

IFIT1 is rapidly evolving and exhibits disparate antiviral activities across 11 mammalian orders

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eLife Assessment

This **important** report describes the changing antiviral activity of IFIT1 across mammals and in response to distinct viruses, likely as a result of past arms races. One of the main strengths of the manuscript is the breadth of mammalian IFIT1 orthologs and viruses that were tested, as well as the thoroughness of the positive selection analysis. Overall the evidence is **convincing**, and the discussion conveys well the limitations due to physical interactions with other IFITs that are not accounted for.

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Abstract

Mammalian mRNAs possess an N7-methylguanosine (m7G) cap and 2'O methylation of the initiating nucleotide at their 5' end, whereas certain viral RNAs lack these characteristic features. The human antiviral restriction factor IFIT1 recognizes and binds to specific viral RNAs that lack the 5' features of host mRNAs, resulting in targeted suppression of viral RNA translation. This interaction imposes significant host-driven evolutionary pressures on viruses, and many viruses have evolved mechanisms to evade the antiviral action of human IFIT1. However, little is known about the virus-driven pressures that may have shaped the antiviral activity of IFIT1 genes across mammals. Here, we take an evolution-guided approach to show that the IFIT1 gene is rapidly evolving in multiple mammalian clades, with positive selection acting upon several residues in distinct regions of the protein. In functional assays with 39 IFIT1s spanning diverse mammals, we demonstrate that IFIT1 exhibits a range of antiviral phenotypes, with many orthologs lacking antiviral activity against viruses that are strongly suppressed by other IFIT1s. We further show that IFIT1s from human and a bat, the black flying fox, inhibit Venezuelan equine encephalitis virus (VEEV) and strongly bind to Cap0 RNAs. Unexpectedly, chimpanzee IFIT1, which differs from human IFIT1 by only 8 amino acids, does not inhibit VEEV infection and exhibits minimal Cap0 RNA-binding. In mutagenesis studies, we determine that amino acids 364 and 366, the latter of which is rapidly evolving, are sufficient to confer the differential anti-VEEV activity between human and chimpanzee IFIT1. These data suggest that virus-host genetic conflicts have influenced the antiviral specificity of IFIT1 across diverse mammalian orders.

Introduction

Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) is an interferon-stimulated gene (ISG) effector that distinguishes self and non-self RNA. Mammalian mRNAs possess an N⁷-methylguanosine (m⁷G) cap and 2′O methylation of the initiating nucleotide, whereas many viral RNAs lack one or both features. IFIT1 binds uncapped 5′-triphosphate RNA (5′-ppp-RNA) and capped but unmethylated RNA (Cap0, an m⁷G cap lacking 2′-O methylation) (1–3). IFIT1 binding to viral RNA inhibits infection by decreasing the amount of accessible RNA from the pool of replicating viral RNA (4) and by interacting with eIF proteins required for translation of viral proteins (5).

This restriction imposes a host-driven evolutionary pressure for viruses to evade IFIT1 through various mechanisms (5). Some encode their own capping and 2′O methyltransferase enzymes, translate through internal ribosome entry sites (IRES), hijack host mRNA caps by cap-snatching, or mask the 5′ end with RNA structures (5). For the alphavirus Venezuelan equine encephalitis virus (VEEV), which has a positive-sense, single-stranded RNA genome, a 5′ UTR structure in the pathogenic Trinidad strain occludes IFIT1 binding, whereas a single-nucleotide change in the attenuated TC-83 strain destabilizes the structure and allows inhibition (1). VEEV TC-83 is sensitive to human IFIT1 and mouse Ifit1B, indicating at least partial conservation of antiviral function by IFIT proteins. Viral countermeasures may therefore drive adaptive changes in IFIT1, yet the scope of this pressure across mammalian IFIT1 remains unclear.

Pathogenic viruses can cause severe disease or death in mammals, imposing a major selective pressure on the host to evolve antiviral defenses. Viruses face reciprocal pressure to evade these defenses. These ‘molecular arms races’ leave signatures of positive selection in many immune genes. Positive selection, or rapid evolution, is denoted by a high ratio of nonsynonymous to synonymous substitutions ($dN/dS > 1$). Computational evolutionary analyses of antiviral ISGs have revealed specific domains and residues that are rapidly evolving (6–8). Several methods can determine if a gene, or part of a gene, is undergoing positive selection. These include Phylogenetic Analysis using Maximum Likelihood (PAML), Fast Unconstrained Bayesian AppRoximation (FUBAR), and Mixed Effects Model of Evolution (MEME). Each one is a codon-substitution model that identifies sites within a gene that evolving under selection. For example, PAML tests whether a neutral-or-purifying model (M7) fits the data significantly worse than one that includes positive selection (M8). Such approaches have pinpointed certain domains and amino acids that determine antiviral function in effectors such as MX1 and TRIM5α (9, 10). In addition to evolutionary pressure from host-pathogen molecular arms races, IFIT1 evolution has also been influenced by gene loss, gene duplication, and recombination with IFIT1B (11). However, the antiviral consequences of these such events have only been tested functionally in a few species, and the breadth of IFIT1 diversity across Mammalia remains unknown (11). Recently, in a screen for antiviral ISGs from bats, we found that IFIT1 from the black flying fox displays particularly strong suppression of VEEV (12). From these prior studies, we hypothesized that evolutionary pressures may have shaped “species-specific” antiviral activity among IFIT1 orthologs. Accordingly, we sought to use evolutionary and functional methods to interrogate differences in antiviral activity and biochemical properties of mammalian IFIT1.

Results

IFIT1 is rapidly evolving in major clades of mammals

Many antiviral effectors exhibit signatures of positive selection (6, 7), prompting us to test whether IFIT1 is similarly evolving across several diverse clades of mammals. Using PAML (13), we determined that IFIT1 exhibits signatures of rapid evolution in primates, bats, carnivores, and ungulates, with the weakest signal in carnivores (Fig 1A). Rodents were excluded because many species lack an IFIT1 ortholog (11). While all lineages shown signs of rapid evolution, carnivore IFIT1s exhibited the least significant likelihood of pervasive positive selection (Fig 1A). Furthermore, each lineage contained at least two codons under selection (Fig 1A). To uncover other rapidly evolving sites, we applied two codon-based models that complement PAML. FUBAR uses a Bayesian framework that assumes selection as pervasive across all branches of the phylogenetic tree (14); it detected at least one positively selected codon in every lineage except carnivores. MEME identifies codons that experience episodic selection on a subset of branches (15); it identified several such sites in each lineage we tested (Fig 1C). These data indicate that positive selection has acted on multiple regions of IFIT1 across Mammalia.

Recombination between paralogous genes can affect analyses of positive selection (16), and the evolution of some IFIT1 sequences has been influenced by recombination with IFIT1B (11). To confirm that the sequences examined were likely IFIT1 orthologs, we aligned all IFIT1 sequences from this study with representative IFIT1B and IFIT3 sequences from several mammals and built a maximum-likelihood tree from their C-terminal regions, as previously described (11). All IFIT1 clustered together, separate from IFIT1B and IFIT3 sequences (Supp Fig 1A), supporting their classification as orthologs. We further evaluated IFIT1 sequences for recombination breakpoints using GARD, a genetic-algorithm approach for recombination detection (16). A single breakpoint was detected in primates (codon 38) and ungulates (codon 188), whereas two were identified in bats (codons 33, 381) and carnivores (codons 14, 370) (Supp Fig 1B).

The presence of GARD breakpoints prompted reanalysis of IFIT1 using only coding regions located after the breakpoint in primates and ungulates or between breakpoints in bats and carnivores. PAML still detected positive selection in every clade except carnivores (Supp Fig 1C), which had the lowest signal of positive selection in our original analysis. Further, nearly all codons flagged in the initial analysis (Fig 1A–C) were recovered when we re-ran FUBAR (Supp Fig 1D) and MEME (Supp Fig 1E) on alignments trimmed after or between the GARD breakpoints. Considering that sequences were trimmed, which removed certain sites previously identified as rapidly evolving, almost all other possible positively selected sites were validated in primates (11/12), bats (14/16), ungulates (30/37), and carnivores (2/4) across PAML, FUBAR, and MEME analyses (Supp Fig 1C–E). To further clarify whether recombination could have impacted these analyses, we used Recombination Detection Program 4 (RDP4) (17) to remove inferred recombinant sequences from the primate alignment. Subsequent PAML, FUBAR, and MEME analyses recovered 10 of the original 14 selected sites (Supp Fig 1F). Importantly, codons 170, 193, and 366 in primates continued to exhibit evidence of rapid evolution in two independent methods in both the GARD breakpoint-trimmed and RDP4-filtered datasets (Supp Fig 1C–F). In bats, codon 193 likewise showed positive selection in two analyses of the between-breakpoint alignment (Supp Fig 1C–E). While signatures of selection differed slightly between the original, the post-or between-breakpoint trimmed, and RDP4-filtered datasets, overall trends and in many cases residue-specific signals were consistent, highlighting the robustness of the evolutionary signatures.

Overall, these analyses indicate that IFIT1 is rapidly evolving across multiple Mammalian clades. Such widespread rapid evolution may result in functional consequences on antiviral activity for an individual species or more broadly across clades of mammals. To address the first prediction, we next tested for functional implications of within-species rapid evolution of IFIT1 using human IFIT1 as a model.

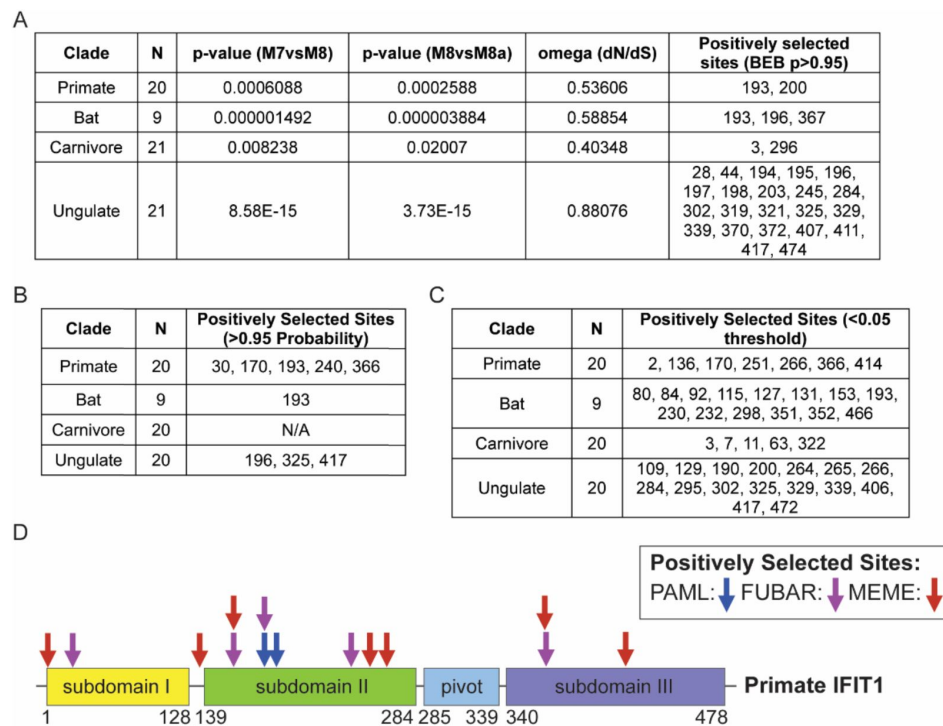


Figure 1.

Evolutionary analysis of IFIT1 from clades of mammals reveals rapid evolution.

A. PAML analysis on NCBI-derived coding sequences that were aligned and then analyzed using CodeML. Likelihood ratio tests were performed to compare model 7 vs model 8 and model 8 vs model 8a to determine the presence of positive selection. N=number of sequences input, and numbers under “positively selected sites” represent residue number of the reference sequence. B. FUBAR analysis on aligned coding sequences using HyPhy software and DataMonkey. N=number of sequences input, and numbers under “positively selected sites” represent residue number of the reference sequence. C. MEME analysis on aligned coding sequences using HyPhy software and the DataMonkey application. N=number of sequences input, and numbers under “sites of episodic positive selection” represent residue number of the reference sequence. D. Diagram of Primate IFIT1 domains and location of positively selected sites determined by PAML, FUBAR, and MEME.

Human IFIT1 residue 193 exhibits mutational resiliency

We focused on three codons in primate IFIT1 – encoding V170, L193, and L366 in the human ortholog – that were rapidly evolving in at least two models (Fig 1D). None of these residues have been associated with RNA-binding or antiviral function (2, 3). In the crystal structure of human IFIT1 bound to RNA, V170 and L366 lie outside the RNA-binding tunnel, whereas L193 resides in the TPR4 loop. This loop sits between α -helices 9 and 10, forms a lid over the 3' exit of the tunnel, and helps link subdomain II to subdomain III (Fig 2A–C) (3). L193 in the TPR4 loop protrudes into the tunnel exit, comes into close contact with RNA (Fig 2C), and is adjacent to R187, a residue essential for RNA binding and antiviral function (2). To test the functional importance of L193, we knocked out endogenous IFIT1 in Huh7.5 cells with CRISPR– Cas9 editing and re-expressed CRISPR-resistant IFIT1 variants carrying every possible amino-acid substitution at position 193. Cells were then infected with VEEV (TC-83 strain) encoding a GFP reporter (VEEV–GFP), and infection was quantified by flow cytometry (Fig 2D). Western blotting determined that all IFIT1 point mutants were expressed to comparable levels (Fig 2D, Bottom). All hydrophobic and positively charged mutants at position 193 inhibited VEEV similarly to the wild-type (193L) IFIT1 (Fig 2D). Polar amino acids introduced at position 193 also retained antiviral function (Fig 2D). Severe loss of function in anti-VEEV activity of IFIT1 was only notable with glycine or proline (~50% loss of antiviral function) or with negatively charged residues (~75–100% loss of antiviral function) (Fig 2D). Thus, codon 193 displays notable functional resiliency, as most substitutions preserve antiviral activity. This result connects our computational analysis to a concrete functional outcome within one species. We next asked whether positive selection also influences interspecies differences in IFIT1 antiviral activity across diverse mammalian orthologs.

IFIT1 orthologs from diverse mammals demonstrate species-specific antiviral activity

We next cloned 39 IFIT1 orthologs—derived from 38 mammalian species, including two predicted equine sequences—into lentiviral expression vectors. These proteins share 60–98% identity with human IFIT1 and span 11 orders within the superorders Euarchontoglires, Laurasiatheria, Afrotheria, and Xenarthra (Fig 3A–B; Supp Table 1). All primate, bat, and ungulate orthologs in this screen were also part of the PAML, FUBAR, and MEME analyses, allowing us to correlate functional differences in antiviral activity with molecular evolutionary analysis. Each construct carried an N-terminal HA tag for western-blot detection. Huh7.5 cells transduced with these vectors were challenged with GFP-expressing VEEV or vesicular stomatitis virus (VSV). These viruses were selected because human IFIT1 restricts them by distinct mechanisms. VSV, a rhabdovirus with a negative-sense RNA genome, is inhibited by IFIT1 binding to its 5'-triphosphate RNA (5'-ppp-N), which sequesters the genome from the replication pool (2). VEEV TC-83 is inhibited by IFIT1-mediated recognition of its Cap0 RNA, which leads to inhibition of translation (1, 18). IFIT1-expressing cells were infected with an MOI of 1 for both VSV–GFP and VEEV–GFP, and infection was quantified by flow cytometry (Fig 3C). Orthologs that strongly reduced infectivity by at least 70% relative to control cells transduced with an empty lentiviral vector were considered hits. Eleven IFIT1 orthologs (African savannah elephant, Chinese pangolin, sheep, white-tailed deer, black flying fox, dromedary camel, orca, Angola colobus, big brown bat, human, and large flying fox) inhibited VEEV (Fig 3C), and five (orca, cape elephant shrew, dolphin, European rabbit, and nine-banded armadillo) inhibited VSV (Fig 3D). We were surprised that hits for each virus spanned diverse clades with minimal overlap. Only orca IFIT1 restricted both viruses, and just 10 of 39 orthologs met the 70% inhibition threshold, indicating that multiple IFIT1 orthologs target neither VEEV or VSV under these experimental conditions. Western blots revealed wide variation in protein abundance (Supp Fig 2), from high expression (e.g., orca, European rabbit) to very low levels (e.g., African green monkey, greater horseshoe bat). Black-capped squirrel monkey IFIT1 also migrated at a lower-than-expected mass, suggesting a truncation or cleavage of the protein product. However, several low-expressing orthologs were potent

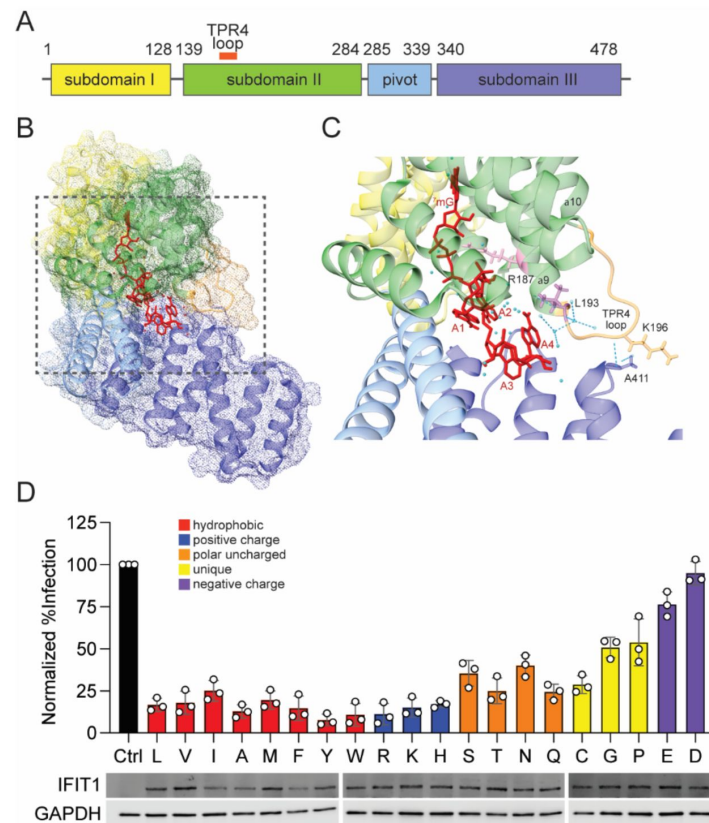


Figure 2.

Mutagenesis of rapidly evolving residue 193 in human IFIT1 reveals mutational resiliency.

A. Depiction of the human IFIT1 protein with the TPR4 loop location (orange) shown. B. Solved crystal structure of IFIT1 bound to RNA with TPR4 loop forming a “lid” over the exit of the RNA-binding tunnel (PDB:5udj). C. Zoom in on the TPR4 loop and RNA-binding tunnel exit illustrating the location of the TPR4 loop and residue 193 in relation to bound RNA in the human IFIT1 crystal structure. D. (Top) Saturating mutagenesis screen in cells expressing human IFIT1 with residue 193 mutated to every possible residue and challenged with VEEV infection. (Top) (Bottom) Western blot from lysates of Huh7.5 cells expressing IFIT1 point mutants.

restrictors, whereas many highly expressed ones were inactive. These data suggest that IFIT1 orthologs may exhibit species-specific antiviral functions independent of differences in protein expression.

We next sought to validate the antiviral phenotypes of a subset of orthologs with more comparable expression. We selected nine orthologs based on protein expression, evolutionary diversity, and variable phenotypes in the screen, including IFIT1 from Chinese pangolin (hereafter referred to as pangolin), orca, sheep, black flying fox, big brown bat, chinchilla, human, chimpanzee, and cape elephant shrew (hereafter referred to as shrew) (**Fig 4A**). Huh7.5 cells were transduced with different doses of lentiviral vectors in an attempt to equalize expression. Western blots showed that eight orthologs were expressed at levels approximately four-fold above to two-fold below human IFIT1. The notable exception was orca IFIT1, which was expressed approximately 15 times higher than human IFIT1 (**Fig 4B**). IFIT1-expressing cells were challenged with VEEV (**Fig 4C**) or VSV (**Fig 4D**) at an MOI of two. VSV was potently inhibited by orca and shrew IFIT1, while no other tested orthologs significantly suppressed infection. Thus, VSV may evade suppression by many mammalian IFIT1s. VEEV was potently inhibited by pangolin, sheep, black flying fox, big brown bat, and human IFIT1, while orca IFIT1 was modestly inhibitory. VEEV was not significantly inhibited by chimpanzee IFIT1 but was suppressed by human IFIT1. This was particularly noteworthy as human and chimpanzee IFIT1 protein sequences differ by only eight amino acids, and one of these (residue 366) was identified as rapidly evolving by both FUBAR and MEME (**Fig 1D**). Together, these validation experiments demonstrate that mammalian IFIT1 exhibits species-specific differences in viral suppression. Furthermore, while expression differences in IFIT1 orthologs are noted and may affect antiviral potency, our data suggest that species-specific differences cannot be fully explained by expression differences. This is exemplified by potent restriction of VEEV by pangolin and big brown bat IFIT1 despite low expression (**Fig 4B** and **4C**). Further, high-expressing sheep IFIT1 only restricted VEEV infection, but not VSV, and orca IFIT1 only modestly reduced VEEV infection despite much higher expression than all other IFIT1s (**Fig 4B-D**).

We next tested this subset of nine IFIT1 orthologs against human parainfluenza virus type 3 (PIV3). PIV3 is a paramyxovirus that, like the rhabdovirus VSV, has a negative-sense single-stranded RNA genome, allowing us to compare each ortholog's activity against two viruses that share a replication strategy. We also selected PIV3 because published reports differ on its susceptibility to human IFIT1, whereas the related PIV5 is consistently IFIT1-sensitive (19–22). PIV3 was significantly restricted by shrew, pangolin, chinchilla, black flying fox, and big brown bat IFIT1 (**Fig 4E**). Suppression of PIV3 by human IFIT1 in our assay was very modest and not statistically significant. Chinchilla IFIT1 exhibited disparate activities, as it did not inhibit VEEV (**Fig 4C**) or VSV (**Fig 4D**), yet significantly protected cells from PIV3 infection (**Fig 4E**).

We then tested these IFIT1 orthologs against another alphavirus related to VEEV, Sindbis virus (SINV) (**Fig 4E**). SINV was largely resistant to most mammalian IFIT1s, consistent with previous human IFIT1 studies (1, 18). However, SINV was potently suppressed by orca IFIT1, and significantly suppressed by pangolin, sheep, and chinchilla IFIT1 (**Fig 4E**), highlighting a pattern of suppression that was distinct across the nine orthologs relative to the alphavirus VEEV. Together, these data demonstrate that IFIT1 orthologs exhibit a range of antiviral phenotypes with a panel of genetically diverse viruses (**Fig 4G**), largely independent of differences in protein expression.

Human, black flying fox, and chimpanzee IFIT1 protein exhibit species-specific RNA binding

Human and black flying fox IFIT1 inhibited VEEV, whereas chimpanzee IFIT1 did not (**Fig 4C**). The black flying fox (*Pteropus alecto*) is a model megabat species that is a reservoir for zoonotic viruses (23–25) and expresses restriction factors with unique antiviral activities (12, 26–

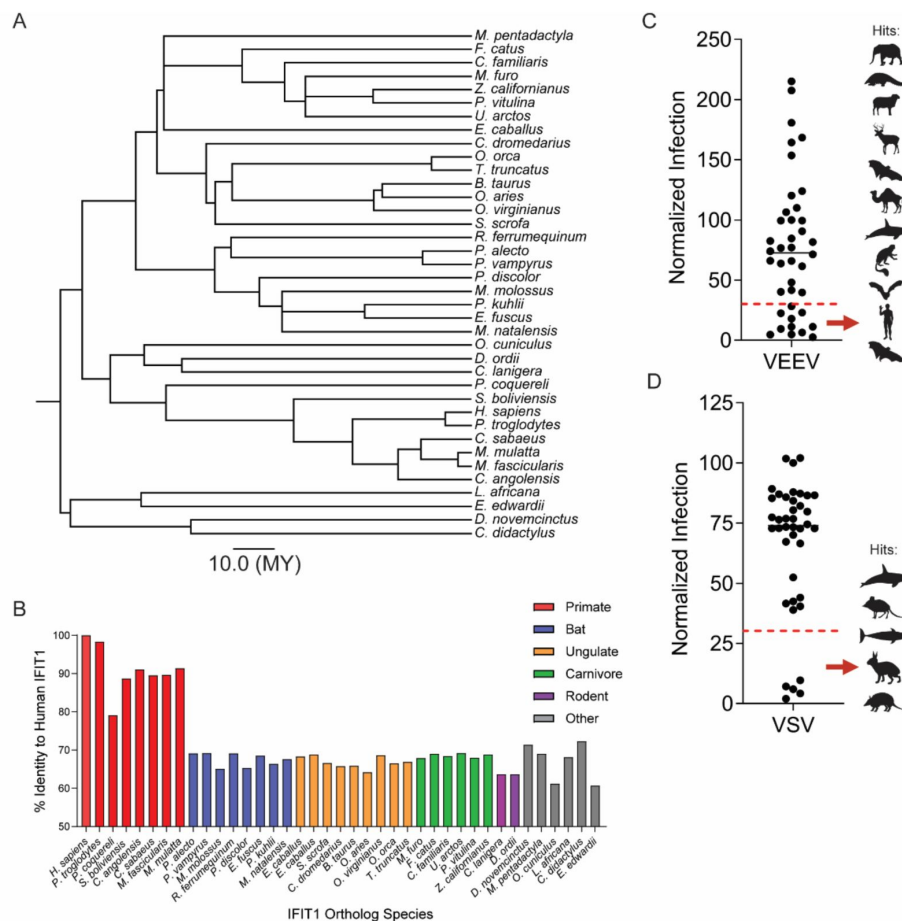


Figure 3.

IFIT1 ortholog screens reveal extensive heterogeneity in mammalian IFIT1 antiviral function.

A. TimeTree illustrating the evolutionary relationship of IFIT1 orthologs selected for ortholog screen. Scale bar represents divergence time of 10 million years. B. Graph of protein sequence identity of IFIT1 orthologs used in screen relative to human IFIT1. C. Dot plot representing the relative infection (compared to control cells) from an ectopic overexpression screen in which Huh7.5 cells expressing 39 different IFIT1 mammalian orthologs were challenged with 1.0 MOI VEEV-GFP for 4 h. Infectivity was quantified by flow cytometry. Red line denotes 30% relative infection. n=2 independent experiments. D. Same as C, for VSV-GFP (1 MOI, 4 h infection). n=3 independent experiments. In C, D, silhouettes represent the species with a relative inhibition of at least 70% in order from most (top) to least (bottom) inhibitory.

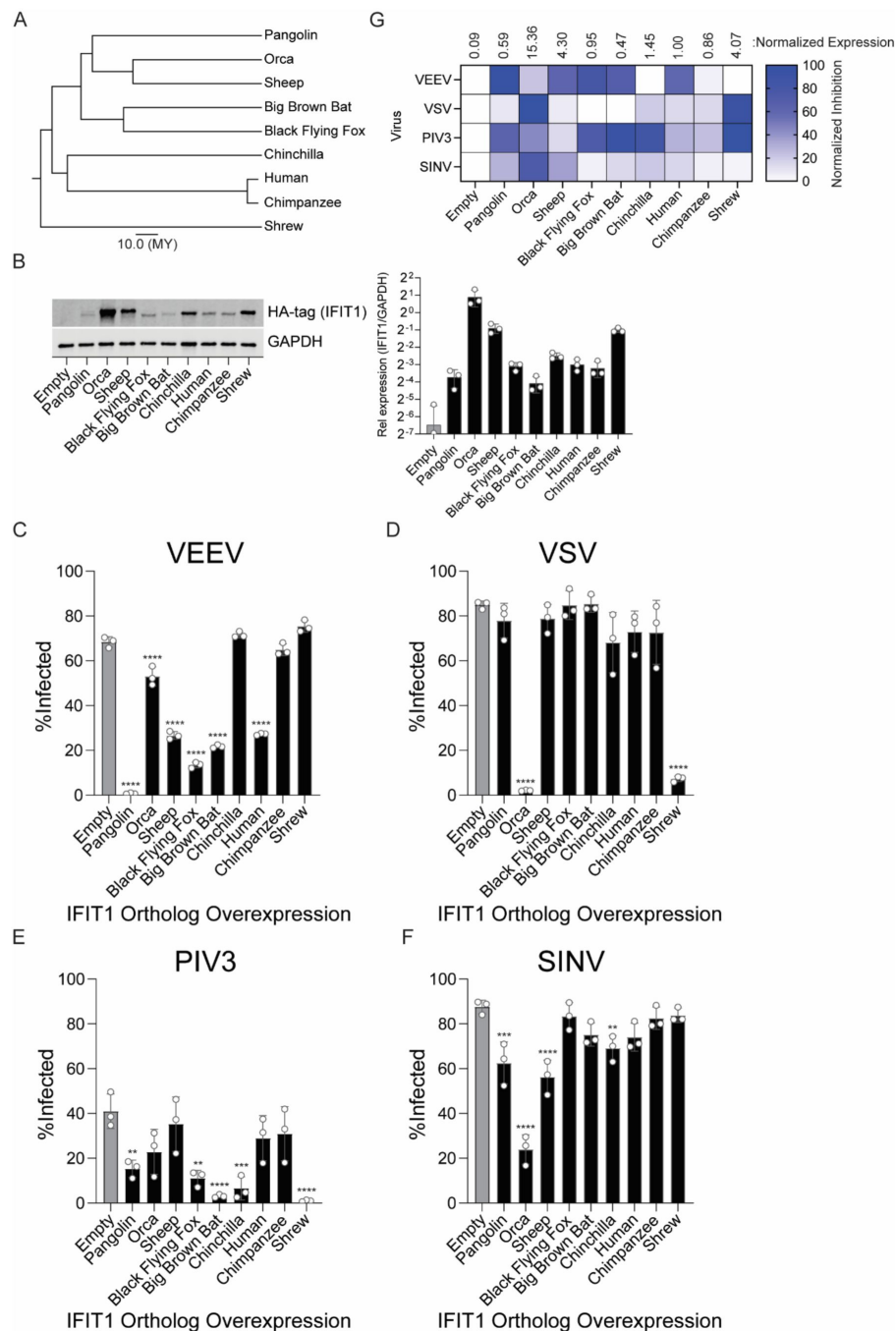


Figure 4.

Validation of selected IFIT1s from ortholog screen.

A. TimeTree illustrating the evolutionary relationship of IFIT1 orthologs selected for screen validation and follow-up. Scale bar represents divergence time of 10 million years. B. Western blot from cells expressing HA-tagged IFIT1 orthologs used in C-F. Image is representative of three independent replicates. Quantification of band intensity was performed using LiCOR Image Studio C. Infection of IFIT1 ortholog-expressing Huh7.5 cells with VEEV-GFP (MOI 2, 4h) D. Infection of IFIT1 ortholog-expressing Huh7.5 cells with VSV-GFP (MOI 2, 4h) E. Infection of IFIT1 ortholog-expressing Huh7.5 cells with PIV3-GFP (MOI 2, 10h) F. Infection of IFIT1 ortholog-expressing Huh7.5 cells with SINV-GFP (MOI 2, 10h). G. Heat map summarizing the infection data shown in C-F and complementary ortholog expression data shown in B. In C-F, data represent mean $\pm$ SD, $n=3$ independent experiments. Statistical significance was determined by one-way ANOVA with Dunnett's test. ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

31 [↗](#)). While human and black flying fox IFIT1 share only 69% identity, human and chimpanzee IFIT1 are over 98% identical (**Fig 3B** [↗](#), **Supp Table 1** [↗](#)). The unique phenotypes of these three species, combined with their well annotated genomes and the ease of purifying IFIT1 in bacteria, prompted us to use them for biochemical studies to examine potential differences in their RNA-binding ability.

Recombinant IFIT1 proteins (**Supp Fig 3** [↗](#)) were tested for Cap0 RNA binding by electrophoretic mobility shift assay (EMSA). A 41-nt transcript corresponding to the VEEV 5' UTR was capped with faustovirus capping enzyme to generate RNAs that resemble physiologic Cap0 termini (32 [↗](#)). After incubation with purified IFIT1 orthologs, protein–RNA complexes were resolved by native PAGE and visualized (**Fig 5A** [↗](#)). Quantification of band shift intensity and area under the curve analysis revealed that black flying fox IFIT1 exhibited the strongest RNA binding activity, followed by human IFIT1 (**Fig 5B** [↗](#), **5C** [↗](#)). Little binding was observed by chimpanzee IFIT1, as band shift intensity was barely above that of a human RNA-binding deficient control (human IFIT1-R187H) (2 [↗](#), 3 [↗](#)) (**Fig 5B** [↗](#), **5C** [↗](#)). These biochemical data are consistent with viral phenotypes (**Fig 4C** [↗](#)), suggesting that species-specific antiviral activities of IFIT1s can be partly explained by RNA binding potential.

Mutagenesis of primate IFIT1 reveals genetic determinants of IFIT1 antiviral function

Human IFIT1 restricts VEEV and binds its Cap0 RNA, whereas chimpanzee IFIT1 shows no VEEV inhibition and little VEEV Cap0 RNA binding. The two proteins differ at only eight residues (**Fig 6A** [↗](#)). We therefore used site-directed mutagenesis to pinpoint which substitutions account for this functional gap. Because three of the eight variant residues—362, 364, and 366—clustered together (**Fig 6A** [↗](#)), we created reciprocal “362/4/6” triple-mutant IFIT1 constructs that swap these positions between chimpanzee and human proteins. One of these (human L366, chimpanzee M366) shows positive selection in two computational models (**Fig 1D** [↗](#)), implying that evolutionary pressure may have shaped the functional divergence between these primate IFIT1 orthologs.

Huh7.5 cells expressing wild type (WT) or mutant IFIT1 were challenged with VEEV-GFP at an MOI of two, and infectivity was quantified by flow cytometry (**Fig 6B** [↗](#)). Western blotting demonstrated near uniform expression of all IFIT1 proteins (**Fig 6B** [↗](#)). WT chimpanzee IFIT1 conferred little protection (approximately 50% infection versus 60% in empty-vector controls), whereas WT human IFIT1 reduced infection to less than 10%. In the human background, only the L366M substitution caused a significant loss of restriction (approximately 4-fold higher infection than WT). Q364R had a modest, non-significant effect, and the 362/4/6 triple mutant nearly abolished activity. In the chimpanzee background, the reciprocal M366L substitution reduced infection by half, R364Q produced a modest, non-significant gain in antiviral activity, and the 362/4/6 triple mutant suppressed VEEV as effectively as WT human IFIT1. These results show that residues N362, Q364, and L366 are necessary for VEEV restriction by human IFIT1, and that their reciprocal substitution is sufficient to endow chimpanzee IFIT1 with potent antiviral activity.

Amino acids 362, 364, and 366 are generally conserved across 20 primate IFIT1 sequences (**Fig 6C** [↗](#)). Residue 366 is typically hydrophobic. Fifteen species, including humans, encode leucine, whereas the other five, including chimpanzee, encode either valine or methionine (**Fig 6C** [↗](#)). This suggests that leucine at position 366, which is rapidly evolving, might be important for antiviral function in primate IFIT1. Additional support for the importance of L366 comes from our findings that the only primate IFIT1, besides those of chimpanzees and humans, which was expressed at appreciable levels during the screen was that of the Angola colobus. This IFIT1 variant also potently inhibited VEEV infection and notably contains a leucine at residue 366, similar to human IFIT1 (**Fig 3C** [↗](#), **Supp Fig 2** [↗](#), **Supp Table 1** [↗](#)). Glutamine is conserved at site 364 in all primate

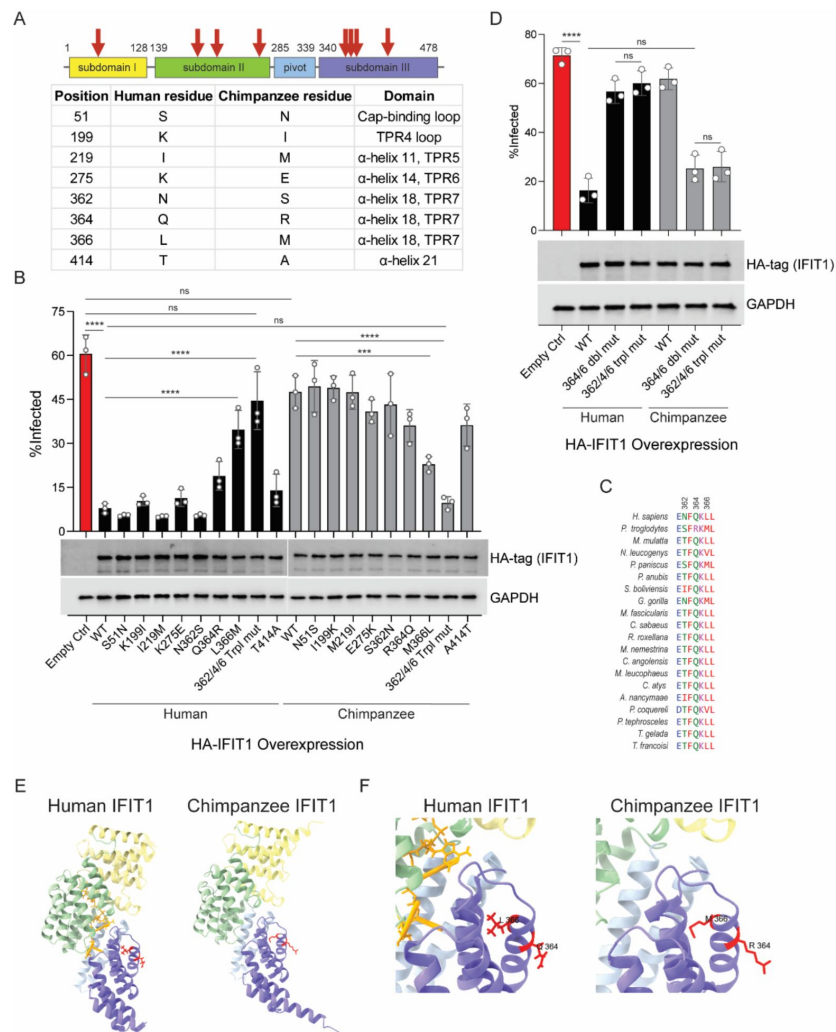


Figure 6.

Mutagenesis uncovers genetic determinants of primate IFIT1 antiviral function.

A. Diagram and chart describing amino acids that differ between human and chimpanzee IFIT1 as well as their domain location. B. Effects of primate IFIT1 mutant expression on VEEV-GFP infection. Huh7.5 cells were infected with an MOI of 2 for 4 h and infectivity was quantified by flow cytometry. Data represent mean $\pm$ SD, $n=3$ independent experiments. Statistical significance was determined by one-way ANOVA with Dunnett's test. ns, $p > 0.05$; ***, $p < 0.001$; and **** $p < 0.0001$. Western blotting for HA-tag (IFIT1) and GAPDH (Loading control) was also performed to determine expression levels of IFIT1 mutants. C. ClustalOmega protein sequence alignment of residues 361-367 of IFIT1 from 20 primate species. D. Effects of primate IFIT1 double or triple mutants on VEEV-GFP infection. Huh7.5 cells were infected with an MOI of 2 for 4 h and infectivity was quantified by flow cytometry. Data represent mean $\pm$ SD, $n=3$ independent experiments. Statistical significance was determined by one-way ANOVA with Dunnett's test. ns, $p > 0.05$; and **** $p < 0.0001$. Western blotting for HA-tag (IFIT1) and GAPDH (Loading control) was also performed to determine relative IFIT1 mutant protein abundance. E. Structure of human IFIT1 bound to RNA (left) or AlphaFold predicted structure of chimpanzee IFIT1 (right) visualizing location of residues 364 and 366 (red) that confer antiviral activity. Graphics were generated using ChimeraX. F. Zoom in of (E).

species aligned except chimpanzee, which encodes an arginine (Q364R) and introduces a positive charge. Point mutants of residue 364 in chimpanzee and human IFIT1 exhibited moderate alterations in anti-VEEV function, though not statistically significant, suggesting that this amino acid may have a minor role in antiviral restriction that synergizes with L366.

To test combined effects of residues 364 and 366, double mutants (referred to as 364/6 double mut) for both chimpanzee and human IFIT1 were generated. Huh7.5 cells expressing WT, 364/6 double mut, or 362/4/6 triple mut IFIT1s of both chimpanzee and human IFIT1 were challenged with VEEV-GFP at an MOI of two, and infection was quantified by flow cytometry (**Fig 6D**). Western blotting demonstrated uniform expression of all human and chimpanzee IFIT1 mutants (**Fig 6D**). As in previous experiments, wild-type human IFIT1 strongly suppressed VEEV infection whereas wild-type chimpanzee IFIT1 did not (**Fig 6D**). However, the 364/6 double mut and 362/4/6 triple mut of human IFIT1 both had a near complete loss of antiviral capacity (**Fig 6D**). These results suggest that residues 364 and 366, but not 362, in human IFIT1 are necessary to confer anti-VEEV function. In contrast, the 364/6 double mutant or the 362/4/6 triple mutant of chimpanzee IFIT1 potently suppressed VEEV infection similar WT human IFIT1. No difference in antiviral potency was observed between chimpanzee IFIT1 with the 364/6 double mut or 362/4/6 triple mut, suggesting again that residue 362 does not play a key role in the differential antiviral properties of human and chimpanzee IFIT1. Together, these data indicate that out of eight amino acids that differ between human and chimpanzee IFIT1, sites 364 and 366, in combination, comprise the underlying differences in anti-VEEV function.

We next examined the location of amino acids 364 and 366 in the solved crystal structure of human IFIT1 bound to RNA and in the chimpanzee IFIT1 structure predicted by AlphaFold. Residues 364 and 366 are located within the α -helix 18 within TPR7 of IFIT1 (**Fig 6A**, **6E**). They are not predicted to contact RNA and are not located in direct proximity of the RNA-binding pocket (**Fig 6E**, **6F**), suggesting they may regulate primate IFIT1 function in an allosteric manner.

Discussion

Here, combining evolutionary and functional data, we conclude that mammalian IFIT1s are rapidly evolving viral restriction factors that exhibit species-specific antiviral activity. Differential antiviral activity of human and chimpanzee IFIT1 led to the unexpected discovery that two residues, 364 and 366, in α -helices 18 within the TPR7 domain of IFIT1 may partially determine primate IFIT1 antiviral capacity. The structures of human and chimp IFIT1 suggest that these amino acids are not directly involved in RNA binding. Thus, they may be involved in conformational changes of IFIT1 during RNA-binding or other allosteric effects that result in a natural loss of function, though additional studies are needed to test this.

Our data also support a model in which the breadth of IFIT1 antiviral phenotypes may be, at least partially, explained by differences in RNA-binding ability. VEEV infection was most potently suppressed by black flying fox IFIT1, significantly inhibited by human IFIT1, and unaffected by chimpanzee IFIT1 (**Fig 4C**). This trend was similarly observed in RNA binding assays with a sequence identical to that of the Cap0 VEEV 5' UTR (**Fig 5C**). These data are consistent with previous studies showing that the ability of IFIT1 to bind viral RNA is required for its antiviral function (4, 33).

The differential antiviral activity between IFIT1B and IFIT1 across several mammals was previously demonstrated by Daugherty et al. (11). This study used phylogenetic approaches to show that gene birth, gene loss and gene conversion occurred throughout vertebrate *IFIT* genes, resulting in extensive genetic diversity (11). Furthermore, through viral infection assays and a yeast growth assay, the authors showed different RNA-binding properties and disparate antiviral functions between IFIT1 or IFIT1B proteins from primates, mouse, and cat (11). Our studies

significantly expand the diversity of mammalian IFIT1s interrogated for antiviral function. Notably, IFIT1s across *Mammalia* are not universally antiviral against VEEV and VSV. In fact, antiviral specificity of IFIT1 orthologs is possibly quite narrow and highly dependent on which virus is being evaluated.

We further show that mammalian IFIT1s spanning diverse orders are rapidly evolving, and that in primates, this is occurring at functionally relevant sites that are not directly implicated in RNA binding. Computational studies identified amino acid 193 in TPR4 loop as rapidly evolving, and scanning mutagenesis showed that this residue is mutationally resilient. This was similarly observed for TRIM5α, in which most mutations within the v1 loop did not affect antiviral potency (34). Alteration of charge within the v1 loop of TRIM5α did play a significant role in determining the antiviral outcome of TRIM5α mutants (34). Similarly, introduction of negatively charged residues at position 193 of human IFIT1 ablated antiviral activity (Fig 2D). Thus, alteration of this single amino acid within IFIT1 may not result in major gain or loss antiviral function, which may maximize the potential for adaptation during evolutionary arms races with viruses. However, we cannot rule out that substitutions at position 193 may influence antiviral function when combined with changes at other sites in IFIT1. Position 193 is also under selection in bats, and multiple codons within or adjacent to the TPR4 loop register as positively selected across all clades examined (Fig 1A-C). Systematic mutagenesis of these residues—alone and in combination—will clarify whether specific side chains or overall loop charge drive species-specific antiviral outcomes.

We note several limitations of our study. [1] All IFIT1 ortholog screens were performed by expressing IFIT1 from distinct species in human cells, which may affect natural antiviral function. For example, some IFIT1 orthologs may bind viral RNA, but not interface with human proteins to effectively suppress translation, resulting in no antiviral activity when expressed in human cells. [2] Our experiments focus on expression of IFIT1 alone, without co-expression of other IFIT proteins. As human IFIT1 antiviral activity and RNA-binding is enhanced by interactions with other IFITs (35, 36), it is possible that the activity of IFIT1 orthologs used in this study is also modulated by co-expression with other IFITs from the cognate species. In particular, human IFIT3 modulates IFIT1 Cap0 RNA-binding and antiviral activity (37).

Therefore, future experiments testing the antiviral potential of co-expressed IFITs from diverse species would be informative. [3] Our biochemical assays focus on IFIT1 binding to Cap0 RNA only. This is due to comparative studies that have demonstrated IFIT1 has the strongest affinity for Cap0 RNA when compared to other RNAs, such as ppp-RNA (3). This allowed us to use human IFIT1 as a positive control for Cap0 RNA-binding with a strong signal-to-noise for comparison to other IFIT1 orthologs. However, it is possible that differences in ppp-RNA binding may underlie differences in the antiviral potency of IFIT1 towards viruses such as VSV and PIV3. Further, viral RNA secondary structure can affect IFIT binding (1, 21); the capacity of IFIT1 orthologs to bind RNA 5' termini may therefore vary with tolerance to structure-based occlusion. [4] This study also does not address the possibility that differential viral evasion of IFIT1 proteins contributes to species-specific antiviral activity. In fact, a recent study has identified species-specific sensitivity of IFIT1 proteins to viral evasion factors encoded by poxviruses (38). [5] We have only provided genetic but not biochemical or structural evidence for the underlying differences between chimpanzee and human IFIT1 RNA-binding and antiviral activity. By mutagenesis assays we demonstrate that two amino acid changes confer antiviral activity between chimpanzee and human IFIT1, suggesting a role for residues 364 and 366 in primate IFIT1. Additional insight into the role of these residues might be gained from molecular dynamics simulations using the crystal structure of human IFIT1 bound to Cap0 RNA as a template. It is also possible that these residues in human IFIT1 may be involved in interactions with other proteins, such as IFIT3, which modulates IFIT1 antiviral activity and Cap0 RNA-binding (37). Indeed, subdomain III of IFIT1 (alpha-helices 17-23), which contains residues 364 and 366 (part of alpha helix 18), showed partial protection from deuterium uptake by hydrogen-deuterium exchange with mass spectrometry (HDX-MS) during

IFIT1-IFIT3 binding experiments (37 [↗](#)). However, residues 364 and 366 themselves were not predicted to directly interact with IFIT3, and to date, structural studies have not demonstrated that these residues are directly involved in IFIT1-IFIT3 interactions. Comparisons with the 364/366 double mutant might determine whether these substitutions alter conformational dynamics in a manner that disfavors IFIT1-IFIT3 interaction and/or RNA binding. [6] Our experiments with chimp IFIT1 suggest that amino acid changes at sites 364 and 366 may have resulted in a natural loss of function for this ortholog. However, we only tested four viruses in infection studies and one viral RNA sequence in RNA-binding studies. Therefore, it is possible that chimpanzee IFIT1 inhibits other viruses, such as those that infect chimpanzees in nature. Future studies may also explore this possibility and uncover other amino acid changes that have resulted in IFIT1 loss of function in certain species. Were a loss of IFIT1 function to occur in chimpanzees or other species, this may have also coincided with a gain of dominant antiviral function in another IFIT or complex of IFIT proteins. However, this has yet to be experimentally tested.

Summarily, our findings underscore the notion that unbiased screening, investigations of restriction factors from non-model mammalian species, and evolutionary-guided approaches may uncover novel insight into host-pathogen interactions.

Materials and methods

Cells and cell culture

Huh7.5 and 293T cells (from C. Rice, The Rockefeller University) were maintained in “complete” DMEM: DMEM (Gibco) supplemented with 10% FBS and 1× non-essential amino acids (NEAA; Gibco). All derived stable cells expressing selectable markers were grown in complete DMEM containing puromycin (Gibco; Huh7.5: 4μg/mL). All cells were incubated at 37°C in 5% CO₂. All cell lines have been STR profiled and confirmed negative for mycoplasma contamination by PCR testing (Venor GeM mycoplasma detection kit, Sigma).

Viruses and viral infections

Venezuelan equine encephalitis virus (TC-83 strain) infectious clone was obtained from I.Frolov. VEEV-GFP stocks were generated as previously described (39 [↗](#)). Briefly, infectious clone plasmid was linearized, and RNA was in vitro transcribed with the mMESSAGE mMACHINE™ SP6 Transcription Kit (Invitrogen) followed by RNA clean-up with the MEGAclear™ Transcription Clean-Up Kit (Invitrogen). BHK-21J cells (8 × 10⁶ cells) were then electroporated with 5μg of in vitro transcribed RNA and plated. Media was changed 18 h after plating, and the remaining supernatant was collected 8 h post-media change. After centrifugation to remove cell debris, virus-containing supernatants were aliquoted and stored at −80°C. Sindbis virus (SINV-GFP, clone S300 from M. Heise) (40 [↗](#)) was generated as previously described. Vesicular stomatitis virus (VSV-GFP) (from J. Rose) and human parainfluenza virus type 3 (PIV3-GFP) (JS strain, from Peter Collins) were propagated by passaging in BHK-21J cells and storing clarified supernatants as virus stocks at −80°C. All viral infections were performed in DMEM supplemented with 1% FBS and 1x non-essential amino acids at a total volume of 0.2mL in 24-well tissue culture plates. After a 1 h incubation period, 0.3mL of complete DMEM was added to all wells until harvest. For analysis of viral infection by flow cytometry, cells were dissociated from the plate with 150μL Accumax (Innovative Cell Technologies) and then fixed in paraformaldehyde at a final concentration of 1%. Cells were allowed to fix for at least 30 min at 4°C and subsequently centrifuged at 1500 × g for 5 min. Supernatant was removed from cell pellets and fixed cells were then resuspended in FACS buffer (PBS [Gibco] supplemented with 3% FBS). Flow cytometry was performed on the Stratadigm S1000EON benchtop flow cytometer, with analysis in FlowJo software.

Cloning and plasmids

IFIT1 coding sequences were obtained from the NCBI database. IFIT1 sequences were cloned into the Gateway expression vector pSCRPSY (41 [DOI](#)). IFIT1 orthologs were synthesized as gBlocks (IDT) with Gateway compatible DNA sequences and an N-terminal HA tag. Human and chimpanzee IFIT1 point mutants were generated using site-directed mutagenesis PCR. Human and chimpanzee IFIT1 362/4/6 triple mutants and 364/6 double mutants were generated by Gibson cloning of gBlocks (IDT). For protein purification, IFIT1 protein coding sequences that were codon-optimized for bacterial expression were cloned into a modified pET28a bacterial expression vector, containing an N-terminal 6X-His tag followed by the yeast Sumo (smt3).

Western Blotting

Cells were lysed with M-PER (Mammalian Protein Extraction Reagent, Thermo Scientific) containing 1x complete protease inhibitor cocktail (Roche) at 4°C for 5 min with intermittent rocking. Lysate was then stored at -80°C. For running SDS-PAGE, lysate was thawed on ice, and mixed to a final concentration of 1x SDS Loading Buffer (0.2 M Tris-Cl pH 6.8, 5% SDS, 25% Glycerol, 0.025% Bromophenol Blue, and 6.25% beta-mercaptoethanol). Samples were run on 12% TGX FastCast acrylamide gels (Bio-Rad) or 4-20% Mini-PROTEAN® TGX™ precast protein gels (BioRad) and transferred to nitrocellulose membranes using a TransBlot Turbo system (Bio-Rad). Membranes were blocked with 5% milk in 1x Tris Buffered Saline, with Tween-20 (TBS-T [20mM Tris, 150mM NaCl, 0.1% Tween-20]) for at least 45min. Subsequent incubation with primary antibody (at dilutions between 1:500 to 1:5000) diluted in 1% milk in TBS-T was performed overnight, rocking at 4°C. Primary antibodies probing for GAPDH (Proteintech Cat# 10494-1-AP, RRID:AB_2263076 or Proteintech Cat# 60004-1-Ig, RRID:AB_2107436), IFIT1 (Proteintech Cat# 23247-1-AP, RRID:AB_2811269) and HA-tagged IFIT1 (Biolegend Cat# 901502, RRID:AB_2565006) were used in this study. After incubation with primary antibodies, membranes were washed 3 times with TBS-T by rocking for 5 min. Secondary antibodies (Goat anti-Rabbit 800CW and Goat anti-Mouse 680RD [LI-COR] at 1:5000 dilutions) diluted in 1x TBS-T were incubated with membranes for at least 45 min. Membranes were then washed 3 times with TBS-T by rocking for 5 min. Proteins were visualized using the LI-COR Odyssey FC imaging system.

Lentivirus production and transduction

To generate lentivirus pseudoparticles, 350,000 293Ts were plated onto poly-lysine coated 6-well plates. Lentiviral packaging plasmids expressing VSV-g and Gag-pol were then mixed with SCRPSY plasmids at a ratio of 0.1µg:0.5 µg:2.5 µg, respectively. Plasmids were incubated for 20 min with a mix of XtremeGENE9 transfection reagent (Roche) and OptiMEM (Gibco). Transfection complexes were then added to 293T cells in 1mL of DMEM supplemented with 3% FBS dropwise. After 6 h of incubation, media was changed to 1.5 mL of DMEM supplemented with 3% FBS. Supernatants containing lentiviral pseudoparticles were harvested at 48 and 72 h post-transfection. Lentiviral pseudoparticles were then processed by adding 4µg/mL Polybrene (Sigma), 20mM HEPES, centrifuging at 1500rpm for 5min to remove cell debris, and storing at - 80°C until transductions.

Lentivirus transductions were performed in 6-well plates after plating 350,000 cells (Huh7.5). Final volume of transductions was either 1mL or 1.5mL of lentivirus mixed with “pseudoparticle” DMEM (DMEM supplemented with 3% FBS, 4µg/mL Polybrene, and 20mM HEPES) After a 6 h incubation at 37°C, 2mL of complete DMEM was added to all transductions. Transduced cells were split into selection media 48h post-transduction and passaged for a minimum of three passages in selection media.

Protein Purification

IFIT1 protein coding sequences that were codon-optimized for bacterial expression were cloned into a modified pET28a bacterial expression vector, containing an N-terminal 6X-His tag followed by the yeast Sumo (smt3). Plasmids were transformed into Rosetta DE3 cells. Transformants were grown in Terrific Broth (TB) with 50 mg/L Kanamycin and 34 mg/L of Chloramphenicol and Antifoam B at 37 °C. At the OD₆₀₀ of 0.4, the temperature was lowered to 18 °C. Protein expression was induced at OD₆₀₀=3 by addition of IPTG to 0.4 mM final concentration. Cells were harvested after an overnight incubation by centrifugation (OD₆₀₀~8.5) and lysed in the lysis buffer (50 mM Tris-HCl pH 8, 500 mM NaCl, 15 mM Imidazole, 1 mM PMSF, 4 mM β-ME) by sonication. Cell lysates were cleared by centrifugation at 35,000 x g for 30 minutes. The cleared lysate was applied onto a gravity column with pre-washed Ni-NTA resin. Resin was washed with 50 mM Tris-HCl pH 8, 1 M NaCl, 30 mM Imidazole, 4 mM β-ME. Proteins were eluted with 50 mM Tris-HCl pH 8, 300 mM NaCl, 300 mM Imidazole, 2 mM DTT. Proteins were treated with Ulp1 protease overnight to remove the Sumo tag and passed over a size-exclusion column (HiLoad 16/600 Superdex 200 pg), equilibrated with 50 mM Tris 8.0, 150 mM NaCl, 1 mM DTT. Peak fractions were pooled, concentrated by ultrafiltration, and stored at - 80 °C until use. For SDS-PAGE gels of purified IFIT1 protein, 500nM of human, black flying fox, chimpanzee, and human R187H mutant IFIT1 were boiled in 4X Laemmli Sample Buffer (BioRad) with 1.25% BME and loaded into a 4-20% Mini-PROTEAN® TGX™ Precast Protein Gel (BioRad). Gels were run and subsequently incubated in Coomassie stain (40% ethanol, 10% glacial acetic acid, 0.1% Coomassie R-250) for 1 hour and destained overnight (10% ethanol, 7.5% glacial acetic acid). Gels were imaged with the ChemiDoc Imaging System (BioRad).

RNA Electrophoretic mobility shift assay

Protein stock concentration of IFIT1 orthologs was determined by diluting protein 1:5 in 1x TBE and measuring A280. Extinction coefficient was determined by ProtParam tool (ExPASy), and subsequently used to calculate and normalize protein concentrations between stocks. Before loading, native gels (10% 19:1 Acrylamide: Bis-acrylamide, 25mM NaCl, 1x TBE, 0.0005% APS, 0.001% TEMED) were pre-run with 1x TBE in a cold room for at least 2.5 h. While gels were pre-running, binding reactions were set up at room temperature after thawing and keeping both RNA and protein stocks on ice. All dilutions of protein and RNA were in 1x reaction buffer (50nM TRIS pH 8.0, 100mM NaCl, 1mM EDTA, 0.01mg/mL Heparin sodium sulfate, 2.5% Glycerol). For binding reactions, 50nM of Cap0 RNA was mixed 1:1 with 0, 80, 160, 240, 320, and 400nM of purified IFIT1 protein in a final volume of 20μL and incubated at room temperature for 20 min.

18 μL of RNA:Protein complexes were then run on pre-run native gels for 2.5 h at 100V in 1x TBE in a cold room. Gels were then stained with 1x SYBR Gold stain (ThermoFisher Scientific) diluted in 1x TBE for 20 min rocking at room temperature and imaged on a ChemiDoc Imaging System (BioRad) with UV transillumination. Band intensity was subsequently analyzed using ImageLab software (BioRad).

In vitro transcription and 5' capping of RNA probes

Uncapped RNA probes were generated using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega). Manufacturer instructions were modified for generating short RNA probes. Briefly, 10μM of DNA oligos corresponding to the T7φ2.5 promoter sequence and the complementary T7φ2.5 promoter sequence followed by the first 41nt of the VEEV TC-83 strain 5' UTR sequence. Oligos were boiled at 95°C for 2-3 min and then slow cooled to room temperature to allow annealing. 1 μM of the annealed DNA oligos served as the template per 20μL T7 transcription reaction. RNA was purified according to manufacturer instructions using phenol:chloroform nucleic acid extraction (Promega).

To generate Cap0 RNA, purified uncapped RNA probes were modified using the Faustovirus Capping Enzyme (FCE) (NEB) using modified manufacturer instructions. Briefly, 100 μ L reactions were composed of 20 μ L of uncapped RNA purified after T7 transcription (described above), 0.2mM SAM, 1mM GTP, 7500U of FCE, 1x FCE reaction buffer, 80U RNasin® Plus Ribonuclease Inhibitor (Promega), and nuclease-free water up to 100 μ L. Reactions were incubated at 42°C for 4.5 h and then another 0.5 h at 50°C. Cap0 RNA was then purified using the same phenol:chloroform nucleic acid extraction as performed after the T7 transcription reaction (Promega). Cap0 RNA was determined to be at least 95% Cap0 RNA (m7GpppA) by targeted LC/MS after Nuclease P1 (NEB) digestion at 37°C for 30 min followed by inactivation of enzyme at 95°C for 2 min. LC/MS was performed by the UT Southwestern Preclinical Pharmacology Core Facility.

Computational Evolutionary Analysis

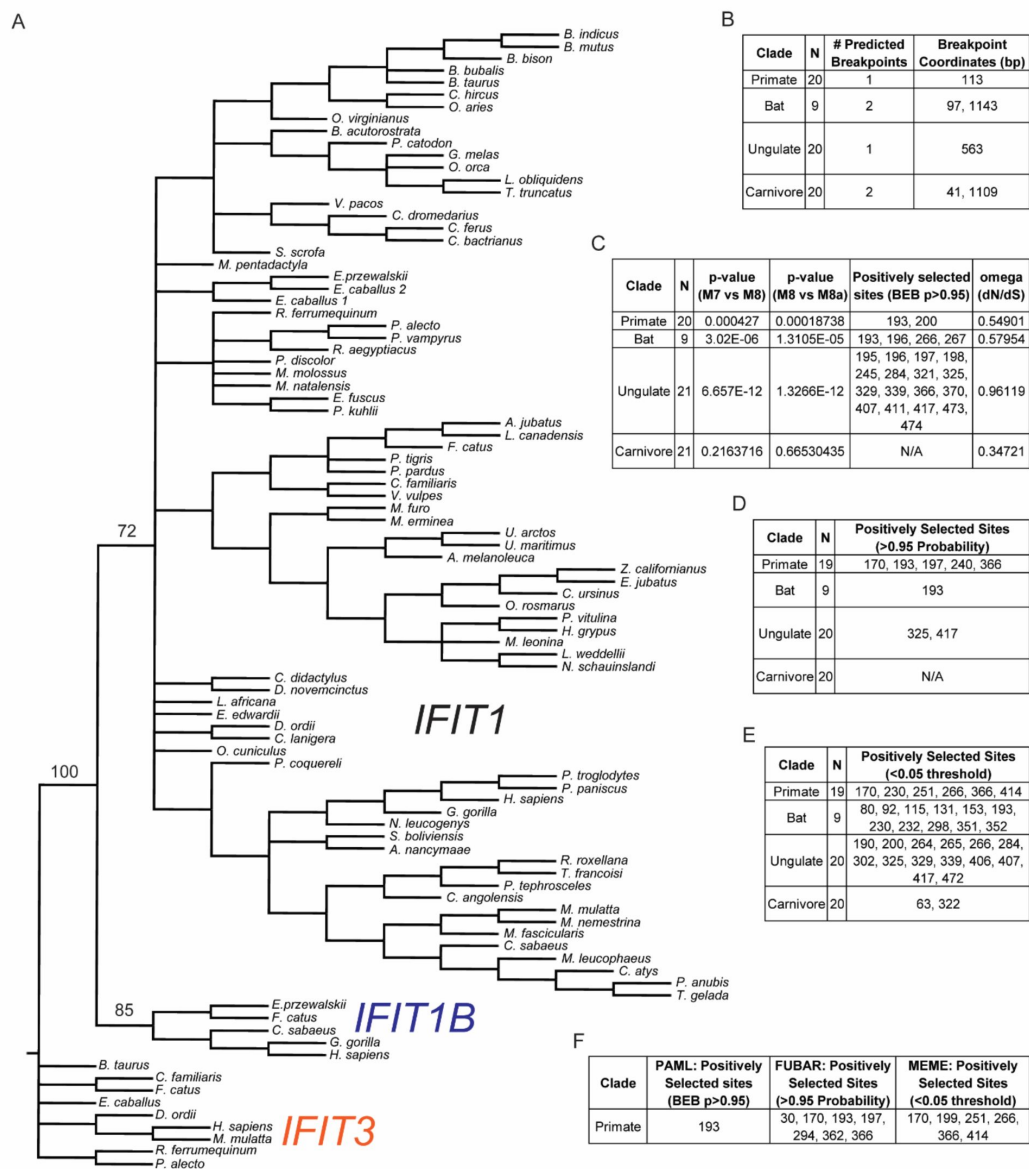
IFIT1 coding sequences for all analyses were obtained from the NCBI database. Alignment of multiple sequences was performed using MUSCLE and ClustalW implemented in MEGA X (42). Maximum-likelihood trees were constructed in MEGA X, with standard settings. Trimmed alignments were then generated (43), and when required, ALTER (44) was used to convert file formats. MEME (45) and FUBAR (14) were performed with HyPhy (46) using the Datamonkey web application (47). To perform PAML analysis, we used default settings with the F3 $\times$ 4 codon frequency table within CodeML. Likelihood ratio tests were performed to compare model 7 versus model 8 and model 8 versus model 8a to determine the presence of positive selection. Bayes empirical Bayes testing was performed to determine specific residues that are adaptively evolving. To detect potential recombination in IFIT1, GARD analysis (16) was performed on aligned coding sequences with HyPhy (46) using the Datamonkey web application (47).

Recombination Detection Program version 4 (RDP4) (17) was also used to remove inferred recombinant sequences from alignment, using default settings. Maximum likelihood tree comparing IFIT1, IFIT1B, and IFIT3 C-terminal sequences was aligned using MUSCLE in MEGA X, followed by maximum likelihood tree generation with 1000 bootstrap replicates in MEGA X (42). Alignment to the C-terminal region of IFITs to distinguish IFIT1 and IFIT1B sequences was previously described (11). Species trees were generated using the TimeTree database (48). FigTree software was then used to make trees shown in figures. Alignments and determination of amino acid identity to human IFIT1 was performed using ClustalOmega (49). Graphics comparing human and chimpanzee IFIT1 structures were generated using ChimeraX (50).

Statistical analysis

All statistical analyses were performed in GraphPad Prism 9. All graphs represent the mean of n=3 independent biological replicates with error bars representing standard deviation. ns, p > 0.05, *, p < 0.05, **, p < 0.01, ***, p < 0.001, and **** p < 0.0001.

Supplementary figures and tables

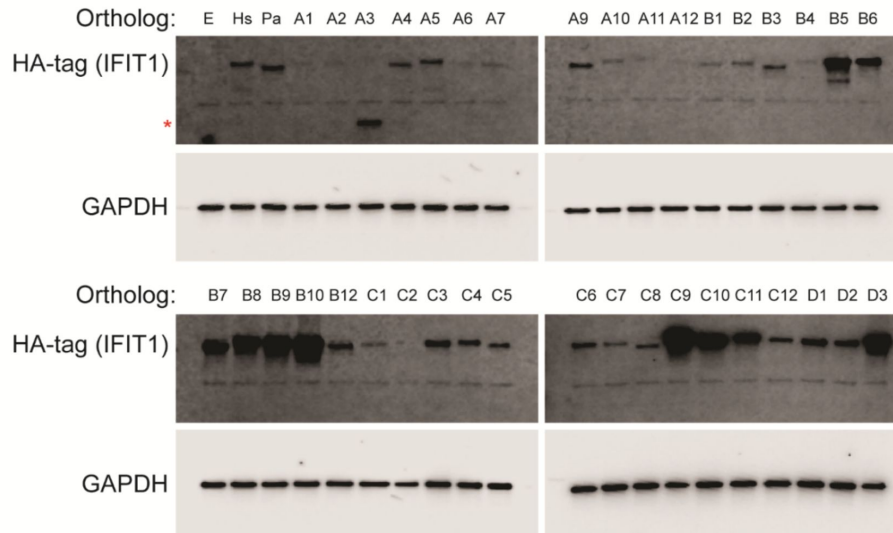


Supplementary Figure 1.

Analysis of IFIT1 for recombination and positive selection.

A. Maximum likelihood tree of C-terminal region of IFIT1, IFIT1B, and IFIT3 coding sequences (corresponding to bases 907-1437 of human IFIT1 coding sequence), generated in MEGA X with 1000 bootstrapping replicates. Select bootstrap values are shown. B. GARD analysis of IFIT1 coding sequences used for evolutionary analysis in [Figure 1](#). C. PAML analysis on regions of IFIT1 coding sequence after (primate and ungulate) or between (bat and carnivore) breakpoints indicated in B. D. FUBAR analysis on regions of IFIT1 coding sequence after (primate and ungulate) or between (bat and carnivore) breakpoints indicated in B. E. MEME analysis on regions of IFIT1 coding sequence after (primate and ungulate) or between (bat and carnivore) breakpoints indicated in B. F. PAML, FUBAR, and MEME analysis on primate IFIT1 coding sequence after removal of inferred recombinant sequences by Recombination Detection Program 4 (RDP4).

A



B

Common Name	Scientific Name	Key	Common Name	Scientific Name	Key
Empty Control	N/A	E	Cow	<i>Bos taurus</i>	B7
Human	<i>Homo sapiens</i>	Hs	Sheep	<i>Ovis aries</i>	B8
Black flying fox	<i>Pteropus alecto</i>	Pa	White-tailed deer	<i>Odocoileus virginianus texanus</i>	B9
African green monkey	<i>Chlorocebus sabaeus</i>	A1	Killer whale	<i>Orcinus orca</i>	B10
Coquerel's sifaka	<i>Propithecus coquereli</i>	A2	Common bottlenose dolphin	<i>Tursiops truncatus</i>	B12
Black-capped squirrel monkey	<i>Saimiri boliviensis</i>	A3	Ferret	<i>Mustela putorius furo</i>	C1
Angola colobus	<i>Colobus angolensis</i>	A4	Cat	<i>Felis catus</i>	C2
Chimpanzee	<i>Pan troglodytes</i>	A5	Dog	<i>Canis lupus familiaris</i>	C3
Crab-eating macaque	<i>Macaca fascicularis</i>	A6	Grizzly bear	<i>Ursus arctos horribilis</i>	C4
Rhesus macaque	<i>Macaca mulatta</i>	A7	Harbor (common) seal	<i>Phoca vitulina</i>	C5
Large flying fox	<i>Pteropus vampyrus</i>	A9	California sea lion	<i>Zalophus californianus</i>	C6
Velvety free tailed bat	<i>Molossus molossus</i>	A10	Nine-banded armadillo	<i>Dasypus novemcinctus</i>	C7
Greater horseshoe bat	<i>Rhinolophus ferrumequinum</i>	A11	Chinese pangolin	<i>Manis pentadactyla</i>	C8
Pale spear-nosed bat	<i>Phyllostomus discolor</i>	A12	European rabbit	<i>Oryctolagus cuniculus</i>	C9
Big brown bat	<i>Eptesicus fuscus</i>	B1	African savannah elephant	<i>Loxodonta africana</i>	C10
Kuhl's pipistrelle	<i>Pipistrellus kuhlii</i>	B2	Cape elephant shrew	<i>Elephantulus edwardii</i>	C11
Natal long-fingered bat	<i>Miniopterus natalensis</i>	B3	Linnaeus's two-toed sloth	<i>Choloepus didactylus</i>	C12
Horse 1	<i>Equus caballus</i>	B4	Ord's kangaroo rat	<i>Dipodomys ordii</i>	D1
Pig	<i>Sus scrofa</i>	B5	Chinchilla	<i>Chinchilla lanigera</i>	D2
Dromedary camel	<i>Camelus dromedarius</i>	B6	Horse 2	<i>Equus caballus</i>	D3

Supplementary Figure 2.

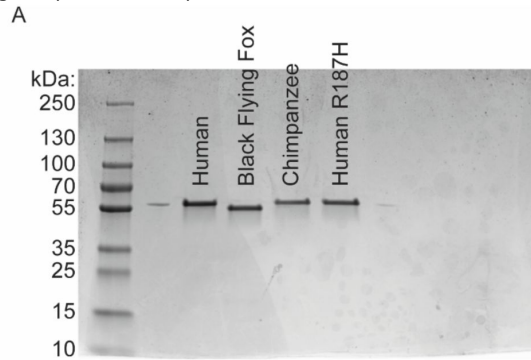
IFIT1 ortholog expression varied during the ortholog screen.

A. Western blot from lysates of cells expressing HA-tagged IFIT1 orthologs for screen in [Fig 1A](#) demonstrating unequal IFIT1 expression patterns. Images are representative of three independent replicates. B. Key denoting which symbols correspond to HA-tagged IFIT1 ortholog. Red asterisk marks the size of the truncated or cleaved protein product in lane A3.

Supplementary Figure 3.

Gel image of recombinant IFIT1 protein.

A. Coomassie stained SDS-PAGE gel of purified IFIT1 protein used for RNA EMSA.



Supplementary Table 1.

Mammalian IFIT1 ortholog characteristics and antiviral activity

Common name	Species	Order	Superorder	% identity to human	Antiviral activity (% of control)					NCBI sequence (CDS)
					VEEV rep1	VEEV rep2	VSV rep1	VSV rep2	VSV rep3	
Human	Homo sapiens	Primate	Euarhontoglires	100	8.7	37.3	60.7	75.3	82.6	NM_001548.5
African green monkey	Chlorocebus sabaeus	Primate	Euarhontoglires	89.5	50.9	28.5	67.5	79.5	73.0	XM_007963500.2
Coquerel's sifaka	Propithecus coquereli	Primate	Euarhontoglires	79.1	110.1	53.1	73.7	81.6	74.9	XM_012659752.1
Black-capped squirrel monkey	Saimiri boliviensis	Primate	Euarhontoglires	88.7	281.6	79.9	94.8	88.5	78.6	XM_003922412.2
Angola colobus	Colobus angolensis	Primate	Euarhontoglires	91	26.5	9.3	69.2	74.5	66.6	XM_011947865.1
Chimpanzee	Pan troglodytes	Primate	Euarhontoglires	98.3	188.0	52.4	59.7	79.7	79.0	NM_001205347.1
Crab-eating macaque	Macaca fascicularis	Primate	Euarhontoglires	89.7	134.2	19.8	86.4	87.1	86.1	XM_015455916.1
Rhesus macaque	Macaca mulatta	Primate	Euarhontoglires	91.4	93.7	54.3	75.0	83.6	72.0	XM_015147649.2
Black flying fox	Pteropus alecto	Chiroptera	Laurasiatheria	69.1	4.9	13.9	106.2	99.2	99.8	XM_006925964.3
Large flying fox	Pteropus vampyrus	Chiroptera	Laurasiatheria	69.2	24.3	32.5	71.4	88.5	69.5	XM_011361637.2
Velvety free tailed bat	Molossus molossus	Chiroptera	Laurasiatheria	65.1	91.1	40.9	93.8	87.1	82.8	XM_036265954.1
Greater horseshoe bat	Rhinolophus ferrumequinum	Chiroptera	Laurasiatheria	69.1	299.4	115.7	87.0	79.7	79.9	XM_033131324.1
Pale spear-nosed bat	Phyllostomus discolor	Chiroptera	Laurasiatheria	65.3	224.7	103.9	82.1	87.9	97.7	XM_028512707.2
Big brown bat	Eptesicus fuscus	Chiroptera	Laurasiatheria	68.5	22.7	22.2	75.0	89.9	96.3	XM_008156938.2
Kuhl's pipistrelle	Pipistrellus kuhlii	Chiroptera	Laurasiatheria	66.4	130.4	68.5	86.7	99.7	73.2	XM_036438510.1
Natal long-fingered bat	Miniopterus natalensis	Chiroptera	Laurasiatheria	67.6	71.5	51.6	67.9	80.3	70.1	XM_016220858.1
Horse 1	Equus caballus	Perissodactyla	Laurasiatheria	68.3	306.3	124.0	79.5	83.6	78.2	XM_005602371.3
Horse 2	Equus caballus	Perissodactyla	Laurasiatheria	68.8	67.7	85.4	101.9	107.9	96.3	XM_023642993.1
Pig	Sus scrofa	Artiodactyla	Laurasiatheria	66.6	131.6	49.6	34.1	44.4	49.0	NM_001244363.1
Dromedary camel	Camelus dromedarius	Artiodactyla	Laurasiatheria	65.8	11.6	10.7	58.1	71.2	90.9	XM_010996286.2
Cow	Bos taurus	Artiodactyla	Laurasiatheria	65.9	63.9	32.4	20.7	42.5	58.3	XM_024986120.1
Sheep	Ovis aries	Artiodactyla	Laurasiatheria	64.2	2.7	7.0	65.3	79.5	57.1	XM_004020283.4
White-tailed deer	Odocoileus virginianus texanu	Artiodactyla	Laurasiatheria	68.6	3.5	9.3	29.0	57.8	45.4	XM_020894471.1
Killer whale (Orca)	Orcinus orca	Artiodactyla	Laurasiatheria	66.5	5.5	16.9	1.2	2.5	2.3	XM_012536578.2
Common bottlenose dolphin	Tursiops truncatus	Artiodactyla	Laurasiatheria	66.9	98.7	66.1	2.9	8.9	6.1	XM_019941810.2
Ferret	Mustela putorius furo	Carnivora	Laurasiatheria	67.9	26.5	54.3	74.0	75.3	90.0	XM_004780758.2
Cat	Felis catus	Carnivora	Laurasiatheria	69	70.3	72.4	84.4	71.5	96.9	XM_003993875.5
Dog	Canis lupus familiaris	Carnivora	Laurasiatheria	68.4	77.2	54.7	39.6	56.4	61.2	XM_843271.5
Grizzly bear	Ursus arctos horribilis	Carnivora	Laurasiatheria	69.2	115.8	53.5	72.7	79.2	80.3	XM_004780758.2
Harbor (common) seal	Phoca vitulina	Carnivora	Laurasiatheria	68	97.5	30.2	73.1	78.1	72.4	XM_032430411.1
California sea lion	Zalophus californianus	Carnivora	Laurasiatheria	68.8	170.9	49.2	87.3	83.8	86.1	XM_035724333.1
Chinchilla	Chinchilla lanigera	Rodentia	Euarhontoglires	63.7	163.9	83.9	89.0	81.9	85.1	XM_026504073.1
Ord's kangaroo rat	Dipodomys ordii	Rodentia	Euarhontoglires	63.7	231.0	76.0	56.2	64.1	79.5	XM_013017244.1
Nine-banded armadillo	Dasypus novemcinctus	Cingulata	Xenarthra	71.4	155.7	57.1	7.0	9.6	12.4	XM_004461422.3
Chinese pangolin	Manis pentadactyla	Pholidota	Laurasiatheria	69	4.3	5.0	80.2	70.1	68.0	XM_036928813.1
European rabbit	Oryctolagus cuniculus	Lagomorpha	Euarhontoglires	61.2	234.8	102.0	2.3	8.9	10.0	XM_008270173.2
African savannah elephant	Loxodonta africana	Proboscidea	Afrotheria	68.1	2.2	2.9	34.1	43.6	47.1	XM_023546938.1
Linnaeus's two-toed sloth	Choloepus didactylus	Pilosa	Xenarthra	72.3	34.7	48.8	25.1	44.4	47.3	XM_037804440.1
Cape elephant shrew	Elephantulus edwardii	Macroscelidea	Afrotheria	60.7	114.6	84.3	3.2	3.8	5.5	XM_006880403.1

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Additional information

Author Contributions

M.B.M, A.M.D., and E.N. performed technical experiments. M.B.M. and I.N.B. performed computational evolutionary analysis. M.B.M and J.W.S conceptualized this study and designed experiments. M.B.M and J.W.S wrote and edited the manuscript. M.B.M and J.W.S obtained funding.

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Reviewer #2 (Public review):

McDougal et al. describe the surprising finding that IFIT1 proteins from different mammalian species inhibit replication of different viruses, indicating that evolution of IFIT1 across mammals has resulted in host species-specific antiviral specificity. Before this work, research into the antiviral activity and specificity of IFIT1 had mostly focused on the human ortholog, which was described to inhibit viruses including vesicular stomatitis virus (VSV) and Venezuelan equine encephalitis virus (VEEV) but not other viruses including Sindbis virus (SINV) and parainfluenza virus type 3 (PIV3). In the current work, the authors first perform evolutionary analyses on IFIT1 genes across a wide range of mammalian species and reveal that IFIT1 genes have evolved under positive selection in primates, bats, carnivores, and ungulates. Based on these data, they hypothesize that IFIT1 proteins from these diverse mammalian groups may show distinct antiviral specificities against a panel of viruses. By generating human cells that express IFIT1 proteins from different mammalian species, the authors show a wide range of antiviral activities of mammalian IFIT1s. Most strikingly, they find several IFIT1 proteins that have completely different antiviral specificities relative to human IFIT1, including IFIT1s that fail to inhibit VSV or VEEV, but strongly inhibit PIV3 or SINV. These results indicate that there is potential for IFIT1 to inhibit a much wider range of viruses than human IFIT1 inhibits. Electrophoretic mobility shift assays (EMSAs) suggest that some of these changes in antiviral specificity can be ascribed to changes in direct binding of viral RNAs. Interestingly, they also find that chimpanzee IFIT1, which is >98% identical to human IFIT1, fails to inhibit any tested virus. Replacing three residues from chimpanzee IFIT1 with those from human IFIT1, one of which has evolved under positive selection in primates, restores activity to chimpanzee IFIT1. Together, these data reveal a vast diversity of IFIT1 antiviral specificity encoded by mammals, consistent with an IFIT1-virus evolutionary "arms race".

Overall, this is a very interesting and well-written manuscript that combines evolutionary and functional approaches to provide new insight into IFIT1 antiviral activity and species-specific antiviral immunity. The conclusion that IFIT1 genes in several mammalian lineages are evolving under positive selection is supported by the data. The virology results, which convincingly show that IFIT1s from different species have distinct antiviral specificity, are the most surprising and exciting part of the paper. As such, this paper will be interesting for researchers studying mechanisms of innate antiviral immunity, as well as those interested in species-specific antiviral immunity. Moreover, it may prompt others to test a wide range of orthologs of antiviral factors beyond those from humans or mice, which could further the concept of host-specific innate antiviral specificity. Additional areas for improvement, which are mostly to clarify the presentation of data and conclusions, are described below.

Strengths:

(1) This paper is a very strong demonstration of the concept that orthologous innate immune proteins can evolve distinct antiviral specificities. Specifically, the authors show that IFIT1 proteins from different mammalian species are able to inhibit replication of distinct groups

of viruses, which is most clearly illustrated in Figure 4G. This is an unexpected finding, as the mechanism by which IFIT1 inhibits viral replication was assumed to be similar across orthologs. While the molecular basis for these differences remains unresolved, this is a clear indication that IFIT1 evolution functionally impacts host-specific antiviral immunity and that IFIT1 has the potential to inhibit a much wider range of viruses than previously described.

(2) By revealing these differences in antiviral specificity across IFIT1 orthologs, the authors highlight the importance of sampling antiviral proteins from different mammalian species to understand what functions are conserved and what functions are lineage- or species-specific. These results might therefore prompt similar investigations with other antiviral proteins, which could reveal a previously undiscovered diversity of specificities for other antiviral immunity proteins.

(3) The authors also surprisingly reveal that chimpanzee IFIT1 shows no antiviral activity against any tested virus despite only differing from human IFIT1 by eight amino acids. By mapping this loss of function to three residues on one helix of the protein, the authors shed new light on a region of the protein with no previously known function.

(4) Combined with evolutionary analyses that indicate that IFIT1 genes are evolving under positive selection in several mammalian groups, these functional data indicate that IFIT1 is engaged in an evolutionary "arms race" with viruses, which results in distinct antiviral specificities of IFIT1 proteins from different species.

Weaknesses:

(1) Some of the results and discussion text could be more focused on the model of evolution-driven changes in IFIT1 specificity. In particular, the majority of the residue mapping is on the chimpanzee protein, where it would appear that this protein has lost all antiviral function, rather than changing its antiviral specificity like some other examples in this paper. As such, the connection between the functional mapping of individual residues with the positive selection analysis and changes in antiviral specificity is not present. While the model that changes in antiviral specificity have been positively selected for is intriguing, it is not supported by data in the paper.

(2) The strength of the differences in antiviral specificity could be highlighted to a greater degree. Specifically, the text describes a number of interesting examples of differences in inhibition of viruses from Figure 3C and 3D, and 4C-F. The revised version has added some clarity by at least providing raw data for 3C and 3D for the reader to make their own comparisons, but it is still difficult to quickly assess which are the most interesting comparisons to make (e.g. for future mapping of residues that might be important).

<https://doi.org/10.7554/eLife.101929.2.sa2>

Reviewer #3 (Public review):

Summary:

This manuscript by McDougal et al, demonstrates species-specific activities of diverse IFIT1 orthologs, and seeks to utilize evolutionary analysis to identify key amino acids under positive selection that contribute to antiviral activity of this host factor. While the authors identify amino acid residues important for antiviral activity of some orthologs, and propose a possible mechanism by which these residues may function, the significance or applicability of these findings to other orthologs is unclear. However, the subject matter is of interest to the field, and these findings contribute to the body of knowledge regarding IFIT1 evolution.

Strengths:

Assessment of multiple IFIT1 orthologs shows the wide variety of antiviral activity of IFIT1, and identification of residues outside of the known RNA binding pocket in the protein suggests additional novel mechanisms which may regulate IFIT1 activity.

Weaknesses:

Given that there appears to be very little overlap observed in orthologs that inhibited the viruses tested, it's possible that other amino acids may be key drivers of antiviral activity in these other orthologs. Thus, it's difficult to conclude whether the findings that residues 362/4/6 are important for IFIT1 activity can be broadly applied to other orthologs, or whether these are unique to human and chimpanzee IFIT1. While additional molecular studies of the impact of these mutations on IFIT1 function (e.g. impact on IFIT complex formation) would lend further insight, as it stands, these findings demonstrate a role for these residues in IFIT1 activity.

<https://doi.org/10.7554/eLife.101929.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public review):

Summary:

McDougal et al. aimed to characterize the antiviral activity of mammalian IFIT1 orthologs. They first performed three different evolutionary selection analyses within each major mammalian clade and identified some overlapping positive selection sites in IFIT1. They found that one site that is positively selected in primates is in the RNA-binding exit tunnel of IFIT1 and is tolerant of mutations to amino acids with similar biochemical properties. They then tested 9 diverse mammalian IFIT1 proteins against VEEV, VSV, PIV3, and SINV and found that each ortholog has distinct antiviral activities. Lastly, they compared human and chimpanzee IFIT1 and found that the determinant of their differential anti-VEEV activity may be partly attributed to their ability to bind Cap0 RNA.

Strengths:

The study is one of the first to test the antiviral activity of IFIT1 from diverse mammalian clades against VEEV, VSV, PIV3, and SINV. Cloning and expressing these 39 IFIT1 orthologs in addition to single and combinatorial mutants is not a trivial task. The positive connection between anti-VEEV activity and Cap0 RNA binding is interesting, suggesting that differences in RNA binding may explain differences in antiviral activity.

Weaknesses:

The evolutionary selection analyses yielded interesting results, but were not used to inform follow-up studies except for a positively selected site identified in primates. Since positive selection is one of the two major angles the authors proposed to investigate mammalian IFIT1 orthologs with, they should integrate the positive selection results with the rest of the paper more seamlessly, such as discussing the positive selection results and their implications, rather than just pointing out that positively selected sites were identified. The paper should elaborate on how the positive selection analyses PAML, FUBAR, and MEME complement one another to explain why the tests gave them different results. Interestingly, MEME which usually provides more sites did not identify site 193 in primates that was identified by both PAML and FUBAR. The authors should also provide

the rationale for choosing to focus on the 3 sites identified in primates only. One of those sites, 193, was also found to be positively selected in bats, although the authors did not discuss or integrate that finding into the study. In Figure 1A, they also showed a $dN/dS < 1$ from PAML, which is confusing and would suggest negative selection instead of positive selection. Importantly, since the authors focused on the rapidly evolving site 193 in primates, they should test the IFIT1 orthologs against viruses that are known to infect primates to directly investigate the impact of the evolutionary arms race at this site on IFIT1 function.

We thank the reviewer for their assessment and for acknowledging the breadth of our dataset regarding diverse IFIT1s, number of viruses tested, and the functional data that may correlate biochemical properties of IFIT1 orthologous proteins with antiviral function. We have expanded the introduction and results sections to better explain and distinguish between PAML, FUBAR, and MEME analyses. Furthermore, we have expanded the discussion to incorporate the observation that site 193 is rapidly evolving in bats, as well as the observation that nearby sites to the TPR4 loop were identified as rapidly evolving in all clades of mammals tested. We also do observe an overall gene dN/dS of <1 , however this is simply the average across all codons of the entire gene and does not rule out positive selection at specific sites. This is observed for other restriction factors, as many domains are undergoing purifying selection to retain core functions (e.g. enzymatic function, structural integrity) while other domains (e.g. interfaces with viral antagonists or viral proteins) show strong positive selection. Specific examples include the restriction factors BST-2/Tetherin (PMID: 19461879) and MxA (PMID: 23084925). Furthermore, we agree that testing more IFIT1-sensitive viruses that naturally infect primates with our IFIT1 193 mutagenesis library would shed light on the influence of host-virus arms races at this site. However, VEEV naturally does also infect humans as well as at least one other species of primate (PMID: 39983680).

Below we individually address the reviewers' claims of inaccurate data interpretation.

Some of the data interpretation is not accurate. For example:

(1) Lines 232-234: "...western blot analysis revealed that the expression of IFIT1 orthologs was relatively uniform, except for the higher expression of orca IFIT1 and notably lower expression of pangolin IFIT1 (Figure 4B)." In fact, most of the orthologs are not expressed in a "relatively uniform" manner e.g. big brown bat vs. shrew are quite different.

We have now included quantification of the western blots to allow the reader to compare infection results with the infection data (Updated Figure 4B and 4G). We have also removed the phrase "relatively uniform" from the text and have instead included text describing the quantified expression differences.

(2) Line 245: "...mammalian IFIT1 species-specific differences in viral suppression are largely independent of expression differences." While it is true that there is no correlation between protein expression and antiviral activity in each species, the authors cannot definitively conclude that the species-specific differences are independent of expression differences. Since the orthologs are clearly not expressed in the same amounts, it is impossible to fully assess their true antiviral activity. At the very least, the authors should acknowledge that the protein expression can affect antiviral activity. They should also consider quantifying the IFIT1 protein bands and normalizing each to GAPDH for readers to better compare protein expression and antiviral activity. The same issue is in Line 267.

We have now included quantification and normalization of the western blots to allow the reader to compare infection results with the infection data (Updated Figure 4B and 4G). Furthermore, we acknowledge in the text that expression differences may affect antiviral potency in infection experiments.

(3) Line 263: "SINV... was modestly suppressed by pangolin, sheep, and chinchilla IFIT1 (Figure 4E)..." The term "modestly suppressed" does not seem fitting if there is 60-70% infection in cells expressing pangolin and chinchilla IFIT1.

We have modified the text to say "significantly suppressed" rather than "modestly suppressed."

(4) The study can be significantly improved if the authors can find a thread to connect each piece of data together, so the readers can form a cohesive story about mammalian IFIT1.

We appreciate the reviewer's suggestion and have tried to make the story including more cohesive through commentary on positive selection and by using the computational analysis to first inform potential evolutionary consequences of IFIT1 functionality first by an intraspecies (human) approach, and then later an interspecies approach with diverse mammals that have great sequence diversity. Furthermore, we point out that almost all IFIT1s tested in the ortholog screen were also included in our computational analysis allowing for the potential to connect functional observations with those seen in the evolutionary analyses.

Reviewer #2 (Public review):

McDougal et al. describe the surprising finding that IFIT1 proteins from different mammalian species inhibit the replication of different viruses, indicating that the evolution of IFIT1 across mammals has resulted in host species-specific antiviral specificity. Before this work, research into the antiviral activity and specificity of IFIT1 had mostly focused on the human ortholog, which was described to inhibit viruses including vesicular stomatitis virus (VSV) and Venezuelan equine encephalitis virus (VEEV) but not other viruses including Sindbis virus (SINV) and parainfluenza virus type 3 (PIV3). In the current work, the authors first perform evolutionary analyses on IFIT1 genes across a wide range of mammalian species and reveal that IFIT1 genes have evolved under positive selection in primates, bats, carnivores, and ungulates. Based on these data, they hypothesize that IFIT1 proteins from these diverse mammalian groups may show distinct antiviral specificities against a panel of viruses. By generating human cells that express IFIT1 proteins from different mammalian species, the authors show a wide range of antiviral activities of mammalian IFIT1s. Most strikingly, they find several IFIT1 proteins that have completely different antiviral specificities relative to human IFIT1, including IFIT1s that fail to inhibit VSV or VEEV, but strongly inhibit PIV3 or SINV. These results indicate that there is potential for IFIT1 to inhibit a much wider range of viruses than human IFIT1 inhibits. Electrophoretic mobility shift assays (EMSAs) suggest that some of these changes in antiviral specificity can be ascribed to changes in the direct binding of viral RNAs. Interestingly, they also find that chimpanzee IFIT1, which is >98% identical to human IFIT1, fails to inhibit any tested virus. Replacing three residues from chimpanzee IFIT1 with those from human IFIT1, one of which has evolved under positive selection in primates, restores activity to chimpanzee IFIT1. Together, these data reveal a vast diversity of IFIT1 antiviral specificity encoded by mammals, consistent with an IFIT1-virus evolutionary "arms race".

Overall, this is a very interesting and well-written manuscript that combines evolutionary and functional approaches to provide new insight into IFIT1 antiviral activity and species-specific antiviral immunity. The conclusion that IFIT1 genes in several mammalian lineages are evolving under positive selection is supported by the data, although there are some important analyses that need to be done to remove any confounding effects from gene recombination that has previously been described between IFIT1 and its

paralog IFIT1B. The virology results, which convincingly show that IFIT1s from different species have distinct antiviral specificity, are the most surprising and exciting part of the paper. As such, this paper will be interesting for researchers studying mechanisms of innate antiviral immunity, as well as those interested in species-specific antiviral immunity. Moreover, it may prompt others to test a wide range of orthologs of antiviral factors beyond those from humans or mice, which could further the concept of host-specific innate antiviral specificity. Additional areas for improvement, which are mostly to clarify the presentation of data and conclusions, are described below.

Strengths:

(1) This paper is a very strong demonstration of the concept that orthologous innate immune proteins can evolve distinct antiviral specificities. Specifically, the authors show that IFIT1 proteins from different mammalian species are able to inhibit the replication of distinct groups of viruses, which is most clearly illustrated in Figure 4G. This is an unexpected finding, as the mechanism by which IFIT1 inhibits viral replication was assumed to be similar across orthologs. While the molecular basis for these differences remains unresolved, this is a clear indication that IFIT1 evolution functionally impacts host-specific antiviral immunity and that IFIT1 has the potential to inhibit a much wider range of viruses than previously described.

(2) By revealing these differences in antiviral specificity across IFIT1 orthologs, the authors highlight the importance of sampling antiviral proteins from different mammalian species to understand what functions are conserved and what functions are lineage- or species-specific. These results might therefore prompt similar investigations with other antiviral proteins, which could reveal a previously undiscovered diversity of specificities for other antiviral immunity proteins.

(3) The authors also surprisingly reveal that chimpanzee IFIT1 shows no antiviral activity against any tested virus despite only differing from human IFIT1 by eight amino acids. By mapping this loss of function to three residues on one helix of the protein, the authors shed new light on a region of the protein with no previously known function.

(4) Combined with evolutionary analyses that indicate that IFIT1 genes are evolving under positive selection in several mammalian groups, these functional data indicate that IFIT1 is engaged in an evolutionary "arms race" with viruses, which results in distinct antiviral specificities of IFIT1 proteins from different species.

Weaknesses:

(1) The evolutionary analyses the authors perform appear to indicate that IFIT1 genes in several mammalian groups have evolved under positive selection. However, IFIT1 has previously been shown to have undergone recurrent instances of recombination with the paralogous IFIT1B, which can confound positive selection analyses such as the ones the authors perform. The authors should analyze their alignments for evidence of recombination using a tool such as GARD (in the same HyPhy package along with MEME and FUBAR). Detection of recombination in these alignments would invalidate their positive selection inferences, in which case the authors need to either analyze individual non-recombining domains or limit the number of species to those that are not undergoing recombination. While it is likely that these analyses will still reveal a signature of positive selection, this step is necessary to ensure that the signatures of selection and sites of positive selection are accurate.

(2) The choice of IFIT1 homologs chosen for study needs to be described in more detail. Many mammalian species encode IFIT1 and IFIT1B proteins, which have been shown to have different antiviral specificity, and the evolutionary relationship between IFIT1 and IFIT1B paralogs is complicated by recombination. As such, the assertion that the proteins

studied in this manuscript are IFIT1 orthologs requires additional support than the percent identity plot shown in Figure 3B.

(3) Some of the results and discussion text could be more focused on the model of evolution-driven changes in IFIT1 specificity. In particular, the chimpanzee data are interesting, but it would appear that this protein has lost all antiviral function, rather than changing its antiviral specificity like some other examples in this paper. As such, the connection between the functional mapping of individual residues with the positive selection analysis is somewhat confusing. It would be more clear to discuss this as a natural loss of function of this IFIT1, which has occurred elsewhere repeatedly across the mammalian tree.

(4) In other places in the manuscript, the strength of the differences in antiviral specificity could be highlighted to a greater degree. Specifically, the text describes a number of interesting examples of differences in inhibition of VSV versus VEEV from Figure 3C and 3D, but it is difficult for a reader to assess this as most of the dots are unlabeled and the primary data are not uploaded. A few potential suggestions would be to have a table of each ortholog with % infection by VSV and % infection by VEEV. Another possibility would be to plot these data as an XY scatter plot. This would highlight any species that deviate from the expected linear relationship between the inhibition of these two viruses, which would provide a larger panel of interesting IFIT1 antiviral specificities than the smaller number of species shown in Figure 4.

We thank the reviewer for their fair assessment of our manuscript. As the reviewer requested, we performed GARD analysis on our alignments used for PAML, FUBAR, and MEME (New Supp Fig 1). By GARD, we found 1 or 2 predicted breakpoints in each clade. However, much of the sequence was after or between the predicted breakpoints. Therefore, we were able to reanalyze for sites undergoing positive selection in the large region of the sequence that do not span the breakpoints. We were able to validate almost all sites originally identified as undergoing positive selection still exhibit signatures of positive selection taking these breakpoints into account: primates (11/12), bats (14/16), ungulates (30/37), and carnivores (2/4). To further validate our positive selection analysis, we used Recombination Detection Program 4 (RDP4) to remove inferred recombinant sequences from the primate IFIT1 alignment and performed PAML, FUBAR, and MEME. Once again, the sites in our original analysis were largely validated by this method. Importantly, sites 170, 193, and 366 in primates, which are discussed in our manuscript, were found to be undergoing positive selection in 2 of the 3 analyses using alignments after the indicated breakpoint in GARD and after removal of recombinant sequences by RDP4. We have updated the text to acknowledge IFIT1/IFIT1B recombination more clearly and include the GARD analysis as well as PAML, FUBAR, and MEME reanalysis taking into account predicted breakpoints by GARD and RDP4. Furthermore, to increase evidence that the sequences used in this study for both computational and functional analysis are IFIT1 orthologs rather than IFIT1B, we have included a maximum likelihood tree after aligning coding sequences on the C-terminal end (corresponding to bases 907-1437 of IFIT1). In Daughterty et al. 2016 (PMID: 27240734) this strategy was used to distinguish between IFIT1 and IFITB. All sequences used in our study grouped with IFIT1 sequences (including many confirmed IFIT1 sequences used in Daughterty et al.) rather than IFIT1B sequences or IFIT3. This new data, including the GARD, RDP4, and maximum likelihood tree is included as a new Supplementary Figure 1.

We also agree with the reviewer that it is possible that chimpanzee IFIT1 has lost antiviral function due to the residues 364 and 366 that differ from human IFIT1. We have updated the discussion sections to include the possibility that chimpanzee IFIT1 is an example of a natural loss of function that has occurred in other species over evolution as well as the potential consequences of this occurrence. Regarding highlighting the strength of differences in antiviral activity between IFIT1 orthologs, we have included several updates to strengthen

the ability of the reader to assess these differences. First, we have included a supplementary table that includes the infection data for each ortholog from the VEEV and VSV screen to allow for readers to evaluate ranked antiviral activity of the species that suppress these viruses. In addition, the silhouettes next to the dot plots indicate the top ranked hits in order of viral inhibition (with the top being the most inhibitory) giving the reader a visual representation in the figure of top antiviral orthologs during our screen. We have also updated the figure legend to inform the reader of this information.

Reviewer #3 (Public Review):

Summary:

This manuscript by McDougal et al, demonstrates species-specific activities of diverse IFIT1 orthologs and seeks to utilize evolutionary analysis to identify key amino acids under positive selection that contribute to the antiviral activity of this host factor. While the authors identify amino acid residues as important for the antiviral activity of some orthologs and propose a possible mechanism by which these residues may function, the significance or applicability of these findings to other orthologs is unclear. However, the subject matter is of interest to the field, and these findings could be significantly strengthened with additional data.

Strengths:

Assessment of multiple IFIT1 orthologs shows the wide variety of antiviral activity of IFIT1, and identification of residues outside of the known RNA binding pocket in the protein suggests additional novel mechanisms that may regulate IFIT1 activity.

Weaknesses:

Consideration of alternative hypotheses that might explain the variable and seemingly inconsistent antiviral activity of IFIT1 orthologs was not really considered. For example, studies show that IFIT1 activity may be regulated by interaction with other IFIT proteins but was not assessed in this study.

Given that there appears to be very little overlap observed in orthologs that inhibited the viruses tested, it's possible that other amino acids may be key drivers of antiviral activity in these other orthologs. Thus, it's difficult to conclude whether the findings that residues 362/4/6 are important for IFIT1 activity can be broadly applied to other orthologs, or whether these are unique to human and chimpanzee IFIT1. Similarly, while the hypothesis that these residues impact IFIT1 activity in an allosteric manner is an attractive one, there is no data to support this.

We thank the reviewer for their fair assessment of our manuscript. To address the weaknesses that the reviewer has pointed out we have expanded the discussion to more directly address alternate hypotheses, such as the possibility of IFIT1 activity being regulated by interaction with other IFIT proteins. Furthermore, we expanded the discussion to include an alternate hypothesis for the role of residues 364 and 366 in primate IFIT1 besides allosteric regulation. In addition, we did not intend to claim or imply that residues 364/6 are the key drivers of antiviral activity for all IFITs tested. However, we speculate that within primates these residues may play a key role as these residues differ between chimpanzee IFIT1 (which lacks significant antiviral activity towards the viruses tested in this study) and human IFIT1 (which possesses significant antiviral activity). In addition, these residues seem to be generally conserved in primate species, apart from chimpanzee IFIT1. We have included changes to the text to more clearly indicate that we highlight the importance of these residues specifically for primate IFIT1, but not necessarily for all IFIT1 proteins in all clades.

Reviewer #1 (Recommendations for the authors):

(1) The readers would benefit from a more detailed background on the concept and estimation of positive selection for the readers, including the M7/8 models in PAML.

We have included more information in the text to provide a better background for the concepts of positive selection and how PAML tests for this using M7 and M8 models.

(2) Presentation of data

a) Figure 3C and 3D: is there a better way to present the infection data so the readers can tell the ranked antiviral activity of the species that suppress VEEV?

We have included a supplementary table that includes the infection data for each ortholog from the VEEV and VSV screen to allow for readers to evaluate ranked antiviral activity of the species that suppress these viruses. In addition, the silhouettes next to the dot plots indicate the top ranked hits in order of viral inhibition (with the top being the most inhibitory). We have updated the figure legend to inform the reader of this information as well.

b) Figure 4C and 4D: consider putting the western blot in Supplementary Figure 1 underneath the infection data or with the heatmap so readers can compare it with the antiviral activity.

We have also included quantification of the western blots performed to evaluate IFIT1 expression during the experiments shown in Figure 4C and 4D in an updated Figure 4B. We have also included normalized expression values with the heatmap shown in an updated Figure 4G so the reader can evaluate potential impact of protein expression on antiviral activity for all infection experiments shown in figure 4.

(3) Line 269-270: as a rationale for narrowing the species to human, black flying fox, and chimp IFIT1, human and black flying fox were chosen because they strongly inhibit VEEV, but pangolin wasn't included even though it had the strongest anti-VEEV activity?

The rationale for narrowing the species to human, black flying fox, and chimpanzee IFIT1 was related to the availability of biological tools, high quality genome/transcriptome sequencing databases, and other factors. Specifically human and chimp IFIT1 are closely related but have variable antiviral activities, making their comparison highly relevant. Bats are well established as reservoirs for diverse viruses, whereas the reservoir status of many other mammals is less well defined. Furthermore, purifying large amounts of high quality IFIT1 protein after bacterial expression was another limitation to functional studies. We have added this information into the manuscript text.

(4) Figure 5A: to strengthen the claim that "species-specific antiviral activities of IFIT1s can be partly explained by RNA binding potential", it would be good to include one more positive and one more negative control. In other words, test the cap0 RNA binding activity of an IFIT1 ortholog that strongly inhibits VEEV and an ortholog that does not. It would also be good to discuss why chimp IFIT1 still shows dose-dependent RNA binding yet it is one of the weakest at inhibiting VEEV.

We appreciate the reviewer's suggestion to include more controls and expand the dataset. While we understand the potential value of expanding the dataset, we believe that human IFIT1 serves as a robust positive control and human IFIT1 R187 (RNA-binding deficient) serves as an established negative control. Future experiments with other purified IFITs from

other species will indeed strengthen evidence linking IFIT1 species-specific activity and RNA-binding.

Regarding chimpanzee IFIT1, we acknowledge there appears to be some dose-dependent Cap0 RNA-binding. However, the binding affinity is much weaker than that of human or black flying fox IFIT1. We speculate that during viral infection reduced binding affinity could impair the ability of chimpanzee IFIT1 to efficiently sequester viral RNA and inhibit viral translation. This reduction in binding affinity may, therefore, allow the cell to be overwhelmed by the exponential increase in viral RNA during replication resulting in an ineffective antiviral IFIT1. In the literature, a similar phenomenon is observed by Hyde et. al (PMID: 24482115). In this study, the authors test mouse Ifit1 Cap0 RNA binding by EMSA of the 5' UTR sequence of VEEV RNA containing an A or G at nucleotide position 3. EMSA shows binding of both the A3 and G3 Cap0 VEEV RNA sequences, however stronger Ifit1 binding is observed for A3 Cap0 RNA sequence. The consequences of the reduced Ifit1 binding of the G3 Cap0 VEEV RNA are observed in vitro by a substantial increase in viral titers produced from cells as well as an increase in protein produced in a luciferase-based translation assay. The authors also show in vivo relevance of this reduction of Ifit1 binding as WT B6 mice infected with VEEV containing the A3 UTR exhibited 100% survival, while WT B6 mice infected with VEEV containing the G3 UTR survived at a rate of only ~25%. Therefore, the literature supports that a decrease in Cap0 RNA binding by an IFIT protein (while still exhibiting Cap0 RNA binding) observed by EMSA can result in considerable alterations of viral infection both in vitro and in vivo.

Minor:

(1) Line 82: "including 5' triphosphate (5'-ppp-RNA), or viral RNAs..." having a comma here will make the sentence clearer.

We have improved the clarity of this sentence. It now reads, "IFIT1 binds uncapped 5'triphosphate RNA (5'-ppp-RNA) and capped but unmethylated RNA (Cap0, an m⁷G cap lacking 2'-O methylation)."

(2) Line 100: "...similar mechanisms have been at least partially evolutionarily conserved in IFIT proteins to restrict viral infection by IFIT proteins".

We have updated the text to improve clarity by revising the sentence to "VEEV TC-83 is sensitive to human IFIT1 and mouse Ifit1B, indicating at least partial conservation of antiviral function by IFIT proteins."

(3) Line 109: "signatures of rapid evolution or positive selection" would put positive selection second because that is the more technical term that can benefit from the more layperson term (rapid evolution).

We have updated this sentence incorporating this suggestion. "Positive selection, or rapid evolution, is denoted by a high ratio of nonsynonymous to synonymous substitutions (dN/dS >1)."

(4) Lines 116-117: "However, this was only assessed in a few species" would benefit from a citation.

We have inserted the citation.

(5) Line 127 heading: "IFIT1 is rapidly evolving in mammals" would be more accurate to say "in major clades of mammals".

We have updated the text to include this suggestion.

| (6) Line 165: *"IFIT1 L193 mutants"*.

We have updated the text to rephrase this for clarity.

| (7) Line 170: *two strains of VEEV were mentioned in the Intro, so it would be good to specify which strain of VEEV was used?*

We have updated the text to clarify the VEEV strain. In this study, all experiments were performed using the VEEV TC-83 strain.

| (8) Line 174: *"Indeed, all mutants at position 193, whether hydrophobic or positively charged, inhibited VEEV similarly to the WT..." It should read "all hydrophobic and positively charged mutants inhibited VEEV similarly to the WT..."*.

We corrected as suggested.

| (9) Line 204: *what are "control cells"? Cells that are mock-infected, or cells without IFIT1?*

We have updated the text to improve clarity. What we refer to as control cells, were cells expressing an empty vector control rather than an IFIT1.

| (10) *Need to clarify n=2 and n=3 replicates throughout the manuscript. Does that refer to three independent experiments? Or an experiment with triplicate wells/samples?*

We have updated the text to say "independent experiments" instead of "biological replicates" to prevent any confusion. All n=2 or n=3 replicates denote independent experiments.

| (11) Line 254: *"dominant antiviral effector against the related human parainfluenza virus type 5..."*

We have updated the text to improve clarity.

| (12) Line 271: *"The black flying fox (Pteropus alecto), is a model megabat species..." scientific name was italicized here but not elsewhere. Remove comma.*

We have updated the text accordingly.

| (13) Line 293: *"...chimpanzee IFIT1 lacked these properties" butchimp IFIT1 can bind cap0 RNA, just at a lower level.*

We have updated the text to acknowledge that chimpanzee IFIT1 can bind cap0 RNA, albeit at a lower level than human IFIT1.

| (14) Figure 6B: *please fix the x-axis labels. They're very cramped.*

We have updated the x-axis labels for figure 6B and figure 6D to improve clarity.

| (15) Line 609: *"...trimmed and aligned"?*

Our phrasing is to indicate that coding sequences were aligned, and gaps were removed to reduce the chance of false positive signal by underrepresented codons such as gaps or short

insertions. We have removed “trimmed” from the text and changed the text to say “aligned sequences” to increase clarity.

Reviewer #2 (Recommendations for the authors):

(1) Numbers less than 10 should be spelled out throughout the manuscript (e.g. line 138).

We have updated the text to reflect the request.

(2) Line 165: "expression of IFIT1 193 mutants" should be rephrased.

We have updated the text to rephrase this sentence for clarity.

(3) A supplemental table or file should be included that contains the accession number and species names of sequences used for evolutionary analyses and for functional testing. In addition, the alignments that were used for positive selection can be included.

We have included a supplemental file containing accession numbers, species names for evolutionary analysis and functional studies. In addition, this table includes the infection data for each IFIT1 homolog for the screen performed in figure 3.

(4) The discussion of potential functions of the C-terminus of IFIT1 should include possible interactions with other proteins. In particular, the C-terminus of IFIT1 has been shown to interact with IFIT3 in a way that modulates its activity (PMID: 29525521). Although residues 362-366 were not shown in that paper to interact with a fragment of IFIT3, it is possible that these residues may be important for interaction with full-length IFIT3 or some other IFIT1 binding partner.

We thank the reviewer for their suggestion. We have expanded the discussion to explore the possibility that residues 364 and 366 of IFIT1 may be involved in IFIT1-IFIT3 interactions and consequently Cap0 RNA-binding and antiviral activity.

(5) The quantification of the EMSAs should be described in more detail. In particular, from looking at the images shown in Figure 5A, it would appear that human and chimpanzee IFIT1 show similar degrees of probe shift, while the human R187H panel shows no shifting at all. However, the quantification shows chimpanzee IFIT1 as being statistically indistinguishable from human R187H. Additional information on how bands were quantified and whether they were normalized to unshifted RNA would be helpful in attempting to resolve this visual discordance.

EMSAs were quantified by determining Adj. Vol. Intensity in ImageLab (BioRad), which subtracts background signal, after imaging at the same exposure and SYBR Gold staining time. To determine Adj. Vol. Intensity, we drew a box (same size for each gel and lane for each replicate) for each lane above the free probe. These values were not normalized to unshifted RNA, however equal RNA was loaded. While the ANOVA shows no significant difference, between human R187H and chimpanzee IFIT1 band shift intensity, this is potentially due to the between group variance in the ANOVA. The increase in the AUC value for chimpanzee IFIT1 is 36.4% higher than R187H.

The AUC of Adj. Vol. Intensity of human IFIT1 band shift is roughly 2-fold more than that of chimpanzee IFIT1. We believe this matches with the visual representation as well, as human IFIT1 has a darker “upper” band in the shift, as well as a clear dark “lower” band that is not well defined in the chimpanzee shift. Furthermore, the upper band of the chimpanzee IFIT1 shift appears to be as intense in the 400nM as the upper band in the 240nM human IFIT1 lane, without taking into account the lower band seen for human IFIT1 as well. We included

this quantification as K_D was unable to be calculated due to no clear probe disappearance and we do not intend for this quantification to act as a substitute for binding affinity calculations, rather to aid the reader in data interpretation.

Reviewer #3 (Recommendations for the authors):

(1) IFIT1 has been demonstrated to function in conjunction with other IFIT proteins, do you think the absence of antiviral activity is due to isolated expression of IFIT1 without these cofactors, and therefore might explain why there was little overlap observed in orthologs that inhibited the viruses tested (Figure 3, lines 209-210).

We do not believe that isolated expression of IFIT1 without cofactors (such as orthologous IFIT proteins) would fully explain the disparities in antiviral activity as many IFIT1s that expressed inhibited either VSV or VEEV in our screen. However, we acknowledge that the expression of IFIT1 alone does create a limitation in our study as IFIT1 antiviral activity and RNA-binding can be modulated by interactions with other IFIT proteins. Therefore, we do believe that it is possible that co-expression of IFIT1 with other IFITs from a given species might potentially enhance antiviral activity. Future studies may shed light on this.

(2) Figure 5 - Calculating the K_D for each protein would be more informative. How does the binding affinity of these IFIT1 proteins compare to that which has previously been reported?

We are unable to accurately determine K_D as there is not substantial diminished signal of the free probe. Therefore, we are only able to compare IFIT1 protein binding between species without accurate mathematical calculation of binding affinity. Our result does appear similar to that of mouse Ifit1 binding to VEEV RNA (PMID: 24482115), in which the authors also do not calculate a K_D for their RNA EMSA.

(3) Mutants 364 and 366 may not have direct contact with RNA, but RNA EMSA data presented suggest that the binding affinity may be different (though this is hard to conclude without K_D data). Additional biochemical data with these mutants might provide more insight here.

We agree that further studies using 364 and 366 double mutant human and chimpanzee protein in EMSAs would provide additional biochemical data and provide insight into the role of these residues in direct RNA binding. We acknowledge this is a limitation of our study as we provide only genetic data demonstrating the importance of these residues.

(4) Given that there appears to be very little overlap observed in orthologs that inhibited the viruses tested, it's possible that other amino acids may be key drivers of antiviral activity in these other orthologs. Thus, it's difficult to conclude whether the findings that residues 362/4/6 are important for IFIT1 activity can be broadly applied to other orthologs. A more systematic assessment of the role of these mutations across multiple diverse orthologs would provide more insight here. Do other antiviral proteins show this trend (ie exhibit little overlap in orthologs that inhibit these viruses). What do you think might be driving this?

We agree that other residues outside of 364 and 366 may be key drivers of antiviral activity across the IFIT1 orthologs tested. We do not hypothesize that this will broadly apply across IFIT1 from diverse clades of mammals as overall amino acid identity can differ by over 30%. However, based on the chimpanzee and human IFIT1 data, as well as sequence alignment within primates specifically, we believe these residues may be key for primate (but not necessarily other clades of mammals) IFIT1 antiviral activity.

Regarding if other antiviral proteins show little overlap in orthologs that inhibit a given virus, to our knowledge such a functional study with this large and divergent dataset of orthologs has not been performed. However, there are many examples of restriction factors exhibiting species-specific antiviral activity when ortholog screens have been performed. For example, HIV was reported to be suppressed by MX2 orthologs from human, rhesus macaque, and African green monkey, but not sheep or dog MX2 (PMID: 24760893). In addition, foamy virus was inhibited by the human and rhesus macaque orthologs of PHF11, but not the mouse and feline orthologs (PMID: 32678836). Furthermore, studies from our lab have shown variability in RTP4 ortholog antiviral activity inhibition towards viruses such as hepatitis C virus (HCV), West Nile virus (WNV), and Zika virus (ZIKV) (PMID: 33113352).

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