

1 **Interferon induction and not replication interference mainly determines anti-**  
2 **influenza virus activity of defective interfering particles**

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18 **interference**

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25 **Abstract**

26 Defective interfering (DI) RNAs arise during influenza virus replication, can be packaged into  
27 particles (DIPs) and suppress spread of wildtype (WT) virus. However, the molecular signatures  
28 of DI RNAs and the mechanism underlying antiviral activity are incompletely understood. Here,  
29 we show that any central deletion is sufficient to convert a viral RNA into a DI RNA and that  
30 antiviral activity of DIPs is inversely correlated with DI RNA length when induction of the  
31 interferon (IFN) system is disfavored. When induction of the IFN system was allowed, it was  
32 found to be the major contributor to DIP antiviral activity. Finally, while both DIPs and influenza  
33 virus triggered expression of IFN-stimulated genes (ISG) only virus stimulated robust expression  
34 of IFN. These results suggest a key role of innate immune activation in DIP antiviral activity and  
35 point towards previously unappreciated differences in DIP- and influenza virus-mediated  
36 activation of the effector functions of the IFN system.

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## 47 **Importance**

48 Defective interfering (DI) RNAs naturally arise during RNA virus infection. They can be  
49 packaged into defective interfering particles (DIPs) and exert antiviral activity by suppressing  
50 viral genome replication and inducing the interferon (IFN) system. However, inhibition of  
51 influenza virus infection by DI RNAs has been incompletely understood. Here, we show that  
52 induction of the IFN system and not suppression of genome replication is the major determinant  
53 of DIP antiviral activity. Moreover, we demonstrate that DIPs induce IFN-stimulated genes (ISG)  
54 but not IFN with high efficiency. Our results reveal unexpected major differences in influenza  
55 virus and DIP activation of the IFN system, a key barrier against viral infection, and provide  
56 insights into how to design DIPs for antiviral therapy.

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## 70 **Introduction**

71 The annually recurring influenza epidemics are a major source of global morbidity and mortality  
72 and intermittent pandemics can have even more severe consequences (1). Influenza therapy and  
73 vaccination are available but suffer from serious shortcomings (1). The success of influenza  
74 therapy with currently licensed drugs, which target the viral proteins neuraminidase (NA), matrix  
75 protein 2 (M2) or polymerase acidic protein (PA), can be compromised by resistance  
76 development (2). Moreover, vaccines against epidemic influenza need to be annually adjusted to  
77 the viruses expected to circulate during the next influenza season and offer little or no protection  
78 against emerging pandemic viruses (1). Thus, the identification of novel targets and strategies for  
79 antiviral intervention is an important task.

80         Influenza viruses contain a segmented, negative sense RNA genome. The genomic  
81 segments are replicated by the viral polymerase, which consists of the subunits polymerase basic  
82 proteins 1 (PB1) and 2 (PB2) as well as PA (3). The error rate of the viral polymerase is high and  
83 can result in the synthesis of genomic segments that harbor deletions (4-10). These defective  
84 segments may interfere with the amplification of wt segments and are thus termed defective  
85 interfering (DI) RNAs (4-6, 8-10). Packaging of DI RNAs into viral particles results in the  
86 formation of DI particles (DIPs), which suppress wt influenza virus spread (4, 5). It has been  
87 proposed that DIPs suppress influenza virus infection by interfering with genome replication (a  
88 process subsequently termed replication interference) and by inducing an interferon (IFN)  
89 response (4, 5, 11-17). However, this concept has not been systematically investigated and the  
90 relative contribution of replication interference and IFN induction to DIP antiviral activity is  
91 unknown.

92         We recently developed a cell culture system that allows production of genetically defined  
93 DIPs based on reverse genetics and a cell line complementing defects in influenza A virus (IAV)

94 genomic segment 1 (18). Here, we used this system as well as a mini-replicon assay (19) to  
95 analyze the contribution of replication interference and IFN induction to the antiviral activity of  
96 DIPs. We report that in the mini-replicon assay any central deletion in segment 1, 2 or 3 converts  
97 these segments into DI RNAs, which suppress replication of diverse target segments. Inhibitory  
98 activity of these DI RNAs was inversely correlated with segment length and a similar correlation  
99 was seen in the context of DIP and IAV infection under conditions which disfavored IAV  
100 inhibition by DIP-dependent induction of the IFN system. If induction of the IFN system was  
101 allowed before IAV infection, it largely accounted for DIP antiviral activity. Finally, DIPs  
102 robustly induced IFN-stimulated gene (ISG) but not IFN expression, indicating that IAV and  
103 DIPs may differ in the activation of the effector functions of the IFN system. Our results suggest  
104 that although interference with genome replication contributes to DIP antiviral activity, the  
105 induction of the IFN system is the major determinant of suppression of virus infection by DIPs.

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## 118 **Results**

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### 120 **DI-244 inhibits segment replication in a mini-replicon assay and inhibition is independent** 121 **of the truncated PB2 open reading frame**

122 We first investigated whether a previously described IAV mini-replicon assay (19) is suitable to  
123 detect inhibition of IAV genome replication by a prototypic segment 1-derived DI RNA, DI-244  
124 (20). This assay is based on a firefly luciferase open reading frame flanked by the 5' and 3' ends  
125 of IAV segment 8, which is amplified in cells upon coexpression of the constituents of the viral  
126 polymerase complex PB1, PB2, and PA, and the viral nucleoprotein (NP) (19). Transfection of  
127 293T cells with plasmids encoding the mini-genome reporter segment and the IAV proteins  
128 mentioned above resulted in luciferase activities in cell lysates that were approximately 1,000-  
129 fold higher than those measured in cells transfected with the reporter alone or transfected with the  
130 full set of plasmids except the PB2 encoding plasmid (Figure 1A). Moreover, cotransfection of  
131 two different amounts of DI-244 encoding plasmid resulted in a concentration dependent  
132 decrease in luciferase activity, indicating that DI-244 inhibited replication of the reporter segment  
133 (Figure 1A). This inhibitory activity was also observed when the PB2 start codon in DI-244 and  
134 two subsequent ATGs (positions 11 and 28) were mutated (Figure 1B). In contrast, transfection  
135 of expression plasmid pCAGGS containing the truncated PB2 ORF of DI-244 or empty pCAGGS  
136 did not reduce luciferase signals (Figure 1B). These results indicate that inhibition of segment  
137 replication by DI-244 can be visualized in the mini-replicon assay and does not require  
138 expression of truncated PB2.

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### 140 **Inhibitory activity of segment 1, 2 and 3-derived DI RNAs is inversely correlated with RNA** 141 **length and is independent of the target segment**

142 It is believed that the short length of DI-244, as compared to wt segment 1, results in faster  
143 amplification of DI-244 and ultimately in suppression of amplification of the wt segment (4, 5). If  
144 correct, one would assume that DI RNA length is a major determinant of antiviral activity. We  
145 explored this possibility by investigating the capacity of a set of ten segment 1-derived RNAs  
146 with nested central deletions to inhibit segment amplification in the mini-replicon assay. All  
147 RNAs tested exerted inhibitory activity and an inverse correlation between RNA length and  
148 inhibitory activity was observed (Figure 1C, Table S1). When the plasmids encoding the DI  
149 RNAs with the smallest and largest deletion were normalized for copy numbers instead of weight  
150 little effect on inhibitory activity was observed. Thus, the inverse correlation between DI RNA  
151 length and inhibitory activity was not due to differences in plasmid copy numbers transfected  
152 (not shown). Finally, further shortening of DI-244 did not augment inhibitory activity (not  
153 shown), suggesting that DI-244 length may be optimal for inhibition of wt segment replication. In  
154 sum, our results show that the ability of segment 1-derived DI RNAs to block replication of a wt  
155 segment is dependent on the DI RNA length.

156 We next explored whether the inverse correlation between length and inhibitory activity is  
157 also observed for segment 2- and 3-derived DI RNAs. For this, we introduced central, nested  
158 deletions in segment 2 and 3 and investigated inhibitory activity in the mini-replicon system. As  
159 for segment 1-derived RNAs, all segment 2- and 3-based RNAs with deletions exerted inhibitory  
160 activity and inhibition inversely correlated with RNA length, although this correlation was more  
161 pronounced for segment 2 as compared to segment 3 (Figure 1D, Table S1).

162 Next, we examined whether the segment 1-, 2- and 3-derived DI RNAs with the largest  
163 deletion (constructs DI-244 (segment 1, S1), DI-156 (segment 2, S2), DI-178 (segment 3, S3),  
164 Table S1) were able to efficiently suppress replication of different IAV segments or were mainly  
165 active against segment 8, which was so far employed in the mini-replicon assay. For this, we

166 added the 5' and 3' ends of segments 1, 2, 4, 6, and 7 to the firefly luciferase sequence and tested  
167 the amplification of these reporter segments in the mini-replicon assay. In the absence of DI  
168 RNAs, all segments were efficiently amplified, as demonstrated by high luciferase activity in  
169 lysates of cells coexpressing PB2, PB1, PA and NP (Figure 1E). Cotransfection of two different  
170 amounts of segment 1-, 2- or 3-derived DI RNAs reduced replication of all reporter segments  
171 efficiently and in a concentration dependent manner (Figure 1E). Thus, in the mini-replicon  
172 assay, introduction of a deletion into an IAV genomic segment is sufficient to convert it into a DI  
173 RNA and length and inhibitory activity of these DI RNAs are inversely correlated.

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#### 175 **Inverse correlation between anti-IAV activity of DIPs and DI RNA length**

176 We recently reported a cell culture system for production of DIPs in the absence of helper virus,  
177 which relies on IAV reverse genetics and DIP producer cell lines stably expressing the PB2  
178 protein (18). We employed this system to generate DIPs with nested deletions in segment 1 and  
179 assessed their ability to inhibit infection of MDCK cells with A/PR/8/34 (PR8). We found that  
180 DI-244, which contains the smallest DI RNA, inhibited PR8 infection with the highest efficiency  
181 and that inhibitory activity of DIPs decreased as DI RNA length increased (Figure 1F). Thus, an  
182 inverse correlation between DI RNA length and inhibitory activity observed in the mini-replicon  
183 assay could be confirmed in the context of DIPs, at least under the conditions tested.

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#### 185 **Preincubation of target cells with DI-244 increases antiviral activity**

186 It has been reported that DIPs can block viral infection by stimulating the IFN system (11, 12).  
187 Therefore, we sought to clarify whether induction of the IFN system could contribute to DI-244  
188 antiviral activity in MDCK cells. Trypsin is used for A/PR8/34 activation but can inactivate IFN $\alpha$   
189 (Figure 2A) (21) and can thus confound analyses of IAV inhibition by the IFN system. Therefore,



190 we switched to A/WSN/33 (WSN) as challenge virus and WSN-derived DIPs, since WSN can  
191 replicate trypsin-independently in cell cultures containing fetal bovine serum (FBS) (22). To  
192 obtain first insights into a potential role of the IFN system in DIP antiviral activity, we reasoned  
193 that if induction of the IFN system was a major determinant of DIP antiviral activity, then time-  
194 of-DIP addition to target cells should have a major impact on the efficiency of IAV inhibition by  
195 DIPs. Thus, addition of DIPs and virus to target cells at the same time should preclude the  
196 establishment of a robust DIP-induced antiviral state prior to IAV infection. In contrast, addition  
197 of DIPs at 24 h before virus should allow for establishment of such an antiviral state and might  
198 thereby boost DIP antiviral activity. Preincubation of target MDCK cells with DI-244 for 24 h  
199 indeed increased DIP antiviral activity as compared to simultaneous addition of DI-244 and IAV,  
200 especially when high doses of DI-244 were analyzed (Figure 2B, left panel). Unexpectedly,  
201 similar results were obtained in the presence of trypsin (Figure 2B, right panel), indicating that  
202 the enhanced antiviral activity of DI-244 upon 24 h preincubation with target cells was likely not  
203 due to induction of IFN $\alpha$  or another trypsin-sensitive antiviral host cell protein.

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### 205 **DI-244 induces anti-IAV activity in A549 cells in a STAT1-independent fashion**

206 In order to more directly assess the contribution of the IFN system to DI-244 antiviral activity,  
207 we employed A549 wt cells and A549 cells which lack STAT1 (signal transducer and activator of  
208 transcription 1, STAT1<sup>-/-</sup>) and are thus defective in IFN-induced signaling. Confirmatory  
209 experiments revealed that IFN $\alpha$ , IAV and DI-244 strongly upregulated *MXI* gene expression in  
210 A549 wt but not *STAT1*<sup>-/-</sup> cells, in keeping with a defective JAK/STAT signaling pathway (Figure  
211 2C). Addition of undiluted and 1:10 diluted DI-244 to A549 cells at 24 h before infection with  
212 WSN resulted in 100 -fold higher antiviral activity as compared to DI-244 added at the same time  
213 as virus (Figure 2D), confirming and extending the data obtained with MDCK cells.

214 Unexpectedly, addition of undiluted DIP to A549 STAT1<sup>-/-</sup> cells still resulted in high antiviral  
215 activity (Figure 2D), although 10-fold diluted DI-244 showed markedly reduced antiviral activity  
216 in STAT1<sup>-/-</sup> cells as compared to wt cells. In contrast, inhibition of vesicular stomatitis virus  
217 (VSV) infection by DI-244 was completely dependent on STAT1, independent of the DIP dose  
218 used (Figure 2D). Finally, we asked whether the antiviral activity of DIPs still depended on the  
219 DI RNA length if DIPs were added to cells before virus. In contrast to what was observed with  
220 MDCK cells in the presence of trypsin, all DIPs with nested deletions in segment 1 inhibited  
221 WSN infection of A549 wt cells with similar efficiency (Figure 2E and data not shown),  
222 indicating that the contribution of replication interference to DIP antiviral activity was minor or  
223 absent under those conditions. Collectively, our findings indicate that DIPs can induce robust,  
224 partially STAT1-independent anti-IAV activity that is not determined by DI RNA length and  
225 markedly more potent than DIP-mediated inhibition of IAV genome replication.

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### 227 **DI-244 induces robust expression of ISGs but not IFN**

228 In order to understand how DIPs activate the IFN system, we compared DIP- and IAV-mediated  
229 stimulation of IFN expression. For this, an IFN bioassay was employed that was based on VSV, a  
230 highly IFN-sensitive virus (23). A549 or A549 STAT1<sup>-/-</sup> effector cells were incubated with IAV,  
231 VSV or DI-244 for 24 h, the supernatants collected and heat and acid treated to inactivate viral  
232 particles but not IFN, which is known to display a certain heat and acid stability. Subsequently,  
233 the supernatants were added to sentinel cells (A549) for 24 h followed by inoculation of the  
234 sentinel cells with a single-cycle reporter VSV replicon and quantification of infection. For  
235 standardization, A549 cells were incubated with recombinant IFN $\alpha$ , infected with the single-  
236 cycle VSV and infection efficiency quantified. Supernatants from IAV exposed A549 wt cells but  
237 not A549 STAT1<sup>-/-</sup> cells potently inhibited subsequent VSV infection (Figure 3A), indicating that

238 IAV induced production of IFN in a STAT1-dependent fashion, as expected. Similar findings  
239 were made with supernatants from VSV exposed cells but antiviral activity was independent of  
240 STAT1 expression (Figure 3A), again in agreement with published data (24). Finally, and  
241 unexpectedly, supernatants from A549 wt cells exposed to DI-244 were not inhibitory and the  
242 same finding was made for supernatant from DI-244 treated A549 STAT1<sup>-/-</sup> cells, indicating that  
243 IFN induction by DI-244 was low or absent (Figure 3A).

244         The ability of DI-244 to inhibit IAV and VSV infection without inducing IFN, as  
245 determined in the bioassay, posed the question how DI-244 alters gene expression in target cells  
246 to block infection. To address this question, A549 cells and A549 STAT1<sup>-/-</sup> were either incubated  
247 with control supernatants or supernatants containing DI-244 or IAV and subjected to RNAseq  
248 analysis. PR8 was employed for these studies, in order to limit viral replication to a single cycle  
249 (since no trypsin was present after virus inoculation) (25). Neither PR8 nor DI-244 induced the  
250 expression of IFN receptors (Figure 3B). In contrast, PR8 but not DI-244 induced expression of  
251 IFN $\beta$  and IFN $\lambda$  (Figure 3B), in agreement with our results obtained in the bioassay. Despite the  
252 differential upregulation of IFNs by PR8 and DI-244 both induced the robust expression of  
253 antiviral ISGs, including MX1, IFITM1 and ISG15 in A549 wt cells, although induction by PR8  
254 was more efficient than that observed for DI-244 (Figure 3B). Moreover, no ISG induction was  
255 observed in PR8 or DIP treated A549 STAT1<sup>-/-</sup> cells, with the exception of ISG15 and RSAD2  
256 (Viperin), the expression of which was induced by PR8 but not DI-244 (Figure 3B). Finally,  
257 results with A549 wt cells were confirmed by qRT-PCR analyses. Induction of IFN $\beta$  and IFN $\lambda$   
258 by DI-244 was detectable but at least 100-fold less efficient as compared to PR8 while  
259 differences in ISG induction were frequently less than 10-fold (Figure 3C). In sum, these results  
260 suggest that DI-244 inhibits viral infection by STAT1-dependent induction of ISG expression  
261 without inducing appreciable expression of IFN.

## 262 **Discussion**

263 DI RNAs arise in IAV infected cell cultures, eggs, animals and patients (4, 5, 20, 26-29). They  
264 inhibit IAV infection and might modulate IAV intra- and interpatient spread and pathogenesis.  
265 However, the mechanism underlying DI RNA antiviral activity and the determinants controlling  
266 whether a defective viral genomic RNA is also interfering are incompletely understood. Here, we  
267 show that any central deletion in segments 1, 2 and 3 of IAV is sufficient to convert these RNAs  
268 into DI RNAs and that inhibitory activity of the respective DI RNAs extends to all tested IAV  
269 genomic RNAs. Moreover, we provide evidence that the contribution of replication interference  
270 to DIP antiviral activity in cell culture is minor as compared to induction of the IFN system.

271 IAV and influenza B virus DI RNAs usually contain deletions relative to the genomic  
272 RNAs they arose from (4, 5), although an exception has recently been reported (30). Moreover,  
273 DI RNAs derived from IAV segments 1-3, which encode the subunits of the viral polymerase,  
274 arise more frequently than those derived from other segments (4-6, 8, 9, 31, 32) and were thus in  
275 the focus of the present study. The almost universal presence of a deletion in DI RNAs suggests  
276 that their shorter length might allow them to out-compete their parental RNAs for resources  
277 required for RNA replication. Although this hypothesis is frequently posited (4, 5), direct  
278 experimental proof is largely lacking. Here we provide this proof by demonstrating that deleting  
279 any internal sequence from segments 1, 2 and 3 is sufficient to generate a DI RNA. Furthermore,  
280 we demonstrate that the inhibitory activity of these DI RNAs is determined by their length, at  
281 least in the absence of an IFN response, and extends to all target segments tested. The latter  
282 observation fits with the finding that DI-244 interferes with replication of several genomic RNAs  
283 in IAV infected cells (33). In sum, deleting the sequences between the conserved 5' and 3' ends  
284 of any IAV RNA, which are required for transcription and translation, will likely generate potent  
285 DI RNAs. In some cases, the truncated open reading frame encoded by such DI-RNAs might

286 contribute to antiviral activity (34) but this was not observed for DI-244, in keeping with  
287 previous results (33).

288         Type I IFNs trigger the expression of about 400 genes, many of which encode proteins  
289 with antiviral activity, including MX1 (35). The present study shows that when conditions are  
290 chosen that allow DIPs to robustly activate the IFN system, DIPs are potent inducers of ISG  
291 expression and the contribution of replication interference to DIP antiviral activity is minor.  
292 Notably, RNAseq analysis revealed that IAV but not DIPs induced type I and type III IFN  
293 expression although both triggered ISG expression in a STAT1-dependent fashion. A potential  
294 explanation for this discrepancy is that DIPs induced IFN expression at levels too low to be  
295 detected by RNAseq but still sufficient to induce ISGs. Indeed, qRT-PCR analysis revealed  
296 modest upregulation of type I and III IFN upon DIP treatment. Alternatively, one might speculate  
297 that DIPs may induce ISGs via an unidentified IFN-independent, STAT1-dependent pathway.  
298 Interestingly, Wang and colleagues also reported that DIPs induce robust levels of ISGs but only  
299 moderate levels of IFN (36) and further research is required to explore why IFN and ISG  
300 induction are not correlated in the context of DIPs. Moreover, it is unclear how undiluted DIPs  
301 exerted anti-IAV but not anti-VSV activity in STAT1<sup>-/-</sup> cells without inducing ISGs or other  
302 cellular genes. Competition of DIPs with IAV for engagement of entry receptors is one  
303 possibility. Collectively, our results underline previous findings that DIPs are potent inducers of  
304 antiviral responses (13-17) and show that DIP antiviral activity due to induction of ISG  
305 expression outweighs that due to replication interference.

306         What are the major implications of our findings for DIP development as antivirals and for  
307 elucidating the role of naturally occurring DIPs in IAV infection? First, it is essential that  
308 antiviral activity of DIPs is examined in IFN competent animal models which express ISGs with  
309 potent anti-IAV activity, particularly MX1. Second, antiviral activity due to replication

310 interference can be attained only if DIPs are added in 100 to 1,000 fold excess relative to virus  
311 (18) and it remains to be examined whether the strong IFN induction under those conditions  
312 exerts unwanted toxic effects in animals and humans. Third, DIP treatment should be more  
313 effective in the prophylactic as compared to the therapeutic setting, since only in the former DIP-  
314 induced IFN can fully contribute to antiviral activity. Fourth, design of DI RNA and analysis of  
315 DI RNAs emerging in patients should focus on the smallest RNAs, since they can be expected to  
316 exert the highest antiviral activity.

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## 319 **Material and Methods**

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### 321 **Plasmids and oligonucleotides**

322 Plasmids for rescue of A/PR/8/34, pHW191-pHW198, and A/WSN/33, pHW181-pHW188, were  
323 previously described (37). Plasmids encoding DI RNAs were generated by splice overlap PCR,  
324 joining 5' and 3'-end sequences of desired length, following a strategy previously described for  
325 DI-244 (18). A multiple cloning site (mcs) for later insertion of a reporter gene was included in  
326 the respective oligonucleotide sequences (Table S2). The PCR products were cloned into  
327 pHW2000-GGAarI by golden gate cloning (38). Start codons in DI-244 were mutated using  
328 splice overlap PCR primer pairs mutIAV-seg1-ATG-for (5'-  
329 TCAATTATATTCAATTTGGAAAGAATAAAAAG -3')/mutIAV-seg1-ATG-rev (5'-  
330 CTTTTATTCTTTCCAAATTGAATATAATTGA-3') and DImut2+3ATG-for (5'-  
331 ACTACGAAATCTAATCTCGCAGTCTCGCACCCGCGAGATACTCACAAAAACCACCGT  
332 GGACCATATCGCCATAATCAAGAAG-3')/DImut2+3ATG-rev (5'-  
333 CTTCTTGATTATGGCGATATGGTCCACGGTGGTTTTTTGTGAGTATCTCGCGGGTGCGA  
334 GACTGCGAGATTAGATTTTCGTAGT-3'). PCR constructs were cloned into pHW2000-  
335 GGAarI as described above.

336 For expression of the truncated PB2 ORF from DI-244, the ORF was amplified from  
337 pHW2000GG-DI244-rep using primers PB2-QCXIP-5N (5'-  
338 CCGCGGCCGCACCATGGAAAGAATAAAAAGAACTAC-3')/PB2-3XBgl (5'-  
339 GGAGATCTCGAGCTAATTGATGGCCATCCGAAT-3') digested with NotI/XhoI and cloned  
340 into NotI/SalI digested pCAGGS-mcs bearing an altered multiple cloning site (XhoI-SacI-  
341 Asp718I-NotI-EcoRV-ClaI-EcoRI-SmaI-SalI-SphI-NheI-BglIII).

342 For generation of empty vector p19polI-GGAarI the insert was amplified from pHW2000-  
343 GGAarI by splice overlap PCR using primers HW2-GG-5Bgl, CCdelE-rev (5'-  
344 CGTCTTTCATTGCCATACGAAACTCCGGATGAGCATTCATCAG-3'), CCdelE-for (5'-  
345 CTGATGAATGCTCATCCGGAGTTTCGTATGGCAATGAAAGACG-3')/ rRNA-Pr(GG)-  
346 3Eco (5'-GCGAATTCTATAGAATAGGGCCAGGTC-3') and cut with BglII and EcoRI for  
347 insertion into BamHI and EcoRI digested p19luc (39).

348 Reporter plasmids for mini-replicon assay have been described (pPolI-Luc (vRNA/FLUAV/NS1  
349 Seg8-NCR) (19) or were newly generated. First, the reporter with segment 8 ends was amplified  
350 with primers fluA AarI-NS-1 and fluA AarI-NS-890R (Table S3) and inserted into vector  
351 p19polI-GGAarI by Golden Gate cloning. To generate reporters with ends derived from other  
352 segments of IAV, the luciferase reporter gene was amplified with primers encoding the respective  
353 untranslated regions (Table S3) and cloned into vector p19polI-GGAarI as described before. All  
354 PCR amplified sequences were confirmed by automated sequence analysis.

355

## 356 **Cells and viruses**

357 293T, A549 wt and A549 STAT1<sup>-/-</sup> cells were maintained in Dulbecco's Modified Eagle Medium  
358 (DMEM; Gibco) containing 10% fetal bovine serum (FBS, Gibco), penicillin (Pen, 100 IU/mL)  
359 and streptomycin (Strep, 100 µg/ml). BHK-21 cells were cultivated in Dulbecco's modified  
360 Eagle medium (DMEM, Pan Biotech) supplemented with 5% fetal bovine serum and pen/strep.

361 293T cell lines stably expressing codon optimized PB2 (293T-PB2opt) were cultured in the  
362 presence of 1 µg/ml puromycin. Madin-Darby canine kidney cells (MDCK) were cultured in  
363 Glasgow's Modified Eagle Medium (GMEM; Gibco) supplemented with 10% fetal bovine serum  
364 (FBS, Gibco) and pen/strep. MDCK cells stably expressing PB2opt were maintained in the  
365 presence of 1.5 µg/ml puromycin. For generation of A549 STAT1<sup>-/-</sup> cells, A549 wt cells were



366 transduced with pLentiCRISPR v2 (Addgene, plasmid 52961), a lentivirus expressing Cas9,  
367 puromycin resistance, and a guide RNA targeting human *STAT1*  
368 (TTCAAGACCAGCGGCCTCTGAGG). Transduced cells were puromycin selected for seven  
369 days and surviving cells were plated in 96-well dishes as single cells and expanded. Clonal  
370 populations were then lysed and whole cell extract was examined for STAT1 expression by  
371 immunoblot. These efforts identified a single clone that demonstrated a complete loss of STAT1  
372 expression, which we refer herein as STAT1<sup>-/-</sup> cells. All cells lines were regularly tested for  
373 mycoplasma contamination.

374         A/PR/8/34 (H1N1) and A/WSN/33 (H1N1) (37, 40) were produced in embryonated  
375 chicken eggs as described previously (41) while A/WSN/33 adapted to growth in A549 cells was  
376 obtained from the strain repository of the IVM Münster and was amplified in A549 cells by  
377 continuous passaging. IAV titers were determined using focus formation assay as described (18,  
378 38, 42). Replication-competent vesicular stomatitis virus (VSV) expressing eGFP and either  
379 wildtype VSV matrix protein (VSV\*) or a matrix protein variant harboring four amino acid  
380 substitutions associated with increased induction of type-I interferon response (VSV\*M<sub>Q</sub>) have  
381 been described elsewhere (43) and were amplified using BHK-21 cells. Further, a VSV  
382 glycoprotein trans-complemented, single-cycle VSV replicon that lacks the genetic information  
383 for VSV-G but instead codes for eGFP and firefly luciferase genes (VSV\*ΔG-FLuc) (44) was  
384 employed and propagated on BHK-G43 cells (45). All VSV variants were titrated on BHK-21  
385 cells and eGFP-positive foci (replication-competent VSV) or eGFP-positive single cells (single-  
386 cycle VSV) were counted as described previously (46).

387

388 **Mini-replicon assay**

389 The mini-replicon assay was performed as described (19). In brief, 293T cells seeded in 12-well  
390 plates at a cell density of  $2 \times 10^5$  cells per well were cotransfected with plasmids encoding PB1  
391 (10 ng), PB2 (10 ng), NP (100 ng), reporter segment encoding firefly luciferase (50 ng) and  
392 plasmid encoding a DI RNA or empty plasmid (amounts indicated in figures or figure legends).  
393 Cells were washed at 6-8 h and harvested at 24 h post transfection. Firefly luciferase activity in  
394 cell lysates was measured using a commercial kit (PJK) and the Plate Chameleon V reader  
395 (Hidex, Turku, Finland) jointly with Microwin 2000 software.

396

### 397 **Production of DIPs**

398 A coculture of  $1.4 \times 10^6$  293T cells and  $0.4 \times 10^6$  MDCK cells each stably expressing PB2opt and  
399 seeded in T-25 flask was cotransfected with plasmids encoding IAV genomic segments 2-8 of  
400 either PR8 or WSN origin and a plasmid encoding a segment 1-derived DI-RNA. After overnight  
401 incubation, cells were washed once with PBS and, for production of A/PR/8/34-derived DIPs,  
402 DMEM infection medium (0.2% MACS BSA, 1% pen/strep) supplemented with TPCK trypsin  
403 ( $0.5 \mu\text{g/ml}$ ) was added. For production of A/WSN/33-derived DIPs, DMEM growth medium (2%  
404 FCS, 1% pen/strep) was added. As a negative control, parental MDCK and 293T cells were  
405 transfected. Supernatants containing A/PR/8/34-derived DIPs were harvested at 4, 6, 8 and 10  
406 days post transfection while supernatants containing A/WSN/33-derived DIPs were harvested at  
407 3, 5, 7 and 9 days post transfection. Supernatants were cleared from debris by centrifugation,  
408 aliquoted and stored at  $-80^\circ\text{C}$  for further use. For some experiments, DIPs were further amplified  
409 in MDCK-PB2opt cells. For this, a total of  $3 \times 10^6$  cells were seeded in T-75 flasks and infected at  
410 an MOI of 0.01 or lower. Upon detection of CPE, supernatants were cleared from debris by  
411 centrifugation and sterile-filtration ( $0.45 \mu\text{m}$  filter), aliquoted and stored at  $-80^\circ\text{C}$  for further use.  
412 Integrity of selected DIP preparations was controlled with segments specific PCR. Infectious

413 titers of supernatants were determined by focus formation assay using MDCK-PB2opt cells as  
414 targets, as described (18, 38, 42).

415

#### 416 **Analysis of antiviral activity of DIPs**

417 For testing the antiviral activity of DIPs in MDCK cells in the presence of trypsin, cells were  
418 seeded at 10,000 cells/well in 96-well plates and coinfecting with DIP (MOI 1, and 10-fold  
419 dilutions) and IAV (A/PR/8/34, MOI 0.001) for 1 h in Glasgow's MEM (GMEM) infection  
420 medium containing trypsin (0.5 µg/ml). Alternatively, DIPs were added 24 h prior to the virus.

421 For analysis of DIP antiviral activity in MDCK cells, A549 wt and A549 STAT1<sup>-/-</sup> cells in the  
422 absence of trypsin, cells were again seeded at 10,000 cells/well in 96-well plates and either  
423 coinfecting with DIP (MOI 5 or 10, and 10-fold dilutions) and IAV (A/WSN/33, MOI 0.1) in  
424 DMEM medium without trypsin or DIPs added 24 h prior to the virus. After 1 h, cells coexposed  
425 to DIPs and virus were washed and culture medium with or without trypsin was added.

426 Supernatants were harvested after 72 h (MDCK) and 96 h (A549 wt and A549 STAT1<sup>-/-</sup>). Viral  
427 titers in culture supernatants were quantified using focus formation assay and MDCK cells, as  
428 described (38, 42).

429

#### 430 **Quantitative RT-PCR analysis**

431 In order to investigate modulation of *MXI* mRNA expression by IAV, DIPs and IFN, a  
432 quantitative RT-PCR assay was performed. For this, A549 cells were seeded at a cell density of 2  
433 ×10<sup>5</sup> cells/well in 12-well plates and inoculated with IAV (MOI 1), DIPs (MOI 1) or pan-IFNα  
434 (100 U/ml, PBL Assay Science) using DMEM infection medium for 1 h (DMEM infection  
435 medium without trypsin was added to cells exposed to IFNα). Then cells were washed once with  
436 PBS and cultured in DMEM infection medium without trypsin for 24 h. To assess the effect of

437 trypsin on *MX1* induction by IFN $\alpha$ , cells were incubated for 24 h with IFN $\alpha$  in the presence of 0,  
438 0.5, 0.05 and 0.005  $\mu\text{g/ml}$  trypsin. At 24 h post treatment, total cellular RNA was extracted using  
439 the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. After determining the  
440 RNA content, 1  $\mu\text{g}$  RNA was used as template for cDNA synthesis employing the SuperScript III  
441 First-Strand Synthesis System (ThermoFisher Scientific), following the protocol for random  
442 hexamers. Subsequently, 1  $\mu\text{l}$  of cDNA (total volume after cDNA synthesis: 20  $\mu\text{l}$ ) was analyzed  
443 by quantitative PCR on a Rotorgene Q device (Qiagen) employing the QuantiTect SYBR Green  
444 PCR Kit (Qiagen). Each sample was analyzed in triplicates for transcript levels - given as cycle  
445 threshold (Ct) values - of  $\beta$ -actin (*ACTB*, internal transcript control) and myxovirus resistance  
446 protein 1 (*MX1*, indicator for IFN induction, target transcript) with primer previously reported by  
447 Biesold and colleagues (47). In order to analyze gene expression, the  $2^{-\Delta\Delta\text{Ct}}$  method was used  
448 (48).

449 The impact of IAV or DIP- on expression of cellular mRNAs was measured by RNAseq  
450 and results for certain mRNAs were confirmed by quantitative RT-PCR. Expression of these  
451 mRNAs was assayed using TB Green™ Premix Ex Taq™ II (Tli RNase H Plus; Takara)  
452 according to manufacturer's instructions with QuantiTect primer assays (Qiagen). For this, RNA  
453 was isolated as described above and reverse transcribed into cDNA with the prime Script RT  
454 reagent kit (Takara). 10 ng of cDNA was used in a 1X reaction consisting of 12.5  $\mu\text{l}$  TB Green  
455 Premix Ex Taq II (Tli RNaseH plus) (2X), 2  $\mu\text{l}$  10X QuantiTect primer assay, and 0.5  $\mu\text{l}$  50X  
456 ROX reference dye in a final reaction volume of 25  $\mu\text{l}$ . PCR reactions were performed in a  
457 StepOne Plus Instrument (Thermo Fisher). The following QuantiTect primer assays were used.  
458 Hs\_IFIT1\_1\_SG (QT00201012), Hs\_ISG20\_1\_SG (QT00225372), Hs\_IFNB1\_1\_SG  
459 (QT00203763). Hs\_MX1\_1\_SG (QT00090895), Hs\_IFNL1\_2\_SG (QT01033564),  
460 Hs EIF2AK2\_1\_SG (QT00022960), Hs\_IFNL2\_1\_SG (QT00222488), Hs\_RR18s

461 (QT00199367), Hs\_ISG15\_1\_SG (QT00072814), Hs\_RSAD2\_1\_SG (QT00005271). 18S RNA  
462 is used as housekeeping gene. Fold gene induction over mock treated control is calculated by the  
463  $\Delta\Delta\text{CT}$  method.

464

#### 465 **Vesicular Stomatitis Virus Replicon-Based Bioassay**

466 To analyze the relative contribution of IFN induction to antiviral activity, a VSV replicon-based  
467 bioassay (44) was performed. This assay is based on the principle that inoculation of effector  
468 cells with virus or DIPs leads to the induction of the innate immune system, resulting in the  
469 release of type-I IFN into the culture supernatant. These supernatants are then used to inoculate  
470 sentinel cells. Here, the type-I IFN will bind to the IFN $\alpha/\beta$  receptors and trigger a signaling  
471 cascade leading to the induction of an antiviral state. Subsequent inoculation of the sentinel cells  
472 with a highly IFN-sensitive VSV replicon containing a luciferase reporter will yield luciferase  
473 activities that inversely correlate with the extent of the induced antiviral state. A549 and A549  
474 STAT1<sup>-/-</sup> cells (= effector cells) were seeded in 12-well plates (200,000 cells/well) and inoculated  
475 with IAV, VSV\*, VSV\*M<sub>Q</sub>, or DIPs (all at MOI of 1) using DMEM infection medium  
476 containing trypsin for 1h. The cells were washed once with PBS and cultured in DMEM infection  
477 medium without trypsin (used for all further steps) for 16-18 hours. Next, supernatant was  
478 harvested and infectious virus was inactivated by addition of 0.1 M HCl and heating the samples  
479 for 30 min to 56 °C. After the samples cooled down to room temperature, alkaline treatment was  
480 performed using 0.1 M NaOH to neutralize the acidic pH. Subsequently, two-fold serial dilutions  
481 of the samples were prepared. In addition, medium containing two-fold serial dilutions of  
482 recombinant pan IFN $\alpha$  (starting at a concentration of 400 U/ml) were treated in the same fashion.  
483 These samples served as reference and were later used to calculate the relative antiviral activity  
484 present in the different supernatants (given as relative IFN $\alpha$  units per ml). The diluted

485 supernatants and IFN $\alpha$  reference samples were added in quadruplicates to a confluent layer of  
486 A549 cells grown in 96-well plates (= sentinel cells) and incubated for 24 h. Thereafter, the cells  
487 were inoculated with VSV\* $\Delta$ G-FLuc reporter virus (MOI of 3) and further incubated for 6 h.  
488 Then, the medium was aspirated and 50  $\mu$ l/well of 1x luciferase lysis buffer was added.  
489 Following an incubation period of 30 min the lysates were transferred into white, opaque-walled  
490 96-well plates and firefly luciferase activity in cell lysates was measured as described above for  
491 the mini-replicon assay.  
492 For normalization, luciferase activity was set as 100 % for cells that received regular culture  
493 medium instead of diluted culture supernatant/IFN $\alpha$  prior to inoculation with VSV\* $\Delta$ G-FLuc.  
494 Using the normalized luciferase values of cells treated with the IFN $\alpha$  reference samples and a  
495 non-linear regression model we then calculated the relative IFN $\alpha$  content (given as units per ml)  
496 for the effector cell supernatants.

497

#### 498 **RNA-seq analysis**

499 For analysis of IAV and DIP mediated modulation of cellular gene expression, A549 wt and  
500 A549 STAT1<sup>-/-</sup> cells were exposed for 1h to DMEM infection medium supplemented with TPCK  
501 trypsin (0.5  $\mu$ g/ml) and containing A/PR/8/34 or DI-244 at an MOI of 1 or were exposed to  
502 control supernatants. Subsequently, cells were washed and cultured with DMEM infection  
503 medium without trypsin. At 24 h post treatment, total cellular RNA was extracted using the  
504 RNeasy Mini kit (Qiagen) following the manufacturer's instructions and subsequently sent for  
505 RNAseq analysis at the Integrative Genomics Core Unit (NIG), Department of Human Genetics,  
506 University Medical Center Göttingen.  
507 RNA-seq libraries were performed using the non-stranded mRNA Kit (Illumina). Quality and  
508 integrity of RNA was assessed with the Fragment Analyzer using the standard sensitivity RNA

509 Analysis Kit (Advanced Analytical). All samples selected for sequencing exhibited an RNA  
510 integrity number of >8. After library generation, we used the QuantiFluor™dsDNA System  
511 (Promega) for accurate quantitation of cDNA libraries. The size of final cDNA libraries was  
512 determined by using the dsDNA 905 Reagent Kit (Advanced Analytical) exhibiting a sizing of  
513 300 bp in average. Libraries were pooled and sequenced on an Illumina HiSeq 4000 (Illumina)  
514 generating 50 bp single-end reads (28-35 Mio reads/sample). The raw read & quality check were  
515 done by transforming sequence images the BaseCaller software (Illumina) to BCL files,  
516 which were demultiplexed to fastq files with bcl2fastq v2.20. The sequencing quality was asserted  
517 using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).  
518 For subsequent data analysis, ISGs with anti-IAV activity were selected based on work by  
519 Schoggins and colleagues (35). ISG expression in IAV- or DIP-treated cells was further  
520 normalized to ISG expression in control-treated cells.

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698 **Figures legends**

699

700 **FIG 1** Antiviral activity of DI RNAs inversely correlates with DI RNA length

701 (A) DI-244 inhibits genome replication in the mini-replicon assay. 293T cells were transfected  
702 with plasmids encoding the viral polymerase proteins, NP, a segment 8-based luciferase reporter  
703 (mini-replicon system) and either empty plasmid or plasmid for expression of DI-244 mRNA (10  
704 and 300 ng) and vRNA. Removing the plasmid encoding PB2 from the transfection mix served as  
705 negative control. Cotransfection of all support plasmids and empty plasmid instead of DI-244  
706 encoding plasmid served as positive control. The average of five independent experiments is  
707 shown, for which the positive control was set as 100%. Error bars indicate standard error of the  
708 mean (SEM).

709 (B) The truncated open reading frame of DI-244 does not contribute to inhibition of genome  
710 replication in the mini-replicon assay. The experiment was carried out as described for panel A  
711 but the cells were cotransfected with a plasmid for expression of DI-244 mRNA and vRNA with  
712 or without the first three ATGs of the PB2 ORF being intact (DI-244, DI-244 mut ATG), a  
713 plasmid for expression of DI-244 mRNA (DI-244 ORF) or empty plasmid pCAGGS. The  
714 average of three independent experiments is shown, for which the positive control was set as  
715 100%. Error bars indicate SEM.

716 (C) The inhibitory activity of segment 1-derived DI RNAs in the mini-replicon assays is  
717 inversely correlated with DI RNA length. The experiment was carried out as described for panel  
718 A but 300 ng of plasmids harboring the indicated segment 1-derived DI RNAs were  
719 cotransfected. The DI RNAs tested were numbered as shown in table S1. The average of five  
720 independent experiments is shown, for which the positive control was set as 100%. Error bars  
721 indicate SEM.

722 (D) The inhibitory activity of segment 2- and 3-derived DI RNAs in the mini-replicon assays is  
723 inversely correlated with DI RNA length. The experiment was conducted as described for panel  
724 A but 300 ng of plasmids harboring the indicated segment 2 and 3-derived DI-RNAs were  
725 cotransfected. The DI RNAs tested were numbered as shown in table S1. The average of three  
726 independent experiments is shown, for which the positive control was set as 100%. Error bars  
727 indicate SEM.

728 (E) The inhibitory activity of DI RNAs in the mini-replicon assays is independent from the origin  
729 of the reporter segment. The experiment was carried out as described for panel A but the  
730 indicated reporter segments and segment 1, 2 and 3-derived DI RNAs were used. The results of a  
731 single representative experiment are shown and were confirmed in an independent experiment.  
732 Error bars indicate standard deviation (SD).

733 (F) Antiviral activity of segment 1-derived DIPs is inversely correlated with DI RNA lengths in  
734 the presence of trypsin. MDCK cells were coinfecting with the indicated DIPs (MOI 1) and  
735 A/PR/8/34 (MOI 0.001) in the presence of trypsin, washed, and cultured in medium with trypsin.  
736 DIP-negative supernatants served as controls. At 72 h post infection, viral titers in culture  
737 supernatants were determined by focus formation assay. The average of four independent  
738 experiments is shown; error bars indicate SEM.

739 In panels A-D statistical significance of differences between values measured for cells  
740 cotransfected with support plasmids and either empty plasmid (+ control) or DI RNA encoding  
741 plasmid was determined using one-way ANOVA with Sidak's (panel A) or with Dunnett's  
742 posttest (panel B-D). In panel F statistical significance of differences between values measured  
743 for cells infected with virus and cells infected with virus in the presence of DIPs was determined  
744 using one-way ANOVA with Dunnett's posttest. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$

745

746 **FIG 2** Induction of the IFN system is a major contributor to DIP antiviral activity

747 (A) Trypsin inactivates IFN $\alpha$ . A549 wt cells were exposed to recombinant IFN $\alpha$  (100 U/ml) in  
748 the presence and absence of serially diluted trypsin (T). Undiluted trypsin (IFN $\alpha$  + T) was added  
749 at a concentration of 0.5  $\mu$ g/ml. After 24 h, cells were harvested, RNA isolated and *MXI*  
750 expression analyzed by quantitative RT-PCR. *MXI* transcripts levels were normalized against  
751 *ACTB* ( $\beta$ -actin) transcript levels. The average of three independent experiments is shown. Error  
752 bars indicate SEM.

753 (B) Pre-exposure of target cells to DIPs boosts DIP antiviral activity independent of trypsin. Left  
754 panel, - Trypsin condition: MDCK cells were either coinfecting with DI-244 (MOI 10) and  
755 A/WSN/33 (MOI 0.1) in the absence of trypsin or DI-244 was added to cells at 24 h before virus.  
756 Cells were washed 1 h after addition of virus and maintained in growth medium without trypsin.  
757 At 72 h post infection, viral titers in culture supernatants were determined by focus formation  
758 assay. Right panel, + trypsin condition: The experiment was carried out as described for the left  
759 panel, but A/WSN/33-derived DIPs (MOI 1) and A/WSN/33 (MOI 0.001) were used and  
760 maintained in infection medium supplemented with trypsin. The average of three independent  
761 experiments is shown in both panels; error bars indicate SEM.

762 (C) STAT1 is required for *MXI* induction by IAV and DIP. A549 cells and A549 STAT1<sup>-/-</sup> cells  
763 were exposed to IFN $\alpha$  (100 U/ml), A/PR/8/34 or DI-244 (all MOI 1, in the presence of trypsin)  
764 for 1 h, washed, incubated for 24 h in the absence of trypsin and *MXI* mRNA expression  
765 quantified using qRT-PCR. The average of five independent experiments is shown. Error bars  
766 indicate SEM.

767 (D) Anti-IAV activity of DI-244 is partially and anti-VSV activity of DIP is fully dependent on  
768 STAT1. Antiviral activity of DI-244 was analyzed as described for the left panel of figure 2B but  
769 A549 wt and A549 STAT1<sup>-/-</sup> cells were used. At 96 h post infection, viral titers in culture

770 supernatants were determined by focus formation assay. The average of six (A/WSN/33) and  
771 three independent experiments (VSV) is shown. Error bars indicate SEM.

772 (E) DI RNA length does not modulate DIP antiviral activity in the context of a functional IFN  
773 system. Antiviral activity of the indicated DIPs was analyzed as described for panel D adding  
774 DIPs 24 h before virus. The average of five independent experiments is shown. Error bars  
775 indicate SEM.

776 In panels B and D statistical significance of differences between values measured for cells  
777 inoculated with DIPs at 24 h before IAV infection and cells to which IAV and DIPs were added  
778 at the same time was determined using two-way ANOVA with Sidak's posttest. In panel E  
779 statistical significance of differences in viral titers obtained on cells treated with different  
780 concentrations of DIPs or without (-) DIPs was determined using one-way ANOVA with  
781 Dunnett's posttest. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$

782

783 **FIG 3** DI-244 robustly induces ISG but not IFN expression

784 (A) DI-244 does not induce IFN expression as determined in a VSV-replicon-based bioassay.  
785 A549 and A549 STAT1<sup>-/-</sup> effector cells were exposed to IAV, VSV or DI-244 and supernatants  
786 collected, heat inactivated, acid treated and added onto A549 sentinel cells followed by infection  
787 with VSV. For calibration, A549 cells were incubated with recombinant IFN $\alpha$ , VSV infected and  
788 infection efficiency was quantified. The average of three independent experiments is shown.  
789 Error bars indicate SEM.

790 (B) DI-244 induces robust ISG but not IFN expression as determined by RNAseq. A549 cells  
791 (top panel) and A549 STAT1<sup>-/-</sup> cells (bottom panel) were incubated with IAV (A/PR/8/34), DI-  
792 244 and control supernatants at an MOI of 1 in the absence of trypsin and subjected to RNAseq  
793 analysis. Expression of selected ISGs is shown. The average of two independent experiments



794 (A549) and three experiments (A549 STAT1<sup>-/-</sup>) is presented. Error bars indicate SEM and black  
795 lines within the floating bars indicate the mean.

796 (C) DI-244 induces robust ISG but not IFN expression as determined by qRT-PCR analysis. The  
797 A549 wt cells described in panel B were subjected to qRT-PCR analysis of ISG expression. The  
798 average of three independent experiments is shown.

799

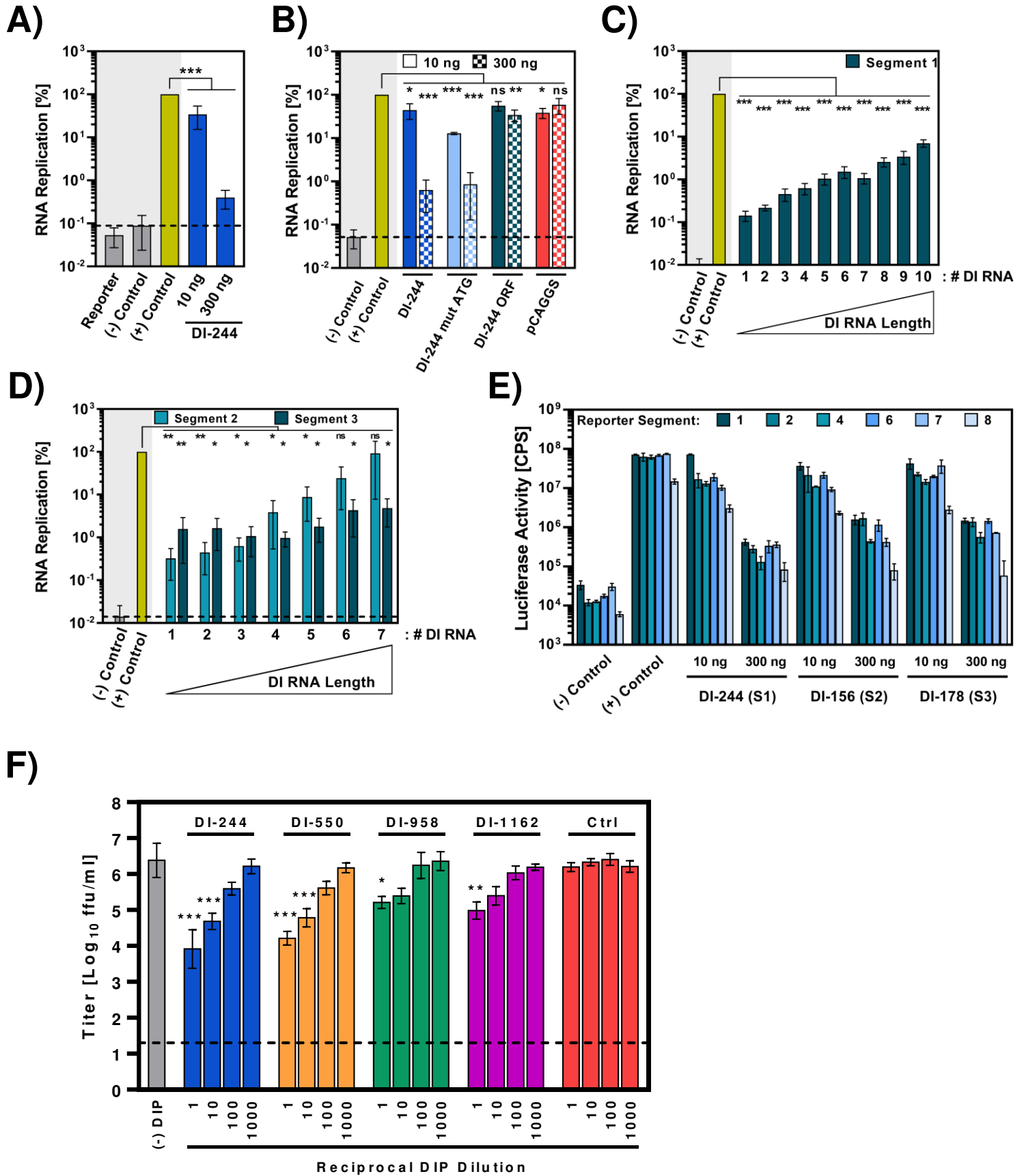
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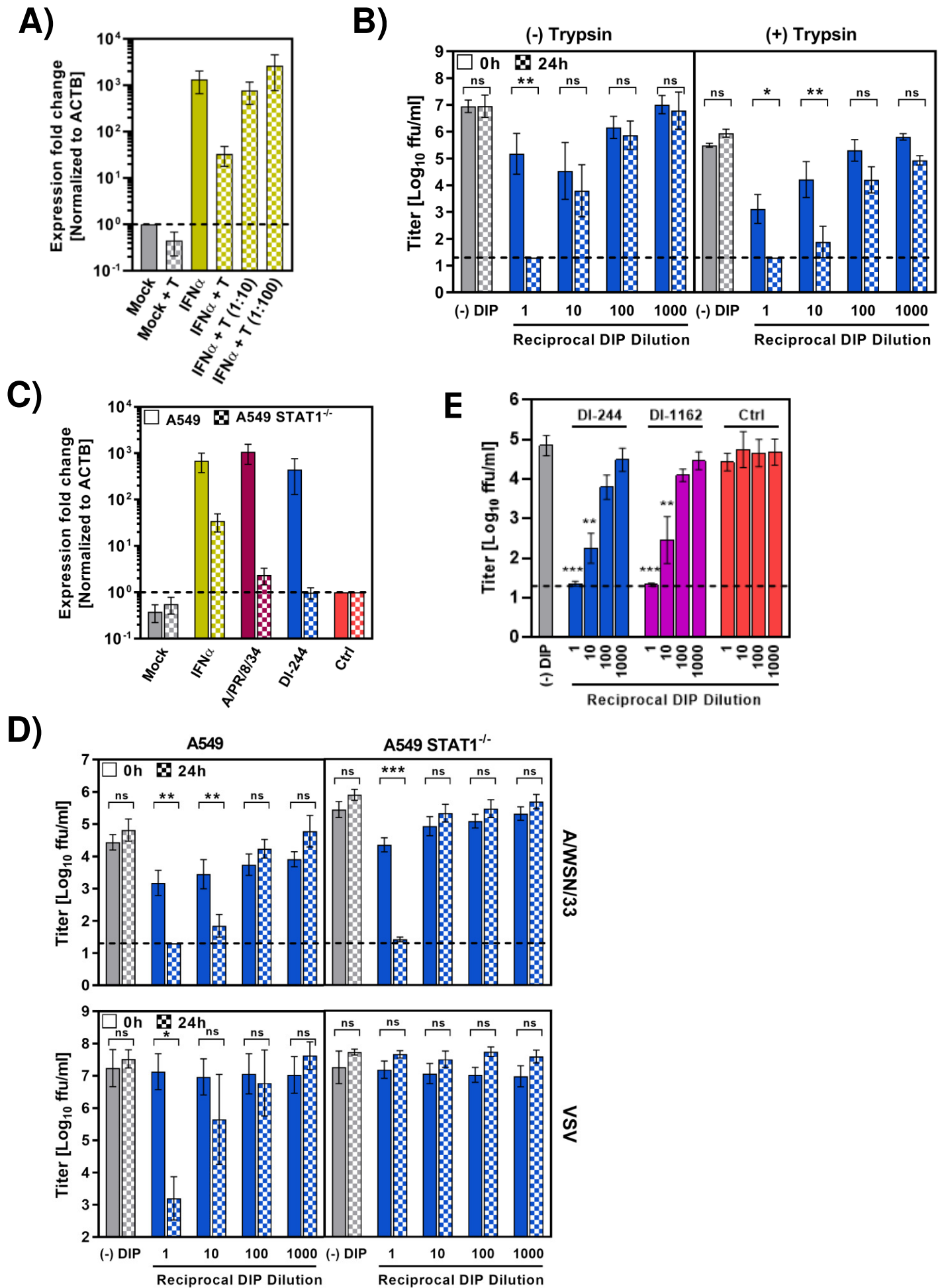
# Figure 1

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## Figure 2

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# Figure 3

