The complementary pairs of N- or C- terminally mKG-fused NS4B and Cardif expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (BiFC signal) and to count BiFC signal-positive cells (B, C). Plasmids expressing p65-mKGN and p50-mKGC individually were used as a BiFC positive control and plasmids expressing N- or C terminally mKG-fused Rluc were used as a negative control. Letters 'N' and 'C' denote complimentary N- and C-terminal fragments of mKG, respectively. Assays were done in triplicate and error bars indicate mean ± SD. Scale bars indicate 10 μm (A). Asterisks indicate P-values less than 0.05 compared with corresponding negative controls.

C. Plasmids expressing mKG fragment-fused STING or NS4B were transfected in HEK293T cells. After 24 hours, the cells were fixed and immunostained with anti-mKG antibody. Mitochondria were stained using Mitotracker and nuclei were stained with DAPI. Cells were observed by confocal microscopy. Scale bars indicate 5 μm.

**Figure 5. Binding of NS4B to STING blocks molecular the interaction between Cardif and STING.**

A, C. NS4B expression plasmid was cotransfected with STING or Cardif expression plasmid into HEK293T cells (A) or Huh7 cells (C). After 24 hours, cell lysates were subjected to immunoprecipitation using anti-HA or anti-Flag and were immunoblotted with anti-myc.

B, D. Cardif and STING expression plasmids were cotransfected with various amounts of NS4B plasmid in HEK293T cells (B) or Huh7 cells (D). After 24 hours, cells lysates were subjected to immunoprecipitation using anti-Flag and were immunoblotted with anti-HA

**Figure 6. Effects on HCV replication levels by STING knock down and NS4B overexpression**

A. Effects of siRNA knock down of STING by siRNA. Huh7 cells were transfected with STING- targeted siRNAs (siRNA STING-1, -2 and -3, respectively) or negative control siRNA (siRNA NTC). Seventy-two hours after transfection, cells were harvested and expression levels of STING protein were detected by immunoblotting. The tf(-) denotes transfection-negative control.

B. Huh7 cells expressing HCV-Feo subgenomic replicon (Huh7/Feo) (27, 28) were transfected with STING-targeted siRNAs or negative control siRNA. Seventy-two hours after transfection, cells were harvested and internal luciferase activities were measured. The Y-axis indicates luciferase activity shown as a ratio of transfection-negative control. Assays were carried out in triplicate and error bars indicate mean+SD. Asterisks indicate P-values less than 0.05 compared with corresponding negative controls.

C. Empty plasmid or plasmid expressing NS4B was transfected into Huh7 cells. After 24 hours, HCV-JFH1 RNA was transfected into these cells. Seventy-two hours after virus transfection, HCV core antigen levels in culture medium were measured. Assays were carried out in triplicate and error bars indicate mean+SD. Asterisks indicate P-values less than 0.05 compared with corresponding negative controls. The tf(-) denotes transfection-negative control.

D. Huh7 cells expressing HCV-Feo replicon (Huh7/Feo) (27, 28) were transfected with NS4B expressing plasmid or empty plasmid (pcDNA). Forty-eight hours after transfection, internal luciferase activities were measured. The Y-axis indicates luciferase activity shown as a ratio of the transfection-negative control. Assays were carried out in triplicate and error bars indicate mean+SD. Asterisks indicate P-values less than 0.05 compared with corresponding negative controls.

**Figure 7. The N-terminal domain of NS4B is essential for suppressing IFN-β promoter activity induced by RIG-I, Cardif, or STING.**

A. Immunoblotting of NS4B and truncated NS4B; NS4B t1-84, NS4Bt85-216. HEK293T cells were transfected with NS4B or truncated NS4B. After 24 hours, the cells were lysed and immunoblot assays were performed. The band indicated by '#' is a truncated NS4B, probably generated via alternative posttranslational processing.

B. Plasmids expressing ΔRIG-I, Cardif, or STING as well as NS3/4A or the indicated truncated form of NS4B were cotransfected with pIFN-β-Fluc and pRL-CMV in HEK293T cells. Dual luciferase assays were performed 24 hours after transfection. Plasmids expressing RIG-IKA, ΔCARD, or pcDNA were used as negative controls. The Y-axis indicates IFN-β-Fluc activity shown as relative values. Assays were carried out in triplicate and error bars indicate mean ± SD. Asterisks indicate P-values less than 0.05 compared with corresponding negative controls.

C. Plasmids expressing NS4Bt1-84-myc of NS4Bt85-261-myc were transfected with or without plasmids expressing HA-STING in HEK293T cells. After 24 hours, the cells were fixed and immunostained. Nuclei were stained with DAPI. Cells were observed by confocal microscopy. Scale bars indicate 5 μm.

**Figure 8. NS4B suppressed IFN-β production pathway independently of and cooperatively with NS3/4A.**

A. Immunoblotting of Cardif and truncated Cardif (Cardif1-508). HEK293T cells were transfected with Cardif or truncated Cardif (Cardif1-508). After 24 hours, the cells were lysed and immunoblot assays were performed.

B. Subcellular localization of Cardif and truncated Cardif (Cardif1-508). HEK293T cells

were immunostaind with anti-Cardif antibody or HEK293T cells were transfected with myc-tagged truncated Cardif (Cardif1-508-myc) and after 24 hours the cells were immunostained with anti-myc. Mitochondria were stained with Mitotracker (red) and nuclei were stained with DAPI (blue). Plasmid expressing myc-tagged truncated Cardif (Cardif1-508) and plasmid expressing HA-tagged STING were transfected into HEK293T cells. The cells were immunostained with anti-myc and anti-HA antibodies and analyzed by confocal laser microscopy. Scale bars indicate 10 μm.

C. Plasmids expressing Cardif or truncated Cardif (Cardif1-508) and pIFN-β-Fluc and pRL-CMV were transfected with or without plasmid expressing NS3/4A or NS4B into HEK293T cells as indicated. Dual luciferase assays were performed 24 hours after transfection. Plasmid expressing ΔCARD or pcDNA was used as a negative control. The Y-axis indicates IFN-β-Fluc activity shown as relative values. Assays were carried out in triplicate and error bars indicate mean ± SD. Asterisks indicate P-values less than 0.05.

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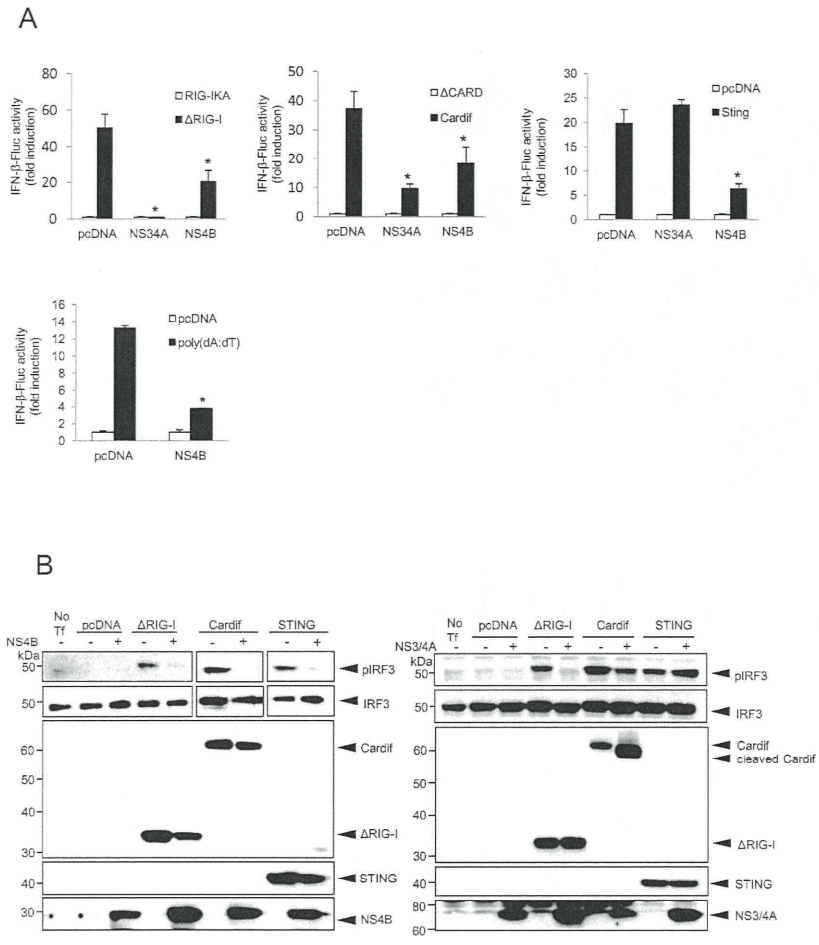


**Figure 1.** Nitta et al.

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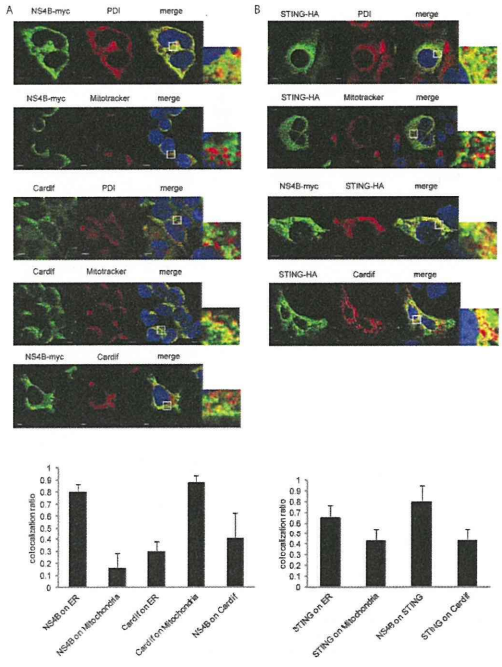


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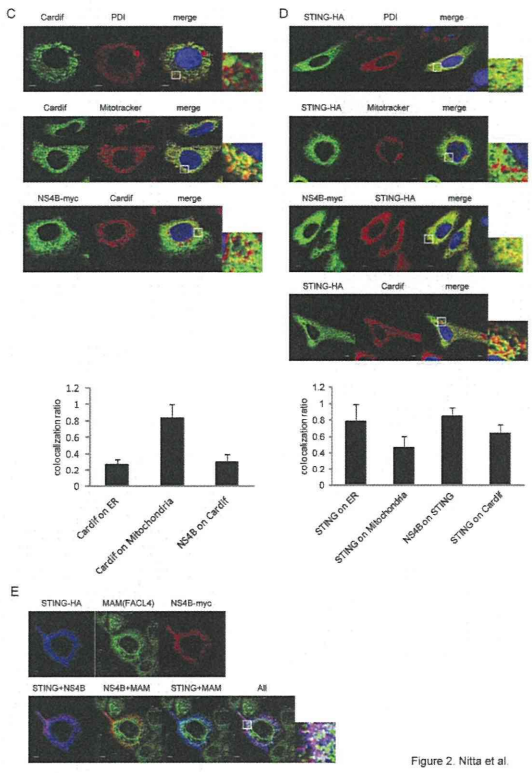


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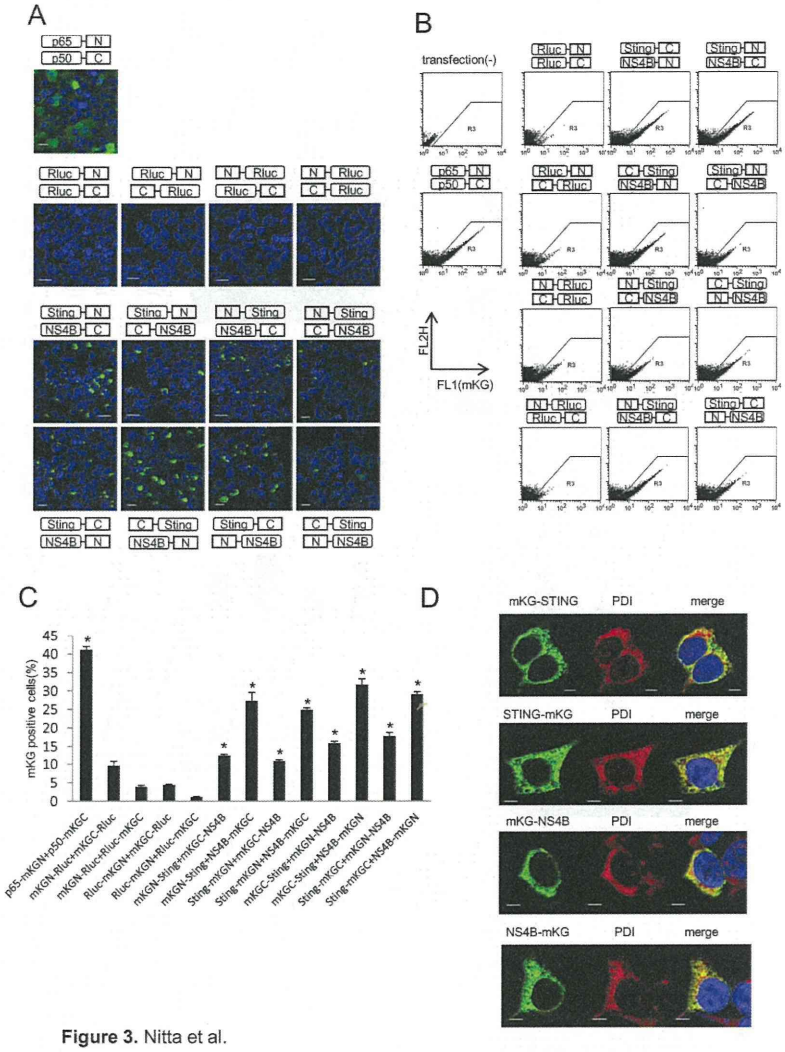


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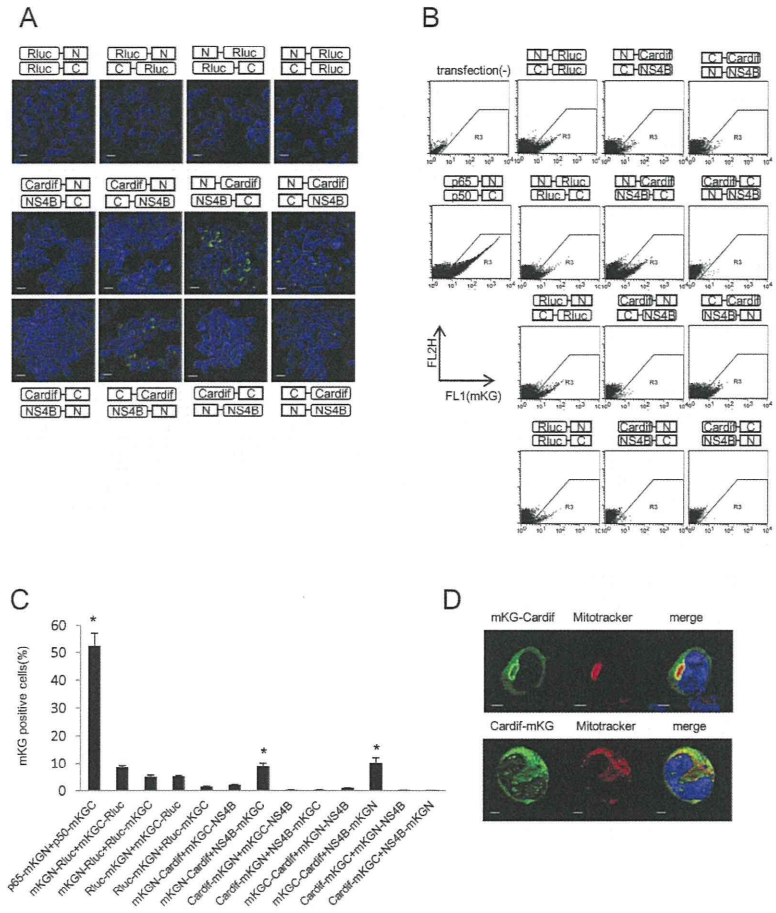


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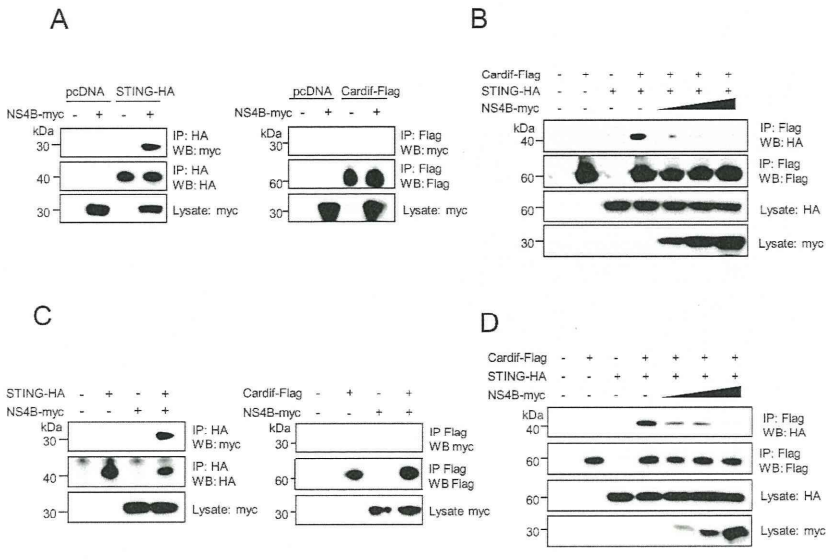


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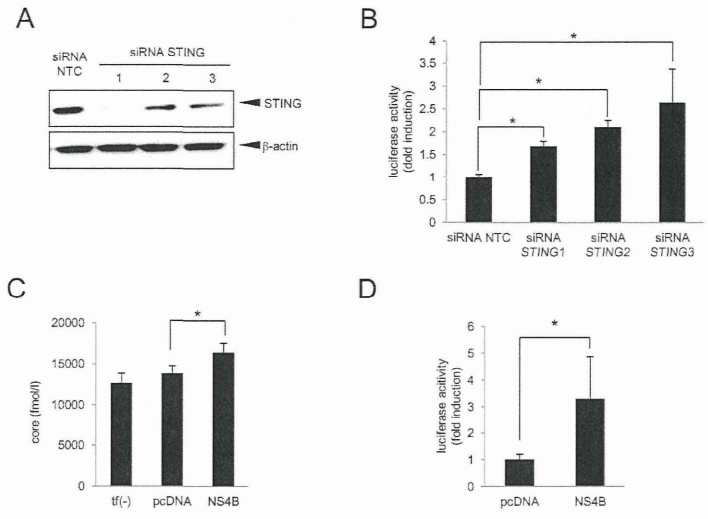


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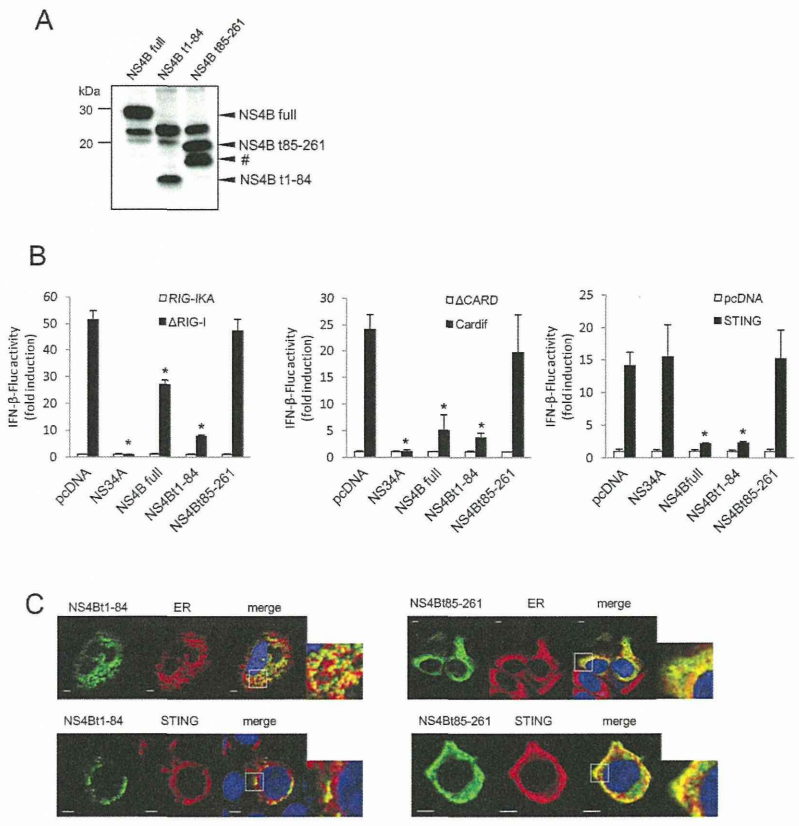


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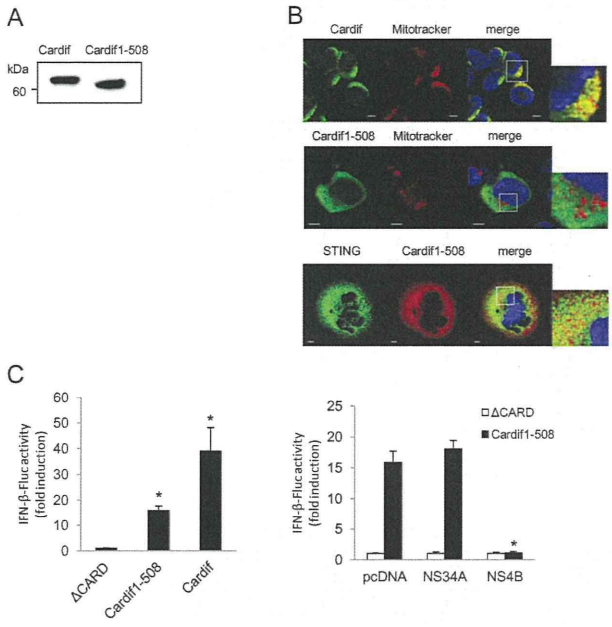


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Figure 3D

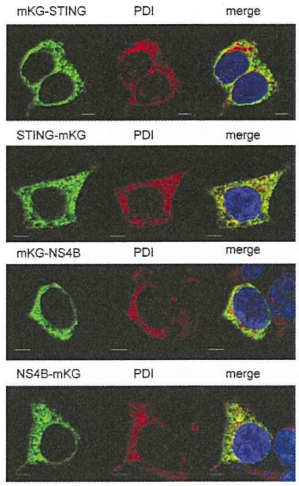



Figure 4D

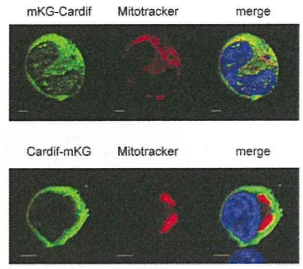



Figure 7C

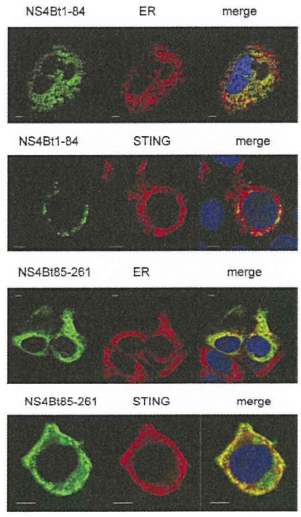
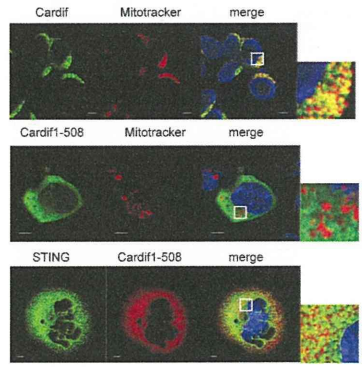



Figure 8B




Supplementary Figure for review Nitta et al.

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