JVI Accepted Manuscript Posted Online 27 November 2019
J. Virol. doi:10.1128/JVI.01535-19
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The *IFNL4* gene is a non-canonical interferon gene with a unique but evolutionarily conserved regulation

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Abstract

Interferon lambda 4 (*IFNL4*) is a recently identified enigmatic member of the interferon lambda family. Genetic data suggest that the *IFNL4* gene acts in a pro-viral and anti-inflammatory manner in patients. However, the protein is *in vitro* indistinguishable from the other members of the interferon lambda family. We have investigated the gene regulation of *IFNL4* in detail and found that it differs radically from that of canonical antiviral interferons. Being induced by viral infection is a defining characteristic of interferons, but viral infection or overexpression of members of the interferon regulatory factor (IRF) family of transcription factors only leads to a minute induction of *IFNL4*. This behavior is evolutionarily conserved and can be reversed by inserting a functional IRF3 binding site into the *IFNL4* promoter. Thus, the regulation of the *IFNL4* gene is radically different and might explain some of the atypical phenotypes associated with the *IFNL4* gene in humans.

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Importance

Recent genetic evidence has highlighted how the *IFNL4* gene acts in a counterintuitive manner as patients with a non-functional *IFNL4* gene exhibit increased clearance of hepatitis C virus but also increased liver inflammation. This suggests that the *IFNL4* gene acts in a pro-viral and anti-inflammatory manner. Those surprising but quite clear genetic data have prompted an extensive examination of the basic characteristics of the *IFNL4* gene and its gene product IFN-λ4. We have investigated the expression of the *IFNL4* gene and found it to be poorly induced by viral infections. A thorough investigation of the *IFNL4* promoter revealed a highly conserved and functional promoter, but also one that lacks the defining characteristic of IFNs, i.e. the ability to be effectively induced by viral infections. We suggest that the unique function of the *IFNL4* gene is related to its non-canonical transcriptional regulation.

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Introduction

Interferons (IFNs) are divided into three families according to their distinct receptor utilization: type I (e.g. IFN-α/β,), type II (IFN-γ) and type III (IFN-λ). Both type I and type III IFNs are potent antiviral cytokines (1). Type II IFN also possesses some antiviral effect but its primary function is to link the innate and adaptive parts of the immune system (2). Type I and type III IFNs play a key role in innate immunity towards viral infection and their expression is induced by viral infection in both immune and non-immune cells (3-5). Humans possess four type III IFN genes known as *IFNL1*, *IFNL2*, *IFNL3* and *IFNL4*. *IFNL1-3* share a high degree of similarity and were identified as a novel family of genes encoding virally induced IFNs by two independent teams in 2003 (6, 7). In the following decade, genome-wide association studies linked clearance of hepatitis C virus (HCV) to genetic variation within the type III IFN loci (8-11), and this subsequently led to the discovery of the *IFNL4* gene when genetic data was compared with RNA-sequencing analysis (12). The DNA sequence similarity between the *IFNL4* gene and the *IFNL1-3* genes is relative low and at the protein level the identity is approximately 28% (13). The *IFNL4* gene is well conserved among mammals, except in rodents where the gene is absent.

Upon identification of the *IFNL4* gene, the authors also identified a dinucleotide variant (ΔG/TT, rs368234815) situated in the first exon of the *IFNL4* gene (12). The *IFNL4*-TT frameshift mutation was introduced during early human evolution, before the "out of Africa" scenario (14). There has subsequently been a positive selection for the *IFNL4*-TT allele resulting in this being the major allele in humans, but with major variations in allele frequency between different human populations. The ΔG/TT variation is associated with the rate of spontaneous HCV clearance as well as the

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92 response to treatment (12). The *IFNL4*-ΔG is the ancestral allele and generates the
93 full-length IFN-λ4 protein, whereas the *IFNL4*-TT allele leads to a frameshift and
94 therefore aborted expression of IFN-λ4. Surprisingly, patients harboring the
95 functional *IFNL4*-ΔG allele have a lower HCV clearance rate compared to those
96 patients, who have the *IFNL4*-TT allele (12). Interestingly, the *IFNL4*-ΔG allele is
97 also associated with lower liver inflammation and fibrosis in HCV-infected patients
98 (15-17) as well as patients with non-alcoholic fatty liver disease (18, 19). Thus, the
99 genetic evidence suggests that *in vivo*, the *IFNL4* gene acts in a pro-viral and anti-
100 inflammatory manner, quite in contrast to the pro-inflammatory and anti-viral effect
101 of other type III IFNs. Here, we will use the term canonical IFNs about all type I and
102 type III IFN genes, except *IFNL4*.
103 Biochemically, the IFN-λ4 protein acts in a manner similar to the proteins encoded by
104 the canonical type III IFN genes. It signals through the canonical IFN-λ receptor
105 complex (20) and induces a set of genes highly similar to that induced by the
106 canonical members of the IFN-λ family (20, 21). Despite its clear antiviral activity *in*
107 *vitro*, the causal role of the IFN-λ4 protein in lower HCV clearance rates is supported
108 by the finding that a single amino acid substitution of a proline to a serine at position
109 70 (IFN-λ4 P70S) in IFN-λ4 substantially affects the antiviral activity of the protein
110 (22). HCV patients harboring the impaired IFN-λ4 S70 variant display lower IFN-
111 stimulated gene (ISG) expression levels but better treatment response rates and better
112 spontaneous clearance rates, when compared to patients coding for the fully active
113 IFN-λ4 P70 variant (22). Finally, comprehensive genome-to-genome analysis in
114 chronically infected HCV patients supported a role for the *IFNL4* gene as the
115 causative gene. In this study, *IFNL4* genotype determined viral load and affected the
116 evolution of HCV quasispecies within patients (23). Thus, the result is a paradox

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where the IFN-λ4 protein is antiviral *in vitro* but appears to be "pro-viral" and "anti-inflammatory" *in vivo*.

Several attempts have been made to measure induction of the *IFNL4* gene during viral infection, both *in vivo* and *in vitro*. Expression of *IFNL4* mRNA was not detected in peripheral blood mononuclear cells from chronically infected HCV patients (24). Similarly, several other studies measured either no or very low *IFNL4* mRNA expression in liver samples from patients with either non-viral liver diseases, chronic hepatitis B virus infection or chronic HCV infection (25-27). Finally, both stimulation with pathogen-associated molecular patterns (PAMP) and viral infection of different hepatoma cell lines or primary human hepatocytes resulted in minimal expression of *IFNL4* but strong expression of the canonical *IFNL3* (28). Together these observations led to speculation that the *IFNL4* promoter is non-functional and that this is part of a general trend during human evolution to abrogate expression of the IFN-λ4 protein.

It is currently unclear why the IFN-λ4 protein became a liability in humans, whereas it is retained in most other mammalian species. To explain the unique genetic observations linked to the *IFNL4* gene, we searched for characteristics of the *IFNL4* gene that differentiates it from the canonical IFNs. Here, we demonstrate that the *IFNL4* promoter is indeed functional and that the mode of gene regulation differs substantially between the *IFNL4* gene and canonical IFNs. Furthermore, we show that the unique features of the *IFNL4* promoter are evolutionarily conserved.

Results

Expression of *IFNL4* mRNA is negligible upon viral infection in different human cell types. The expression of most IFNs is rapidly induced following a viral infection.

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142 Upon infection of human A549 cells with Sendai virus (SeV), expression of *IFNL1*
143 mRNA increased more than 100,000-fold within 5 - 6 hours before it appeared to
144 reach a plateau (Fig. 1A). The expression of *IFNL4* mRNA also increased
145 significantly within the first 5 - 6 hours of infection but far less than that of *IFNL1*. At
146 8 hours after infection, the expression level of *IFNL4* appeared to reach a plateau and
147 was 300-fold lower than for *IFNL1*. To further investigate the rather low expression
148 level of *IFNL4* mRNA, we compared the expression of this gene as well as *IFNL1* and
149 *IFNB1* in four different human cell lines with or without viral infection. Based on the
150 earlier results, we chose to measure mRNA levels at 6 hours after infection or mock-
151 infection. As expected, infection with SeV led to a robust expression of *IFNB1* and
152 *IFNL1* mRNA in A549, THP-1 and PC-3 cells and a moderate expression of *IFNL1*
153 but not *IFNB1* in HepG2 cells (Fig. 1B). In contrast, the expression of *IFNL4* was
154 much lower than that of the two canonical IFNs and even after viral infection,
155 expression of *IFNL4* rarely exceeded the baseline expression of *IFNB1* and *IFNL1*.
156 With the exception of the HepG2 cells, the absolute expression of *IFNB1* and *IFNL1*
157 was a 1000-fold higher than that of *IFNL4* following viral infection. Of note, in both
158 A549 and HepG2 cells, the *IFNL1* and *IFNL4* mRNA levels were close to the
159 detection limit in the absence of viral infection, and for several of the replicates, no
160 product was detected within 40 cycles and those measuring points were thus omitted
161 (Fig. 1B). Cell lines sometimes exhibit minor or major defects in innate immune
162 pathways and therefore we tested the expression of *IFNL4* mRNA in primary human
163 airway epithelial cell (hAEC) cultures stimulated with poly(I:C) and compared it to
164 that of *IFNB1* and *IFNL1* mRNAs (Fig. 1B). In these cells, poly(I:C) stimulation led
165 to a robust expression of *IFNL1* mRNA whereas *IFNB1* mRNA levels were relatively
166 high even in the absence of poly(I:C) and did not increase further after poly(I:C)

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167 treatment. In contrast, *IFNL4* mRNA levels were below the detection limit both with
168 and without poly(I:C) stimulation.

169 The overall low expression of *IFNL4* mRNA was surprising to us because a
170 substantial expression of the *IFNL4* gene was previously reported to occur in
171 epithelial cells infected by human metapneumovirus (hMPV) (29). Therefore, we
172 repeated this experiment where we measured induction of *IFNLs* transcripts in A549
173 cells infected by an influenza A virus (IAV) variant lacking its NS1 gene (IAV ΔNS1),
174 or by hMPV. In contrast to previously published data, hMPV infection did not lead to
175 any substantial expression of *IFNL4* mRNA in our experiments but did induce the
176 expression of *IFNL1* mRNA (Fig. 1C). As expected, infection with IAV ΔNS1
177 resulted in a strong induction of *IFNL1* mRNA but only a minor induction of *IFNL4*
178 mRNA. Altogether, *IFNL4* expression is negligible compared to that of *IFNL1* in
179 A549 cells after hMPV or IAV ΔNS1 infection (Fig. 1C). This leads us to suggest that
180 *IFNL4* is a non-canonical IFN as it lacks the otherwise defining characteristic of IFNs,
181 i.e. their strong induction upon viral infection.

182

183 **The *IFNL1* but not the *IFNL4* promoter is activated by viral infection or**
184 **overexpression of MAVS.** To create a system which allowed us to characterize the
185 *IFNL4* promoter in detail, we cloned a 2,186 bp fragment of the *IFNL1* promoter and
186 a 2,387 bp fragment of the *IFNL4* promoter in front of a firefly luciferase gene (Fig.
187 2A). Because IFN regulatory factor 3 (IRF3) is a crucial transcription factor
188 governing expression of canonical IFNs, we used two ways of activating IRF3:
189 overexpression of the adaptor protein MAVS, which signals upstream of IRF3, or
190 infection with SeV. First, HEK293T cells were transfected with either the *IFNL1* or
191 *IFNL4* promoter reporter construct along with increasing amounts of a construct

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192 encoding MAVS (Fig. 2B). The results show that the *IFNL1* promoter is potently
193 induced by MAVS overexpression whereas this is not the case for the *IFNL4*
194 promoter. Even at the highest amount of MAVS-encoding construct used, the *IFNL1*
195 promoter gives an approximately 230-fold stronger luciferase activity than the *IFNL4*
196 promoter. Next, we repeated the experiment using SeV infection instead of
197 overexpression of MAVS, with the similar result that the *IFNL1* but not the *IFNL4*
198 promoter was potently induced by SeV infection (Fig. 2C). This is consistent with the
199 previous results showing a very low *IFNL4* mRNA expression level in human cell
200 lines.
201
202 **The *IFNL1* but not the *IFNL4* promoter is activated by IRF3 and IRF7 following**
203 **SeV infection.** The transcription factors IRF3 and IRF7 both play an important role in
204 controlling expression of canonical IFNs. However, they are member of a larger
205 family consisting of nine members, IRF1-9. It is possible that activation of the *IFNL4*
206 promoter requires other members of the IRF family than those needed for expression
207 of the canonical IFNs. Therefore, we transfected expression constructs encoding the
208 different IRF members into IRF3 knock out HEK293T cells together with either the
209 *IFNL4* or the *IFNL1* promoter reporter construct and then infected the cells with SeV
210 before measuring luciferase activity. Overexpression of both IRF3 and IRF7 led to a
211 small, yet significant induction of the *IFNL4* promoter upon virus stimulation.
212 However, this induction is negligible compared to that of the *IFNL1* promoter (Fig.
213 2D) and underlines the unique regulatory requirement of the *IFNL4* promoter. In
214 contrast, the *IFNL1* promoter was induced by both IRF3 and IRF7 as expected (Fig.
215 2E).
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Is the behavior of the *IFNL4* promoter evolutionarily conserved?

The above experiments showed that the human *IFNL4* promoter is functionally different from the human *IFNL1* promoter. However, since there has been a selection against a functional *IFNL4* gene in humans (SNP ΔG/TT, rs368234815), it is possible that low *IFNL4* promoter activity is a result of an evolutionary pressure to reduce *IFNL4* expression. If this is the case, the low activity of the *IFNL4* promoter should be specific to humans as the selection against an active form of the *IFNL4* gene (ΔG/TT, rs368234815) is a recent event in human evolution (14). To investigate this, we tested the expression of *IFNL4* mRNA in pigs as well as the activity of *IFNL4* promoters from several mammalian species. We used primary porcine airway epithelial cell cultures stimulated with poly(I:C) and measured *IFNL2* and *IFNL4* mRNA levels by semi-quantitative PCR (Fig. 3). The expression of the porcine *IFNL2* gene is clearly induced by poly(I:C) after 30 cycles whereas *IFNL4* specific primers only yielded a faint band after 40 cycles, indicating a rather low expression of *IFNL4* following poly(I:C) stimulation. This suggests that the difference in expression of *IFNL4* compared to that observed for canonical IFNs is not unique to humans and accordingly seems not to be the result of the recent selection against a functional *IFNL4* gene in humans.

This observation prompted us to investigate the activity of the *IFNL4* promoter from a number of mammalian species. The leucine rich repeat and fibronectin type III domain containing 1 (*LRFN1*) and syncollin (*SYCN*) genes, which flank each side of the type III IFN loci, are evolutionarily conserved among mammals (Fig. 4A). We used those genes to mark the borders of the type III IFN loci and then analyzed the genomic loci in a number of mammalian species to identify *IFNL4* orthologues. We analyzed the genomic loci from human, African green monkey, sheep, pig and the

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house mouse because in these species the type III IFN loci are fully sequenced. The *IFNL1* gene is present in humans and African green monkey but is absent in sheep, mouse, and pig, whereas the *IFNL2* and/or *IFNL3* genes can be found in all five species (Fig. 4A). Many mammals seem to have two highly similar *IFNL4* genes, but in humans, one of these *IFNL4* genes has become a pseudogene whereas mice have no *IFNL4* genes. As for sheep and pigs, sheep possess two *IFNL4* genes like many other ungulates while pigs only possess one *IFNL4* gene. Thus, humans and pigs have a single *IFNL4* gene while African green monkeys and sheep have two *IFNL4* genes. We performed an evolutionary analysis of the *IFNL4* genes we identified and concluded that independent duplications of the *IFNL4* gene must have occurred several times during mammalian evolution. This makes it difficult to precisely destinguish between true paralouges and ortologues genes and we therefore chose those *IFNL4* genes which had the same orientation as the human *IFNL4* gene for our investigation.

To describe if the functionality of the *IFNL4* promoter is evolutionary conserved, we cloned the *IFNL4* promoters from African green monkey, sheep and pig and added them to our comparison. As a control, we also cloned the *IFNL2* or *IFNL3* promoter from these species, except from African green monkey where it was not possible due to technical limitations. To compare the different *IFNL4* promoters, HEK293T cells were transfected with the different type III IFN promoter reporter constructs along with a construct encoding MAVS (Fig. 4B). Our comparison showed that the *IFNL4* promoter is induced to a much lower degree by overexpression of MAVS than the *IFNL1*/*IFNL2*/*IFNL3* promoters in all four species that we have tested here. The same results are also seen when we stimulated cells with SeV infection instead of MAVS overexpression (Fig. 4C). One concern by using non-human promoters in HEK293T

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cells is the compatibility of those promoters with the human system. However, we consistently saw strong induction of the *IFNL1/IFNL2/IFNL3* genes and furthermore the data from the primary pig airway epethelial cells was in agreement with the data obtained using the pig promoters in the HEK293T cells. Altogether, the results suggest that the behavior of the *IFNL4* promoter appears to be a conserved among mammals.

The *IFNL4* promoter is highly conserved and forms a separate evolutionary clade. The evolutionarily conserved behavior of the *IFNL4* promoter prompted us to examine the degree of sequence conservation within the *IFNL4* promoter region. Figure 5A shows a nucleotide alignment of the first 600 base pairs (bp) of the *IFNL4* promoters, counting from the translational start site, from those species examined in the previous promoter activity assay. We chose the translational start site to anchor this alignment for two reasons: *i*) it is clearly defined for all species and *ii*) regulatory elements can be found both upstream and downstream of the transcriptional start site. The analysis demonstrated a high degree of conservation suggesting that there has been a significant selective pressure on this region throughout mammalian evolution.

Next, we calculated an evolutionary tree from a set of *IFNL4* and *IFNL1* promoter sequences. The promoter sequences form two clearly separated clades according to which genes they belong to, confirming our previous conclusion that the *IFNL4* promoter is both evolutionary and functional distinct from the canonical IFN promoters (here represented by the *IFNL1* promoter) (Fig. 5B). We also calculated a tree based upon the protein sequences of IFN-λ1 and IFN-λ4 from the same set of species, and as previously observed (3), the IFN-λ4 sequences form a separate and distinct clade (Fig. 5C).

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The basic *IFNL4* promoter is functional and can be activated by an enhancer element present in the *IFNL1* promoter. A promoter region can, somewhat simplified, be described as consisting of a core promoter where the RNA polymerase II complex assembles, and a series of upstream and/or downstream regulatory elements. To test if the core promoter of *IFNL4* is functional, we constructed chimera A combining 100 bp of proximal *IFNL4* promoter and upstream 1,989 bp of *IFNL1* promoter (Fig 6A). Chimera A is active upon overexpression of MAVS, whereas the inverse chimera B with the *IFNL1* core promoter fused to the upstream regulatory elements of the *IFNL4* promoter was largely inactive, indicating that the *IFNL4* core promoter can be activated by enhancer elements present in the *IFNL1* promoter. To determine if the IRF binding site of the *IFNL1* promoter functions as an enhancer in the context of the *IFNL4* promoter, we constructed chimera C. In chimera C, the IRF binding motif from the *IFNL1* promoter was inserted into the *IFNL4* promoter in the same position as it occupies in *IFNL1* promoter, which is 100 bp upstream of the transcription start site (TSS) (Fig 6B). Chimera C is active whereas chimera C with a mutated IRF site (mutIRF) is inactive (the IRF binding site was mutated by changing nucleotides CAGTTTC of this motif into AAGCAGA). Thus, by inserting a functional IRF binding site into context of the *IFNL4* promoter, the promoter becomes inducible by viral infection. These results confirm that the *IFNL4* promoter is a potentially functional core promoter sequence.

A non-functional and out of place IRF binding site is found within the *IFNL4* promoter. Above, we demonstrated that insertion of the IRF site from the *IFNL1* promoter into the *IFNL4* promoter resulted in a virus-inducible activation of the *IFNL4* promoter. However, one putative IRF site exists within the *IFNL4* promoter.

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317 Replacing the canonical IRF site found in the *IFNL1* promoter with the putative IRF
318 binding site from the *IFNL4* promoter resulted in chimera D (Fig. 6C). Chimera D
319 exhibited only minimal activity upon MAVS overexpression implying that the IRF
320 binding site of *IFNL4* is not functional within the context of the *IFNL1* promoter. Not
321 only does the sequence of the IRF binding sites differ between the *IFNL1* and *IFNL4*
322 promoters but their positions differ as well. While the IRF binding site in the *IFNL1*
323 promoter is located relatively close to the TSS (100 bp from the TSS), the IRF
324 binding site in the *IFNL4* promoter is located further upstream (224 bp from the TSS).
325 This is more distant to the TSS compared to most canonical IFN promoters. In
326 chimera E, we inserted the IRF motif of the *IFNL1* promoter into the position of the
327 putative IRF binding site already found within the *IFNL4* promoter. Thus, chimera C
328 and E differ only by the position of the inserted IRF binding site, yet only chimera C
329 is activated by MAVS overexpression, which shows that the position of the IRF
330 binding site does matter (Fig. 6C). In conclusion, whereas the *IFNL4* promoter
331 contains a putative IRF binding site this seems to be non-functional.
332
333 **The *IFNL4* promoter contains functional NF-κB sites**. Efficient induction of IFN
334 normally requires collaboration between the transcription factors IRF3 and NF-κB
335 and we therefore searched for NF-κB binding sites at the proximal end of the *IFNL4*
336 promoter. Two such sites were identified. To test if these sites potentially could be
337 involved in conferring transcriptional activity, we mutated them individually or
338 together in context of the transcriptionally active chimera C (Fig. 6D). Both mutNF-
339 κB1 and mutNF-κB1/2 have reduced transcription whereas mutNF-κB2 has a minimal
340 effect (Fig. 6D). This indicates that the NF-κB1 site is required for transcriptional
341 activity of chimera C, whereas the NF-κB2 site is less important.

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343 **Both IRF3 and RNA polymerase II are recruited to the *IFNL4* promoter in**
344 **response to viral infection.** To investigate if the IRF3 transcription factor and RNA
345 polymerase II are recruited to the promoter of *IFNL1* and *IFNL4*, we performed
346 chromatin immune precipitation (ChIP) assays on A549 cells infected by SeV. For
347 this purpose, we used antibodies against IRF3 and RNA polymerase II as well as IgG
348 as a control (Fig. 7A-C). The data show that IRF3 is recruited to the promoter region
349 of both *IFNL1* and *IFNL4* but with a tendency towards lower enrichment of IRF3 on
350 the *IFNL4* promoter region after virus infection as compared to *IFNL1* (Fig. 7A).
351 Moreover, we found that RNA polymerase II occupies both promoters after SeV
352 infection but more RNA polymerase II was detected at the *IFNL1* promoter (Fig. 7B).
353 Thus, decreased IRF3 and RNA polymerase II recruitment can account for at least
354 some of the low response to viral infection exhibited by the *IFNL4* promoter as
355 compared to the *IFNL1* promoter.

356

357 **Discussion**

358 Genetic data has attributed unique properties to the *IFNL4* gene. It protects against
359 liver fibrosis (17, 19) and hence it is assumed to help dampening or controlling
360 inflammation in both HCV infected patients and patients with non-alcoholic steatosis
361 (NASH) (15). Furthermore, its ablation facilitates both spontaneous and treatment-
362 induced HCV clearance (12). These observations are counterintuitive when taking
363 into account that IFNs are normally both antiviral and pro-inflammatory and suggest
364 that the *IFNL4* gene is functionally distinct from the canonical IFNs. We have
365 conducted a series of experiments to biochemically characterize the IFN-λ4 protein,
366 but found that the biochemical properties of the IFN-λ4 protein are highly similar to

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367 those of other members of the IFN-λ family, i.e. the IFN-λ4 protein signals through
368 the same receptor and induces a set of genes highly similar to that induced by the
369 IFN-λ3 protein (20, 21).

370 A defining characteristic of IFNs is their ability to be induced by viral infection
371 through the transcription factors IRF3 or IRF7 (30). Here we demonstrate that while
372 the *IFNL4* gene has a putative IRF3/7 binding site, this site is either non-functional or
373 functions very poorly resulting in little or no transcription of the *IFNL4* gene in
374 response to viral infections. This agrees well with previous data showing very low
375 levels of *IFNL4* mRNA in the liver of HCV infected patients (25) but contradicts
376 other findings claiming that *IFNL4* mRNA expression is induced by viral infection *in*
377 *vitro* (29, 31).

378 A previously published study showed that hMPV infection led to a strong induction
379 (approximately 40,000-fold) of *IFNL4* mRNA expression in A549 cells when
380 compared to non-infected cells (29). However, when we tested *IFNL4* gene
381 expression in hMPV infected A549 cells, we found that the *IFNL4* expression was
382 weak and much lower than *IFNL1* expression under the same conditions. A different
383 study showed induction of the *IFNL4* mRNA, measured as fold induction, in SeV
384 infected PC-3 cells (31). We measured *IFNL4* mRNA levels in PC-3 cells under
385 similar conditions and although there was an induction of the *IFNL4* mRNA in
386 infected versus non-infected cells, the expression level was low and comparable to the
387 level of *IFNL1* mRNA found prior to viral infection. During a virus infection, large
388 amounts of canonical IFNs are produced and in that context the rather marginal
389 *IFNL4* expression upon viral infection is unlikely to have significant role. Hence, we
390 do not believe that the low level of *IFNL4* mRNA produced by PC-3 or A549 cells is
391 of physiological relevance.

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We performed a series of assays on different *IFNL4*/*IFNL1* promoter chimeras using a luciferase reporter system in HEK293 cells. Those data clearly show that inserting a functional IRF3 site at the proper position renders the *IFNL4* promoter virus-inducible. We speculate that the *IFNL4* promoter is regulated by yet unknown stimuli, and the evolutionary conservation of both the sequence of the *IFNL4* promoter region as well as its functional characteristics supports this. Our efforts to identify such a stimulus has so far been in vain, but our preliminary data suggest that neither ER stress, LPS nor activation of the inflammasome leads to activation of the *IFNL4* gene.

Other examples of non-canonical regulation of IFN genes exist. The *IFNE* gene, in contrast to the other type I IFN genes, is not induced by viral infection but is specifically expressed in the female reproductive tract where it protects against infection. Furthermore, the expression of the *IFNE* gene is regulated by estrogen (32).

To sum up, we conclude that the regulation of the *IFNL4* gene is a conserved feature among mammals and that the *IFNL4* gene is a non-canonical member of the type III IFN family as it is regulated in a unique way that differs from that of the canonical IFNs.

Materials and methods

Cells

Cell lines used in this study were PC-3 (prostate cancer cell line), A549 (human lung epithelial cells), HepG2 (liver hepatocellular carcinoma), THP-1 (human monocytic cells) and IRF3 knock out HEK293T cells (human embryonic kidney cells). PC-3 cells were cultured in F12K medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. THP-1 cells were cultured in the RPMI-1640 medium supplemented with 10% FBS, 100

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417 U/ml penicillin and 100 µg/ml streptomycin. All other cell lines were cultured in
418 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100
419 U/ml penicillin and 100 µg/ml streptomycin.
420 Well-differentiated human airway epithelial cell cultures from three biological donors
421 were isolated, established and maintained as previously described (33). Primary
422 porcine tracheobronchial airway epithelial cells were isolated from post-mortem
423 material obtained from SPF-pigs. Isolation of cells was performed with protease and
424 DNase digestion and primary porcine tracheobronchial cells were cultured as
425 previously described (33) but with a few modifications. For cellular differentiation,
426 the human epidermal growth factor concentration was increased ten-fold in the air-
427 liquid interface medium, whereas retinoic acid was two-fold more concentrated. The
428 epithelial layer was allowed to differentiate for at least four weeks prior to stimulation
429 experiments.
430
431 **Plasmids**
432 The different promoter sequences used in this study were cloned from genomic DNA
433 using standard cloning techniques. The sources were THP-1 cells for the human
434 *IFNL1* and *IFNL4* promoters, Vero cells for the African green monkey IFNL4
435 promoter, Texel sheep peripheral blood mononuclear cells for the sheep *IFNL3* and
436 *IFNL4* and Göttingen minipig tissue for the pig *IFNL2* and *IFNL4* promoters. Because
437 the transcriptional start site has not been experimentally verified for most of these
438 genes, we chose to always include the 5' UTR in the cloned sequence. The sequences
439 were cloned into the multiple cloning site of the pGL3.1-Basic plasmid (Promega)
440 and afterwards the remaining sequence between the inserted promoter sequence and
441 the translational start site of the firefly luciferase gene was deleted by site-directed

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442 mutagenesis. The different chimeras and mutants of the human *IFNL1* and *IFNL4*
443 promoters were constructed using standard cloning techniques and site-directed
444 mutagenesis. In chimera A, nucleotides -1 to -100 of the *IFNL1* promoter were
445 replaced by nucleotides -1 to -100 of the *IFNL4* promoter. In chimera A, nucleotides -
446 1 to -100 of the *IFNL4* promoter were replaced by nucleotides -1 to -98 of the *IFNL1*
447 promoter. In chimera C, nucleotides -101 to -132 of the *IFNL4* promoter were
448 replaced with -101 to -134 of the *IFNL1* promoter. In chimera C (mutIRF),
449 nucleotides -129 to -123 from the *IFNL1* promoter in chimera were changed from
450 CAGTTTC to AAGCAGA. In chimera D, nucleotides -117 to -132 of the *IFNL1*
451 promoter were replaced with nucleotides -225 to -235 of the *IFNL4* promoter. In
452 chimera E, nucleotides -225 to -235 in the *IFNL4* promoter were replaced with
453 nucleotides -117 to -132 of the *IFNL1* promoter. In chimera C (mutNF-κB1),
454 nucleotides -69 to -66 of the *IFNL4* promoter in chimera C was changed from ACCC
455 to CCCT. In chimera C (mutNF-κB2), nucleotides 32 to 35 of the *IFNL4* promoter in
456 chimera C was changed from CCCA to GGGT. Chimera (mutNF-κB1/2) is a
457 combination of the two previous chimeras.
458 The plasmids used for expressing IRF2, -4 and -8 (pEF-IRF2, pSV-LS-IRF4 and
459 pICSBP) were kind gifts from professor Takashi Fujita (Kyoto University, Japan)
460 whereas the plasmids for expression of IRF3 and MAVS (pcDNA3-IRF3 #32713 and
461 pEF-BOS-MAVS #27224) were from Addgene. The plasmids used for expressing
462 IRF1, -5, -6 and -7 were constructed in our laboratory. The sequences of all plasmids
463 used in this study were verified by Sanger sequencing (GATC Biotech) and detailed
464 information about them is available upon request.
465
466 **Infections and stimulations**

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467 For qPCR assays, cells were seeded in 6-well tissue culture plates at a density of
468 $6x10^5$ per well. For the THP-1 cells, phorbol 12-myristate 13-acetate (PMA) was
469 added to a final concentration of 100 nM to trigger differentiation into macrophages.
470 After resting for 24 hours, the cells were infected with 40 HAU SeV (Cantell strain)
471 per well for 6 hours. Infection of A549 cells with hMPV was performed as previously
472 described (29).
473 For luciferase assays, cells were infected 24 hours post transfection with 20 HAU SeV
474 (Cantell strain) per well for 24 hours.
475 For ChIP assays, cells were seeded in 150 mm tissue culture dishes at a density of 1 x
476 10^7 cells per dish. After resting for 24 hours, the cells were infected with 560 HAU
477 SeV (Cantell strain) per dish for 4 hours.
478 For qPCR and semi-qPCR, human or porcine airway epithelial cells were treated with
479 10 µg/ml poly(I:C) via the basolateral surface for a duration of 18 hours prior to cell
480 lysis and total RNA extraction.
481
482 **Quantitative real-time and semi-quantitative PCR**
483 Total RNA was extracted with the E.Z.N.A. Total RNA Kit I (Omega Bio Tek)
484 according to the manufacturer's instructions. The cDNA synthesis was performed
485 with 0.5 µg RNA using RevertAid Reverse Transcriptase and random hexamer primer
486 according to the manufacturer's instructions (ThermoFisher Scientific). The cDNA
487 was quantified by qPCR using SYBR Green I (Roche) and a LightCycler 480
488 Instrument II (Roche). The following primers were used: human *IFNB1*, forward
489 ACGCCGCATTGACCATCTAT and reverse GTCTCATTCCAGCCAGTGCT;
490 human *IFNL1*, forward TTCCAAGCCCACCACAACTG and reverse
491 GTGACTCTTCCAAGGCGTCC, human *IFNL4*, forward

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492 CGGCCTGCCTTGAGCTG and reverse GGGTTTGTGACGCCTCTTCT; human
493 *HPRT1*, forward CCCTGGCGTCGTGATTAGTG and reverse
494 CACCCTTTCCAAATCCTCAGC. The cycling parameters were 95°C for 10 min
495 followed by 40 cycles of 95°C for 10 s, 60°C for 5 s and 72 °C for 4 s. The crossing
496 points of the amplification curves were determined using the second derivative
497 method on the LightCycler 480 Instrument II software 1.5 (Roche). The
498 hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) gene was used as a
499 reference gene. The level of mRNA was normalized against internal HPRT mRNA
500 content. Relative mRNA levels of each target gene were calculated using the
501 following formula: 2^-(Ct (target)-Ct (control)).
502
503
504 **Semi-quantitative PCR**
505 The following primers were used: human *IFNL1*, forward
506 GGAAGCAGTTGCGATTTAGCC and reverse GACTCTTCCAAGGCGTCCCT;
507 human *IFNL4*, forward TTGGCTTCCCTGACGTCTCT and reverse
508 CTCTTCCTCGTAGCGGTCCC; pig *IFNL2*, forward
509 GTCCCTCTTGGAGGACTGGA and reverse CTGAGCTGGGACACAGGC; pig
510 *IFNL4*, forward GATGTCCGTCGCCTCTTGTA and reverse
511 GCGTCTCTTCCTCATAGTGGT. The PCR was performed as described above for
512 qPCR and run for 20, 30, or 40 cycles. The PCR products were run on a 1% agarose
513 TBE gel with a GeneRuler 100 bp Plus DNA Ladder (ThermoFisher Scientific)
514
515 **Transfections**
516 For transfection, cells were seeded in 12-well tissue culture plates at a density of

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517 4×10^5 cells per well. After resting for 24 hours, and then transfected 24 hours later
518 using polyethylenimine (PEI). For all transfections, a total of 2 µg plasmid was gently
519 mixed with 6 µg PEI in a total volume of 200 µl and incubated 15 minutes at room
520 temperature before adding it drop wise to the cells. The 2 µg plasmid always included
521 50 ng plasmid constitutively expressing *Renilla* luciferase and 1,000 ng pGL3.1
522 plasmid containing a type III IFN promoter. Unless otherwise stated, 400 ng plasmid
523 expressing one of the IRFs or MAVS was included in those experiments that required
524 expression of these proteins. To reach the desired total of 2 µg plasmid for each
525 transfection, an empty pcDNA3.1 plasmid was added accordingly.
526
527 **Luciferase assays**
528 At 24 hours after transfection/infection, cells were lysed with Passive Lysis Buffer
529 (Promega) and firefly and *Renilla* luciferase activity in the lysate was then measured
530 using the Dual-Luciferase Reporter Assay System (Promega) according to the
531 manufacturer's instructions.
532
533 **Chromatin immunoprecipitation (ChIP)**
534 ChIP was performed as previously described (34). The antibodies used were Mouse
535 IgG Isotype Control (ThermoFisher Scientific), IRF-3 (D83B9) Rabbit mAb (Cell
536 Signaling), Mouse Anti-RNA polymerase II CTD repeat YSPTSPS antibody [8WG16]
537 (abcam) and Rabbit Anti-Histone H3 antibody (abcam). The co-immunoprecipated
538 DNA was quantified by qPCR as described above except that the cDNA synthesis
539 step was omitted. The following primers were used: human *IFNL1*, forward
540 TGAGGCCAGTTGGCTGAAAG and reverse GGAGCCTGATGAGGGAACAG;
541 human *IFNL4*, forward TCAACACTACACAAGGGCTGG and reverse

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CGGTTTCCAAATTGTCTCTGTCC; *GAPDH*, forward GCGTGTAAGGGTCCCCGTCCT, and reverse GTTCAACTGGGCACGCACCGA

Alignments and phylogenetic trees

All the sequences used for alignments and phylogenetic trees were extracted from the corresponding genomic reference sequences from NCBI. The accession numbers of these are NC000019 (human), NC023647 (African green monkey), NC010448 (domesticated pig), NC019471 (domesticated sheep), NW007907093 (polar bear) and NW006804147 (western European hedgehog). Sequences were aligned using ClustalW and phylogenetic trees were generated using the Neighbor-Joining method in MEGA7.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 8.2.

Acknowledgements

This study was supported by grants from the Chinese Scholarship Council (HZ), the Swiss National Science Foundation, grant number 179260 (RD), the Danish Council for Independent Research, grant number 7016-00331B (RH), the Riisfort Foundation (RH) and the Toyota-Foundation (RH). The authors would like to thank professor Peter Stäheli, University of Freiburg, for the kind gift of RNA from hMPV and IAV ΔNS1 infected A549 cells as well as Martin Kristian Thomsen, University of Aarhus, for the kind gift of Göttingen minipig tissue.

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Author contributions

HZ, MM, ET-D, KGW, NHH, JV-N, LL, RD and HHG designed, performed and analyzed experiments. RD, ALN, HHG and RH supervised the research. HZ, ET-D, HHG and RH conceived the project and prepared the manuscript. All authors commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest

Figure Legends

Figure 1. Expression of the *IFNL4* gene is not virus-inducible. (**A**) A549 (an adenocarcinomic alveolar basal epithelial cell line) cells were infected with SeV and the expression levels of *IFNL4* and *IFNL1* genes were quantified by RT-qPCR at the indicated time points. Data were calculated relative to internal expression of *HPRT*. The experiment was performed in biological triplicates and data are presented as scatter plot with mean ± SD (n=3). (**B**) A549, HepG2 (a hepatocellular carcinoma cell line), macrophage-like differentiated THP-1 (a monocyte-like cell line before differentiation), and PC-3 (a prostate cancer cell line) cells were mock-infected or infected with SeV for 6 hours while human airway epithelial cell (hAEC) cultures were mock-treated or treated with poly(I:C) for 18 hours. Expression levels of *IFNB1*, *IFNL4* and *IFNL1* genes were quantified by RT-qPCR. Data were calculated relative to internal expression of *HPRT*. The dashed line indicates the detection limit in those

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589 cases where mRNA levels could not be determined for some replicates because they
590 were too low. The experiment was performed in biological sextuplicates (left panel)
591 or triplicates (right panel) and data are presented as scatter plot with mean ± SD (n=3
592 or 6). Statistical significance was determined using an unpaired t test. *, $0.01 < P <$
593 0.05; ns, $P \geq 0.05$. (**B**) A549 cells were mock-infected or infected with hMPV or IAV
594 ΔNS1 for 18 hours. Expression levels of *IFNL1* and *IFNL4* mRNA were assessed by
595 semiquantitative PCR and vizualised by agarose gel electrophoresis. The experiment
596 was performed in biological duplicates.

597

598 **Figure 2. The *IFNL4* promoter is not activated by the same stimuli that readily**
599 **activate the *IFNL1* promoter. (A)** The promoter regions of the human *IFNL1* and
600 *IFNL4* genes corresponding to 2,186 and 2,387 bp, respectively, upstream of their
601 translation start site were inserted in front of the firefly luciferase coding sequence in
602 the pGL3.1-Basic plasmid. (**B**) HEK293T cells were co-transfected with pGL3.1
603 plasmid containing the *IFNL1* or *IFNL4* promoter, a plasmid constitutively expressing
604 the *Renilla* luciferase gene and increasing amounts of a plasmid expressing MAVS.
605 At 24 hours post transfection, firefly/*Renilla* luciferase activity was quantified. The
606 experiment was performed in biological triplicates and the data are presented as bar
607 chart and scatter plot with mean ± SD (n=3). (**C**) HEK293T cells were co-transfected
608 with pGL3.1 plasmid containing the *IFNL1* or *IFNL4* promoter and a plasmid
609 constitutively expressing the *Renilla* luciferase gene. At 24 hours post transfection,
610 the cells were infected with SeV for another 24 hours before quantifying
611 firefly/*Renilla* luciferase activity. One representative out of two independent
612 experiments is shown, each with biological triplicates. The data are presented as bar
613 chart and scatter plot with mean ± SD (n=3). (**D-E**) IRF3 knock out HEK293T cells

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614 were co-transfected with pGL3.1 plasmid containing the *IFNL1* (**D**) or *IFNL4* (**E)**
615 promoter in front of a firefly luciferase gene, a plasmid constitutively expressing the
616 *Renilla* luciferase gene and a plasmid constitutively expressing IRF1, IRF2, IRF3,
617 IRF4, IRF5, IRF6, IRF7 or IRF8. At 24 hours post transfection, the cells were
618 infected with SeV for another 24 hours before quantifying firefly/*Renilla* luciferase
619 activity. One representative out of two independent experiments is shown, each with
620 biological triplicates. The data are presented as bar chart and scatter plot with mean ±
621 SD (n=3). Statistical significance was determined using an unpaired t test. ****; $P < 0.0001$, ***, $0.0001 < P < 0.001$; **, $0.001 < P < 0.01$; *, $0.01 < P < 0.05$; ns, $P \geq 0.05$.
622
623
624

625 **Figure 3. The *IFNL4* gene is poorly expressed in primary porcine epithelial cells.**
626 Porcine airway epithelial cells (pAECs) were treated with 10 µg/ml poly(I:C) via the
627 basolateral surface for 18 hours. Expression levels of *IFNL2* and *IFNL4* mRNA were
628 assessed by semiquantitative PCR and vizualised by agarose gel electrophoresis. The
629 experiment was performed in biological triplicates.

630

631 **Figure 4. The weak functionality of the *IFNL4* promoter is evolutionary**
632 **conserved among mammals.** (**A**) Overview of the type III IFN loci in human,
633 African green monkey, sheep, mouse, and pig. The type III IFN genes illustrated by
634 different colors share a common synteny in mammals and are flanked on each side by
635 the leucine rich repeat and fibronectin type III domain containing 1 (*LRFN1*) and
636 syncollin (*SYCN*) genes. Black arrows indicate the direction of transcription for each
637 gene. (**B**) HEK293T cells were co-transfected with pGL3.1 plasmid containing the

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638 *IFNL4* or *IFNL1/2/3* promoter from human, African green monkey, pig or sheep, a
639 plasmid constitutively expressing the *Renilla* luciferase gene and a plasmid
640 constitutively expressing MAVS. At 24 hours post transfection, firefly/*Renilla*
641 luciferase activity was quantified. One representative out of two independent
642 experiments is shown, each with biological triplicates. The data are presented as
643 scatter plot with mean ± SD (n=3). (**C**) HEK293T cells were co-transfected with
644 pGL3.1 plasmid containing the *IFNL4* or *IFNL1/2/3* promoter from human, African
645 green monkey, pig and sheep and a plasmid constitutively expressing the *Renilla*
646 luciferase gene. At 24 hours post transfection, the cells were infected with SeV for
647 another 24 hours before quantifying firefly/*Renilla* luciferase activity. One
648 representative out of two independent experiments is shown, each with biological
649 triplicates. The data are presented as scatter plot with mean ± SD (n=3). Statistical
650 significance was determined using an unpaired t test. ****; $P < 0.0001$, ***, $0.0001 <$
651 $P < 0.001$; **, $0.001 < P < 0.01$; *, $0.01 < P < 0.05$; ns, $P \geq 0.05$.

652

653 **Figure 5. Conservation of the *IFNL4* promoter among mammals.** (**A**) Alignment
654 of the proximal/core promoter and the 5' UTR of *IFNL4* from human, African green
655 monkey, pig and sheep. The transcriptional start site (TSS) as well as putative IRF
656 and NF-κB binding sites are indicated in the human sequence. (**B**) Phylogenetic tree
657 of *IFNL1* and *IFNL4* from human (*H. sapiens*), African green monkey (*C. sabaeus*),
658 sheep (*O. aries*), pig (*S. scrofa*), western European hedgehog (*E. europaeus*), polar
659 bear (*U. maritimus*) based on the sequence of their proximal/core promoter and 5'
660 UTR. The sequences were aligned using ClustalW and the tree was generated by the
661 Neighbor-Joining method. (**C**) Same as in (B) but using the amino acid sequences of
662 the proteins instead. Both trees are drawn to scale, with branch lengths measured in

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the number of substitutions per site.

Figure 6. Insertion of a functional IRF binding site in the *IFNL4* promoter renders it virus-inducible. (**A-D**) HEK293T cells were co-transfected with a pGL3.1 plasmid containing the *IFNL1* or *IFNL4* promoter or chimeras thereof, a plasmid constitutively expressing the *Renilla* luciferase gene and a plasmid constitutively expressing MAVS. At 24 hours post transfection, firefly/*Renilla* luciferase activity was quantified. One representative out of two independent experiments is shown, each with biological triplicates. The data are presented as scatter plot with mean ± SD (n=3). (**A**) In chimera A and B, the first 100 bp upstream of the TSS has been swapped between the *IFNL1* and *IFNL4* promoters as indicated by the colors on the drawing. (**B**) In chimera C, the IRF binding site from IFNL1 was inserted into the *IFNL4* promoter at the same relative position it occupied in *IFNL1*. In chimera C (mutIRF), the *IFNL1* IRF binding site in chimera C was mutated to render it inactive. (**C**) In chimera D, the IRF binding site in *IFNL1* was replaced with the putative IRF binding site from *IFNL4*. In chimera E, the putative IRF binding site in *IFNL4* was replaced with the IRF binding site from *IFNL1*. (**D**) In chimera C (mutNF-κB1), the NF-κB binding site located upstream of the TSS in *IFNL4* was mutated to render it inactive. In chimera C (mutNF-κB2), the NF-κB binding site located downstream of the TSS in *IFNL4* was mutated to render it inactive. In chimera C (mutNF-κB1/2) both NF-κB binding sites were simultaneous mutated. Statistical significance was determined using ANOVA and Dunnett's T3 multiple comparison test. ***, $0.0001 < P < 0.001$; **, $0.001 < P < 0.01$; ns, $P \geq 0.05$.

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Figure 7. Recruitment of IRF3 and RNA polymerase II to the *IFNL4* and *IFNL1* promoters. A549 cells were mock-infected or infected with SeV for 4 hours before performing ChIP with antibodies against IRF3 (**A**), RNA polymerase II (**B**) and IgG as control (**C**). One representative out of two independent experiments is shown, each with biological duplicates. The data are presented as bar chart and scatter plot with mean.

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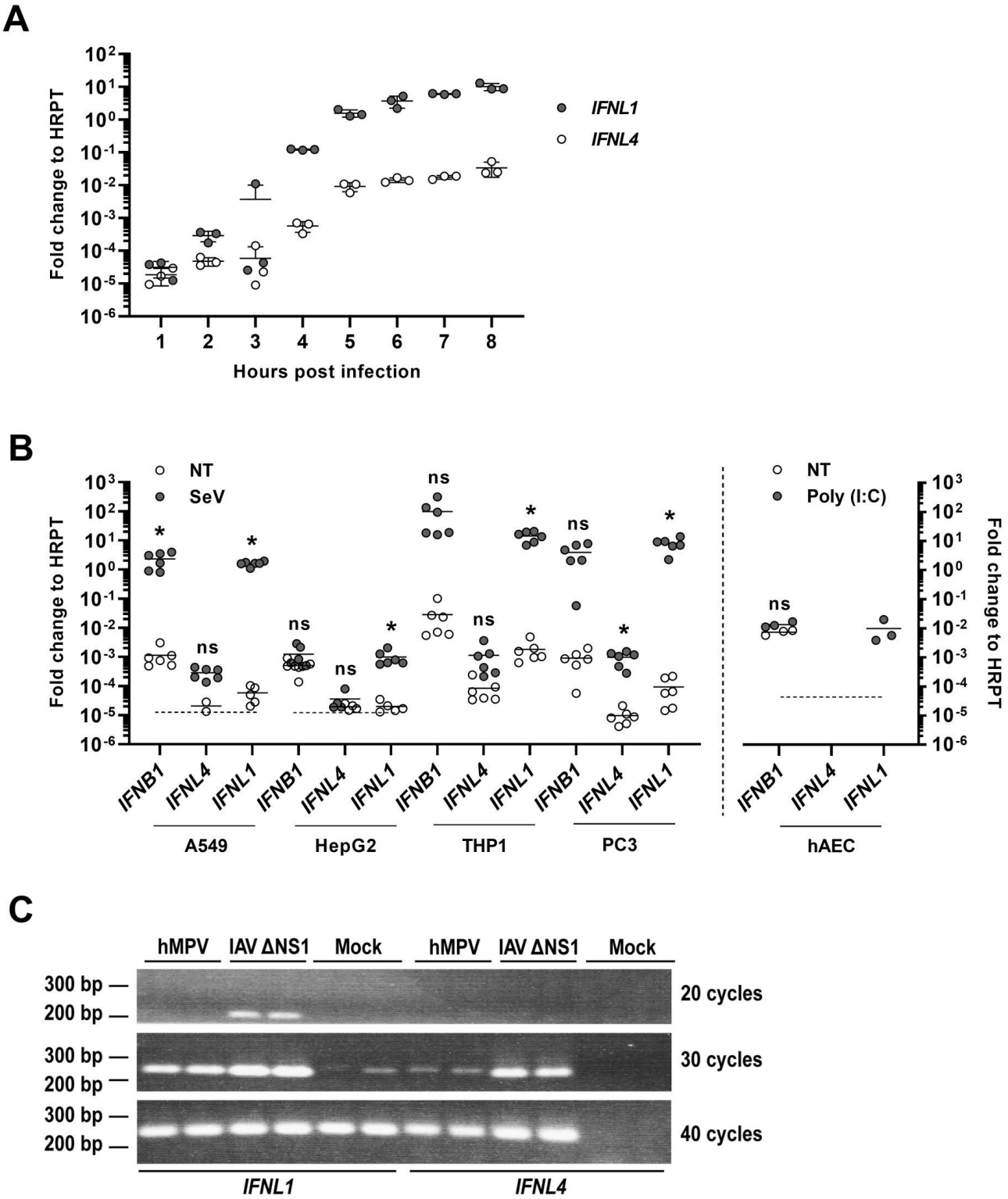

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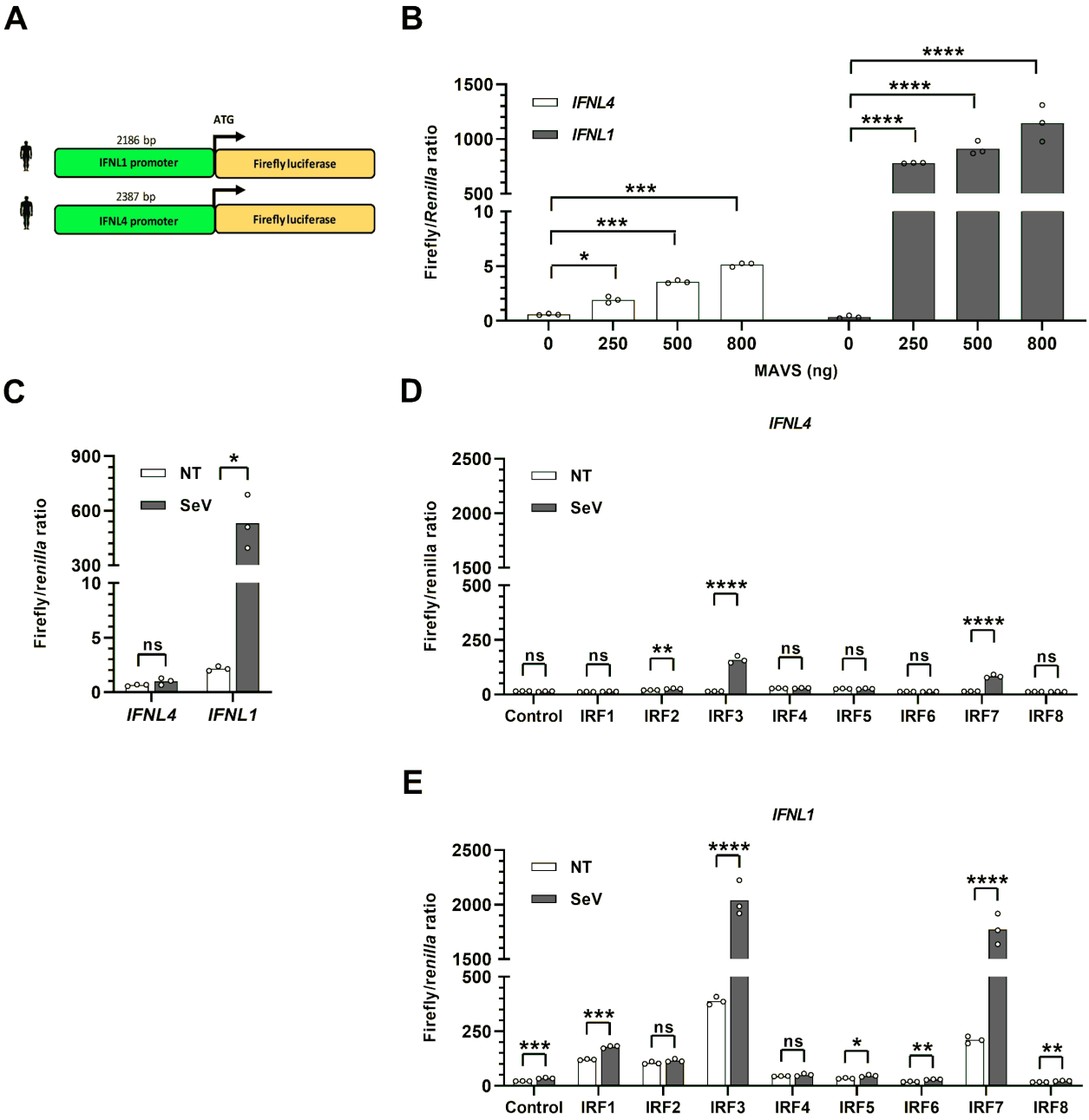

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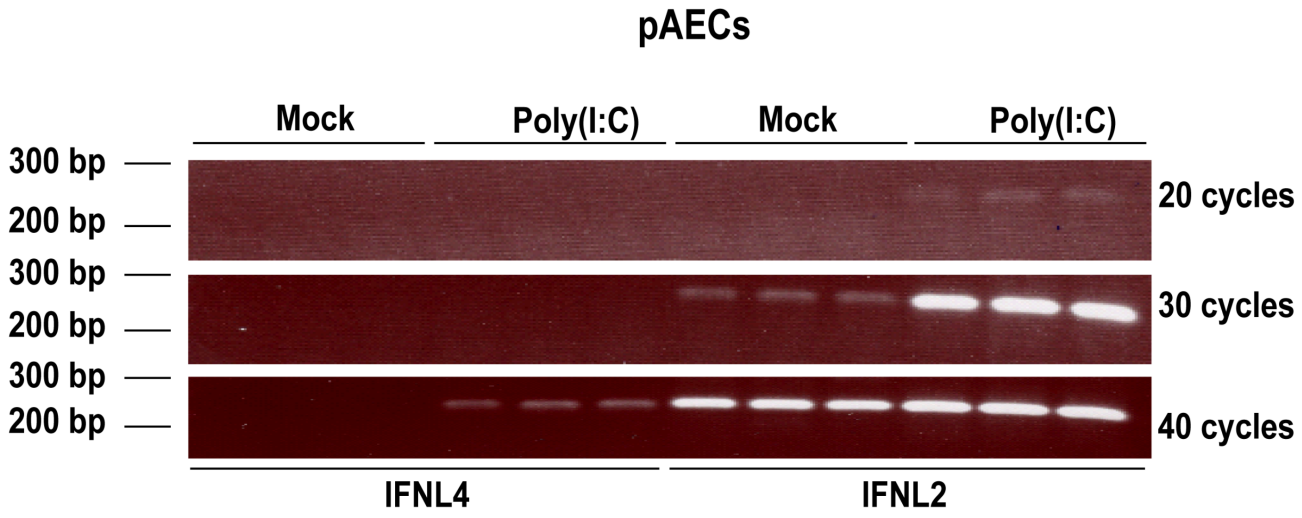

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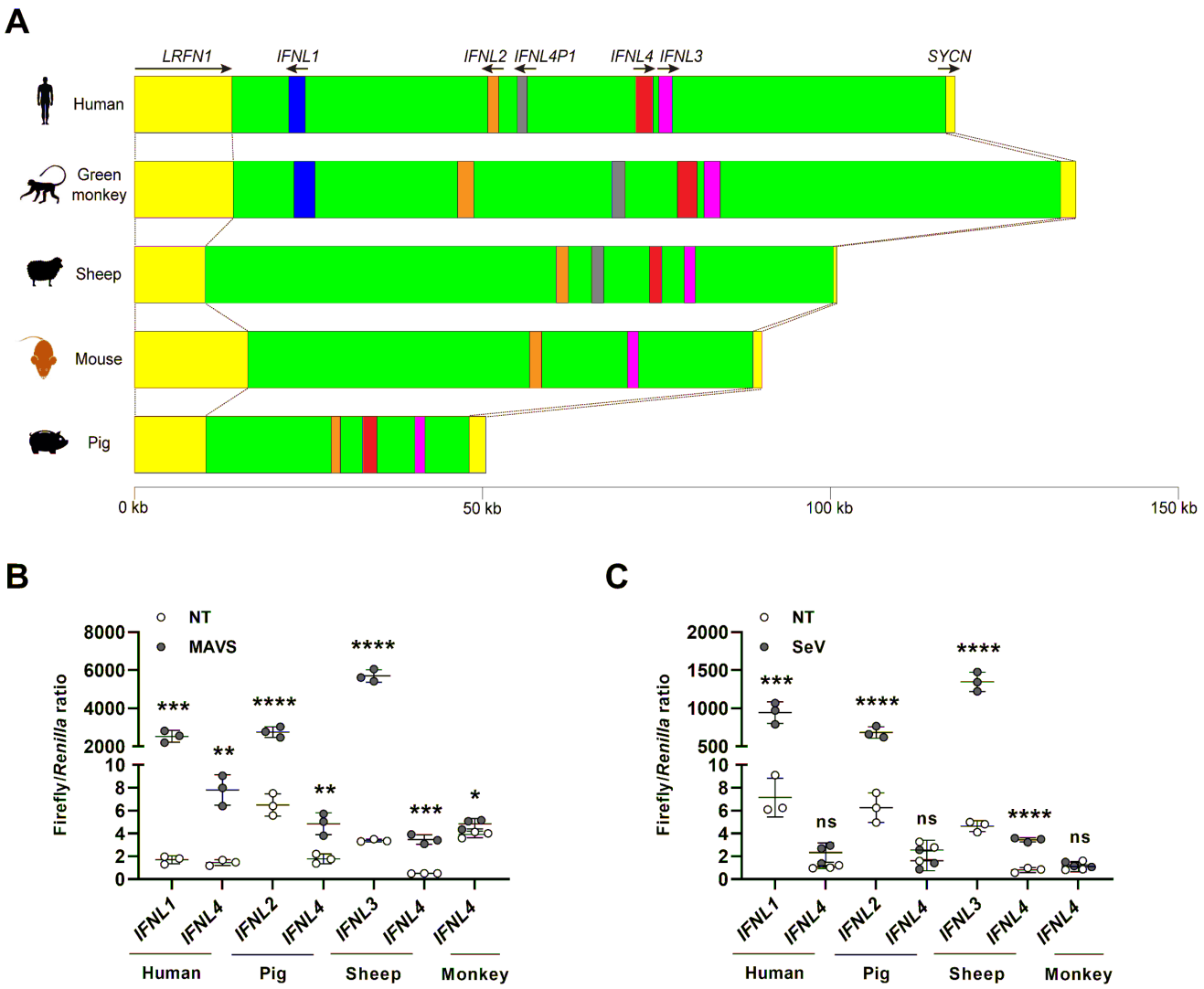

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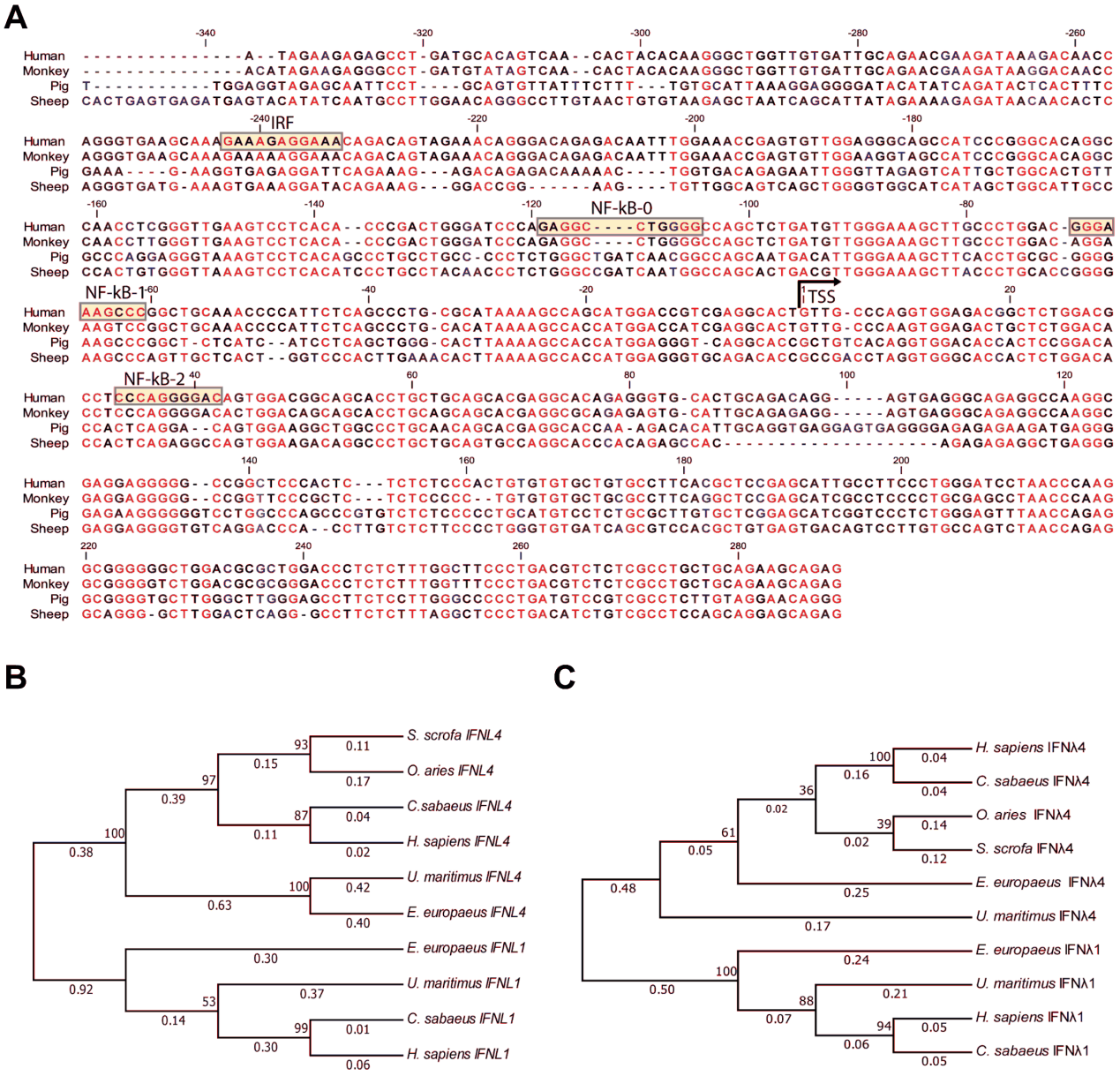

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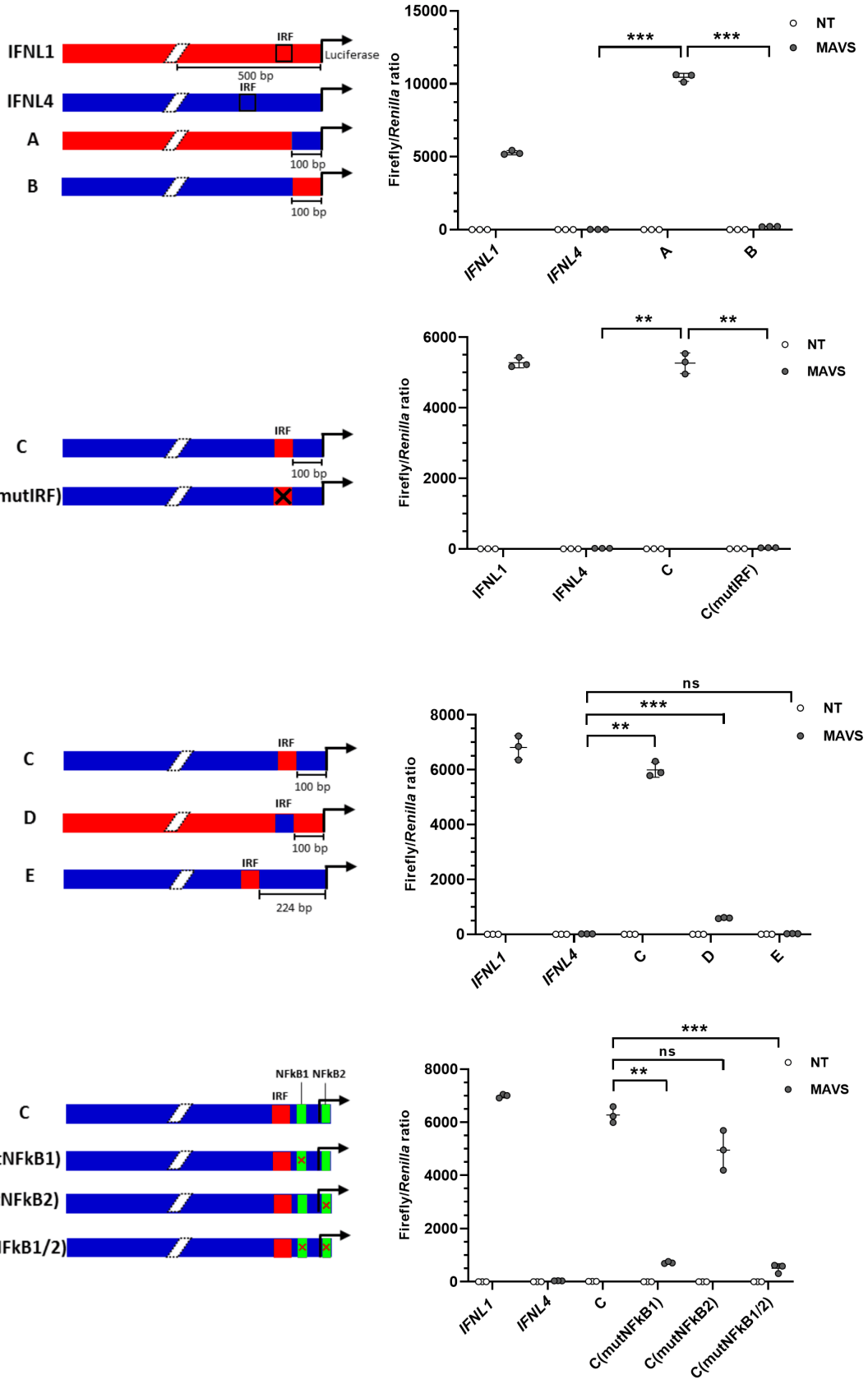

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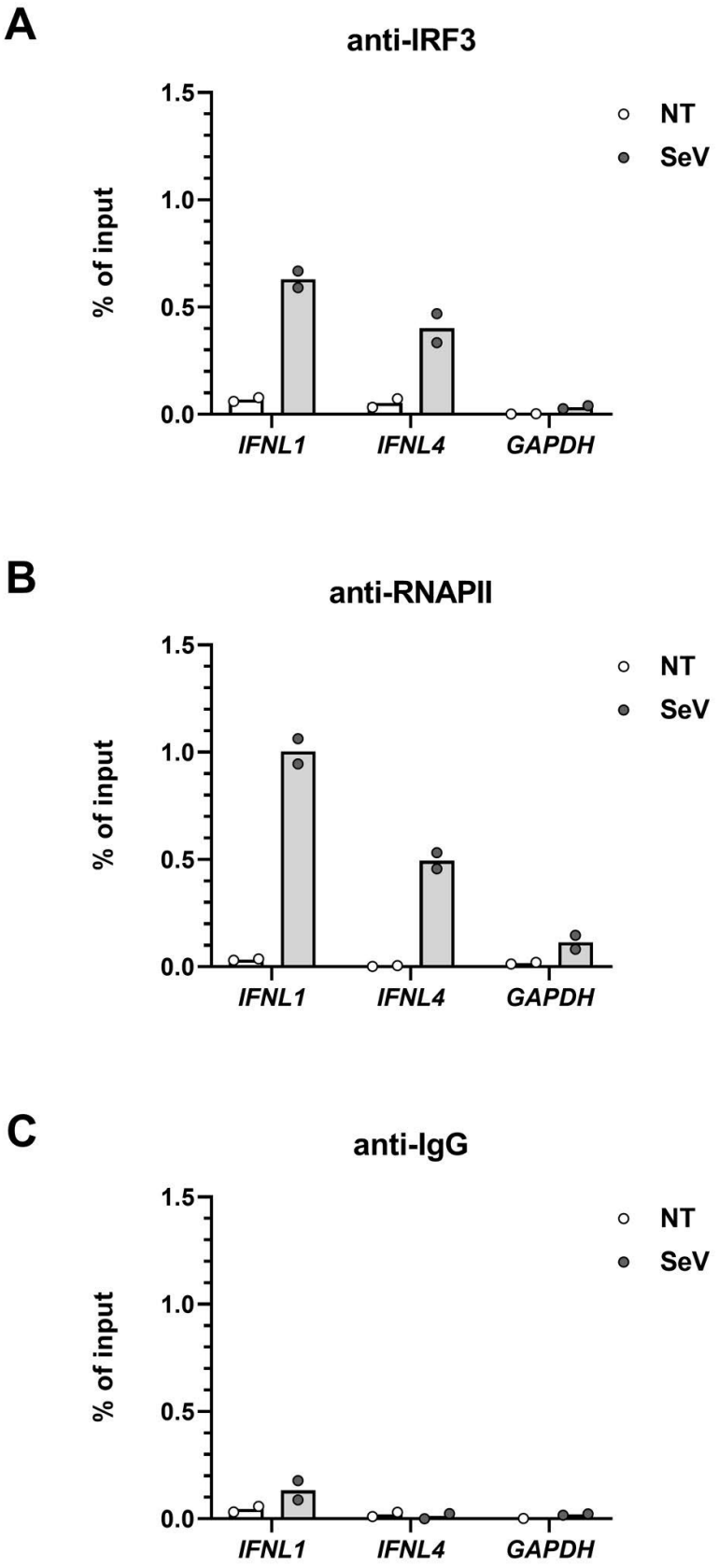

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