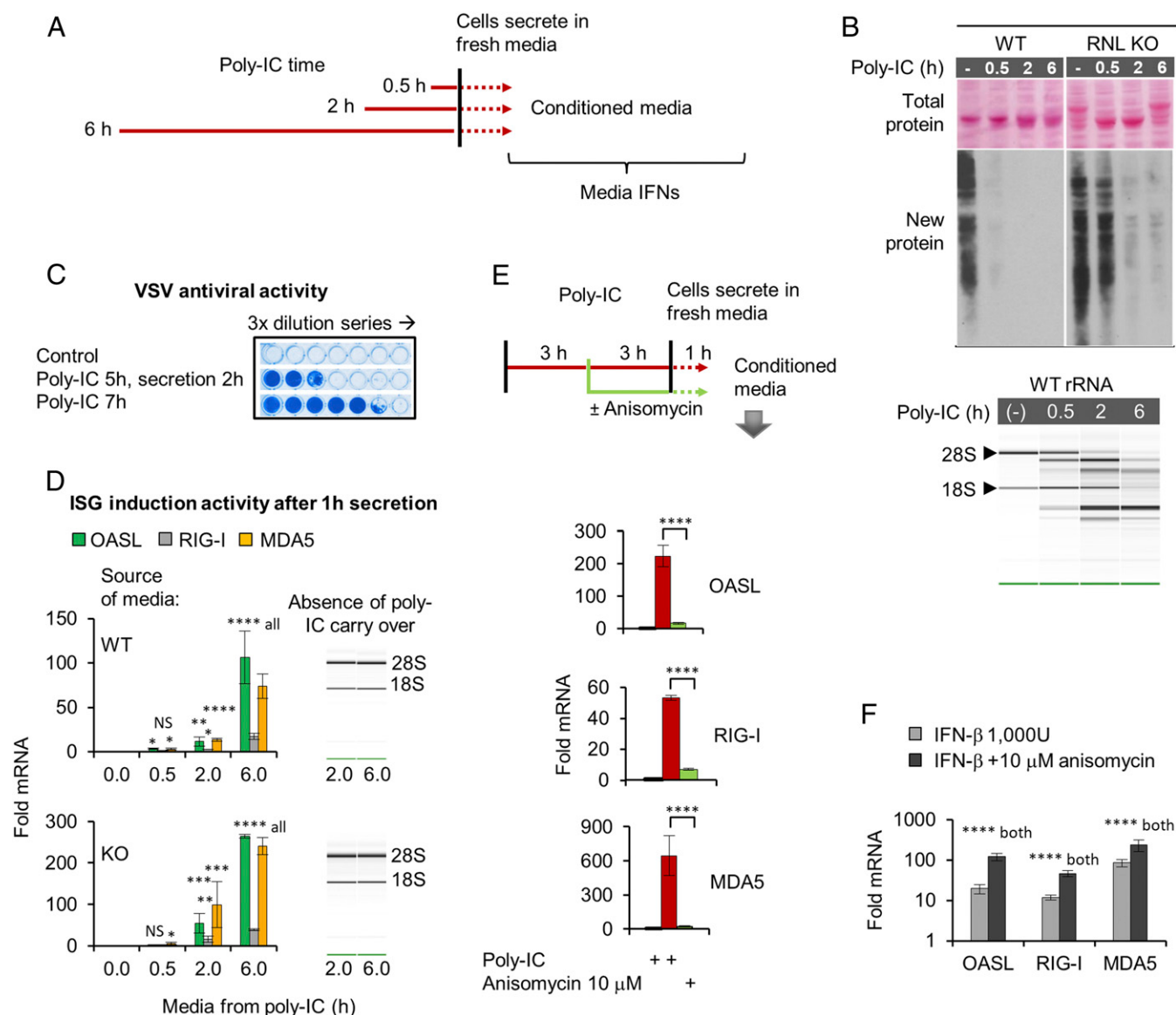


Fig. 5. IFN synthesis after 2-5A-induced global translation shutdown. (A) Diagram of the IFN secretion experiment. (B) Puromycin Western blot (Upper) and RNA nano-ChIP (Lower) analysis of translation and 28S rRNA cleavage in poly-IC-treated (1 μ g/mL) A549 cells. Images are representative of four independent experiments. (C) Antiviral activity of conditioned media from poly-IC-treated (1 μ g/mL) A549 cells (Upper). Condensed results from three additional replicates are shown in *SI Appendix, Fig. S8*. (D) qRT-PCR analysis of ISG expression in WT and RNL-KO A549 cells treated with conditioned media from poly-IC-treated (1 μ g/mL) A549 cells (Lower). Data are mean $\pm$ SE pooled from three biological replicates. (Inset) RNA nano-ChIP of intact rRNA is representative of all experiments. NS, nonsignificant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. (E) qRT-PCR analysis of ISG expression in A549 cells treated with anisomycin after translational arrest by 1 μ g/mL poly-IC but before the transcriptional IFN response. Data are mean $\pm$ SE pooled from three biological replicates. **** $P \leq 0.0001$. (F) Effect of anisomycin treatment on transcriptional IFN signaling. **** $P \leq 0.0001$.

increase of IFN potency (*SI Appendix, Fig. S9*). Therefore, IFNs remained relatively insensitive to RNase L, while basal translation was inhibited by approximately 1,000-fold.

To test whether IFN arises from actively ongoing translation rather than from other potential mechanisms (e.g., delayed secretion of pretranslated IFN stores), we used pulse treatment with a translation inhibitor, anisomycin (Fig. 5E). In this setting, cells were first treated with poly-IC for 3 h, which stopped protein synthesis but did not yet activate a strong transcriptional IFN response. Then anisomycin was added to arrest all protein synthesis, and the cells were kept for another 3 h. Control cells were maintained for the same duration without anisomycin. During the last hour, the media was changed to remove poly-IC, but anisomycin treatment was continued to keep the cells

translationally arrested. An assay of IFN activity in the media revealed that anisomycin treatment after the 2-5A-induced global translational inhibition but before the IFN response blocked IFN production (Fig. 5E and *SI Appendix, Fig. S8C*). A control experiment showed that anisomycin was compatible with IFN sensing by naïve cells (Fig. 5F). Of note, anisomycin had a mild stimulatory effect on ISG mRNAs due to an unknown mechanism; this effect acted in the opposite direction of blocking IFNs and thus did not affect the suitability of anisomycin as a control in our tests. The observation that translation inhibitors, such as anisomycin or cycloheximide, can induce cytokines has been reported previously (40, 41). Taken together, our experiments indicate that IFNs are indeed translated when the bulk of protein synthesis remains silenced by 2-5A.

resembling translation initiation arrest. A similar polysomal disaggregation is observed in the ISR (51) and during inhibition of the 5'-cap complex assembly by blockers of the kinase mTOR (52). Our analysis of mRNA sequences shows that the 5'-UTRs of the IFNs are small (~50–100 nt) and have few uORFs and m6A motifs. In this respect, IFN mRNAs more closely resemble mRNAs of basal proteins than the feature-packed mRNAs of stress proteins, such as ATF4 and BIP (*SI Appendix, Fig. S11*). Most likely, IFNs evade the action of RNase L by a mechanism that does not rely on uORFs and m6A elements. Mechanisms based on regulation of the ribosomal activity are also unlikely, because translational inhibition by RNase L does not correlate with the extent of rRNA cleavage (5). Taken together, these considerations limit the scope of possible mechanisms and suggest that selective IFN translation may result from transcriptional induction of their mRNAs, analogous to the mRNA “superinduction” phenomenon that facilitates cytokine production in cells infected with the bacterium *Legionella pneumophila* (53).

Evidence for Long-Range Communication Between OASs and RNase L. Our observations of subcellular 2-5A dynamics suggest a possible explanation for the bipartite organization of the OAS-RNase L system. The effector (RNase L) and the dsRNA-sensing moiety (the OASs) in the 2-5A system are separated. This arrangement is in contrast with the single-protein structure of another dsRNA sensor, PKR, which encodes the dsRNA-binding domain and the effector kinase domain in the same polypeptide. Our data indicate that the bipartite arrangement of OASs/RNase L may have a function in sensing dsRNA at a distance from the site of

RNase L action. The range of OAS-RNase L communication depends on the efficiency of 2-5A diffusion, which occurs at rates sufficient for 2-5A equilibration in the cytosol, and between the nucleus and the cytosol fast compared with the rate of 2-5A production. These quantitative considerations suggest that RNase L is poised to sense dsRNA located at remote sites in the cytosol and in the nucleus.

Methods

The reporter protein was designed by fusing two halves of Fluc encoding amino acids 1–416 (N-Fluc) and 417–550 (C-Fluc) to the N and C termini, respectively, of the ANK domain of human RNase L (residues 21–325). For cell work, an N-terminal FLAG tag was added. PKKKRKVE and LQLPLRLTLD were the NLS and NES sequences cloned immediately after the N-terminal FLAG tag. Luminescence measurements were performed with a Berthold Technologies microplate reader. IFN proteins were detected using hamster CHO cell lines with both subunits of either type I or type III IFN human receptor expressed. The methodology is described in detail in *SI Appendix, Materials and Methods*.

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