Corrections

GENETICS

The authors note that, within the supporting information Web link “http://intron.ccam.uchc.edu/Graveley/Publications/Publications.html” should be removed. Tables S1–S6 have been added to the online publication. The online version has been corrected.

www.pnas.org/cgi/doi/10.1073/pnas.1304972110

MEDICAL SCIENCES

The authors note that the following statement should be added as a new Acknowledgments section: “This work was supported by National Institute of General Medical Sciences Grant GM073626 (to D.S.K.).”

www.pnas.org/cgi/doi/10.1073/pnas.1306394110

IMMUNOLOGY

The authors note that on page 5917, right column, second full paragraph, line 12 “5′-GTG TAT GGA GGA AAA CCC TAT TTC TTA ACT-3′” should instead appear as “5′-GTG TAT GGA GGA AAA CCC TAT TCT TAA CT-3′”.

www.pnas.org/cgi/doi/10.1073/pnas.1306250110

BIOCHEMISTRY, ENVIRONMENTAL SCIENCES

The authors note that Table 1 appeared incorrectly. Within the Name column, “CARP8” should instead appear as “CARP4,” and “CARP9” should instead appear as “CARP5.” These errors do not affect the conclusions of the article.

www.pnas.org/cgi/doi/10.1073/pnas.1305081110
Table 1. Thirty-six predicted proteins in *S. pistillata* SOM samples detected by LC-MS/MS and their bioinformatics analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene</th>
<th>Accession no.</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>g11108</td>
<td>KC509948</td>
<td>Protocadherin fat-like</td>
</tr>
<tr>
<td>P2</td>
<td>g11187</td>
<td>KC436347</td>
<td>CARP4</td>
</tr>
<tr>
<td>P3</td>
<td>g12510</td>
<td>KC342189</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>P4</td>
<td>g9651</td>
<td>KC342190</td>
<td>Viral inclusion protein</td>
</tr>
<tr>
<td>P5</td>
<td>g1674</td>
<td>KC150884</td>
<td>Hemicentin</td>
</tr>
<tr>
<td>P6</td>
<td>g1666</td>
<td>KC149520</td>
<td>Actin</td>
</tr>
<tr>
<td>P7</td>
<td>g1601</td>
<td>KC342191</td>
<td>Actin</td>
</tr>
<tr>
<td>P8</td>
<td>g654</td>
<td>KC342192</td>
<td>Major yolk protein</td>
</tr>
<tr>
<td>P9</td>
<td>g10811</td>
<td>KC000002</td>
<td>Protocadherin fat-like</td>
</tr>
<tr>
<td>P10</td>
<td>g11107</td>
<td>KC509447</td>
<td>Cadherin</td>
</tr>
<tr>
<td>P11</td>
<td>g3727</td>
<td>KC342193</td>
<td>Actin</td>
</tr>
<tr>
<td>P12</td>
<td>g2385</td>
<td>JX891654</td>
<td>——</td>
</tr>
<tr>
<td>P13</td>
<td>g6918</td>
<td>KC342194</td>
<td>Sushi domain-containing</td>
</tr>
<tr>
<td>P14</td>
<td>g951</td>
<td>KC342195</td>
<td>Collagen - alpha</td>
</tr>
<tr>
<td>P15</td>
<td>g1532</td>
<td>KC43648</td>
<td>CARP5</td>
</tr>
<tr>
<td>P16</td>
<td>g1702</td>
<td>KC342196</td>
<td>——</td>
</tr>
<tr>
<td>P17</td>
<td>g2472</td>
<td>KC149521</td>
<td>Glyceraldehyde 3-phosphatase dehydrogenase</td>
</tr>
<tr>
<td>P18</td>
<td>g810</td>
<td>KC342197</td>
<td>Collagen - alpha</td>
</tr>
<tr>
<td>P19</td>
<td>g2041</td>
<td>KC342198</td>
<td>Contactin-associated protein</td>
</tr>
<tr>
<td>P20</td>
<td>g6066</td>
<td>KC342199</td>
<td>MAM domain anchor protein</td>
</tr>
<tr>
<td>P21</td>
<td>g18277</td>
<td>KC479163</td>
<td>Zona pellucida</td>
</tr>
<tr>
<td>P22</td>
<td>g19762</td>
<td>KC493649</td>
<td>——</td>
</tr>
<tr>
<td>P23</td>
<td>g1057</td>
<td>KC000004</td>
<td>Protocadherin</td>
</tr>
<tr>
<td>P24</td>
<td>g5888</td>
<td>KC479164</td>
<td>Vitellogenin</td>
</tr>
<tr>
<td>P25</td>
<td>g1220</td>
<td>KC479165</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>P26</td>
<td>g441</td>
<td>KC479166</td>
<td>Vitellogenin</td>
</tr>
<tr>
<td>P27</td>
<td>g18472</td>
<td>KC479167</td>
<td>Integrin - alpha</td>
</tr>
<tr>
<td>P28</td>
<td>g1651</td>
<td>KC149519</td>
<td>Late embryogenesis protein</td>
</tr>
<tr>
<td>P29</td>
<td>g3777</td>
<td>KC479168</td>
<td>Tubulin - beta</td>
</tr>
<tr>
<td>P30</td>
<td>g1056</td>
<td>KC000003</td>
<td>Myosin regulatory light chain</td>
</tr>
<tr>
<td>P31</td>
<td>g2020</td>
<td>KC479169</td>
<td>Neurexin</td>
</tr>
<tr>
<td>P32</td>
<td>g5540</td>
<td>KC479170</td>
<td>Kielin/chordin like</td>
</tr>
<tr>
<td>P33</td>
<td>g8985</td>
<td>KC479171</td>
<td>Flagellar associated protein</td>
</tr>
<tr>
<td>P34</td>
<td>g1714</td>
<td>KC479172</td>
<td>MAM/IDL receptor domain</td>
</tr>
<tr>
<td>P35</td>
<td>g7349</td>
<td>EU532164.1</td>
<td>Carbonic anhydrase (STPCA2)</td>
</tr>
<tr>
<td>P36</td>
<td>g3890</td>
<td>KC479173</td>
<td>Zonadhesion-like precursor</td>
</tr>
</tbody>
</table>

Returned sequences with e-values ≤ 10^{-10} are presented in order of decreasing e-value. “Protein name” is the best BLAST hit in NCBI. “Gene” is the code number in our *S. pistillata* gene prediction model. The “+” and “-” represent presence and absence, respectively, of similar sequences in comparison species.

*Sequence similarity is greater than 70%.
†Most similar sequence by bit score.
‡Indicates export signal.
Association of RIG-I with innate immunity of ducks to influenza

Megan R. W. Barber, Jerry R. Aldridge, Jr., Robert G. Webster, and Katherine E. Magor

Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada; and Division of Virology, Department of Infectious Diseases, St. Jude Children’s Research Hospital, Memphis, TN 38105

Contributed by Robert G. Webster, February 16, 2010 (sent for review December 21, 2009)

Ducks and wild waterfowl perpetuate all strains of influenza viruses in nature. In their natural host, influenza viruses typically cause asymptomatic infection and little pathology. Ducks are often resistant to influenza viruses capable of killing chickens. Here, we show that the influenza virus sensor, RIG-I, is present in ducks and plays a role in clearing an influenza infection. We show evidence suggesting that RIG-I may be absent in chickens, providing a plausible explanation for their increased susceptibility to influenza viruses compared with ducks. RIG-I detects RNA ligands derived from uncapped viral transcripts and initiates the IFN response. In this study, we show that the chicken embryonic fibroblast cell line, DF-1, cannot respond to a RIG-I ligand. However, transfection of duck RIG-I into DF-1 cells rescues the detection of ligand and induces IFN-β promoter activity. Additionally, DF-1 cells expressing duck RIG-I have an augmented IFN response resulting in decreased influenza replication after challenge with either low or highly pathogenic avian influenza virus. Implicating RIG-I in the antiviral response to an infection in vivo, we found that RIG-I expression is induced 200 fold, early in an innate immune response in ducks challenged with the H5N1 virus A/Vietnam/1203/04. Finding this natural disease resistance gene in ducks opens the possibility of increasing influenza resistance through creation of a transgenic chicken.


The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU363349 and GU36432).

Association of RIG-I with innate immunity of ducks to influenza

All strains of influenza A virus are perpetuated in the duck reservoir (1). Although rare, when influenza viruses cross over from the avian reservoir into humans they may evolve into pandemic strains. After emerging in 1996, the highly pathogenic avian influenza (HPAI) H5N1 viruses have evolved into multiple clades and subclades and have spread to Europe and Africa, probably by wild birds (2). Although H5N1 viruses do not frequently infect humans they are often highly pathogenic and cause death in otherwise healthy individuals. Among 445 laboratory-confirmed human H5N1 infections, the mortality rate is 60% (3).

In 2002, H5N1 viruses began killing wild waterfowl (4, 5), including ducks, which is highly unusual and a disruption of the normal ecology (1). Typically, ducks do not show signs of disease upon infection with influenza. H5N1 viruses may rapidly adapt, becoming less pathogenic to ducks (6, 7), suggesting that virus evolution helps maintain the balance between virus and host. Many strains of highly pathogenic H5N1 now cause asymptomatic infection in ducks, making them a “Trojan horse” in the spread of these influenza viruses (6–9). In contrast, many of these same H5N1 viruses cause 100% mortality in chickens within hours or days. We therefore reasoned that superior innate immunity might protect the duck during this critical period.

The molecular basis of the natural resistance of ducks to influenza infection is unresolved. A successful innate immune response to influenza infection involves a robust, yet transient induction of IFN-stimulated antiviral genes. RIG-I is a cytoplasmic RNA sensor (10), and triggering by influenza virus leads to production of IFN-β and expression of downstream IFN-stimulated antiviral genes (11). A hallmark of lethal influenza virus infection is interference with expression of RIG-I and downstream genes (12). Further, RNA viruses have been shown to be more virulent and replicate to higher levels in mice lacking RIG-I (13). Considering this, we speculated that the outcome of influenza infection in birds may also be determined by RIG-I and the downstream subset of IFN-stimulated genes. Here we show that the duck, the natural host of influenza, has an intact and functional RIG-I that is induced upon infection with H5N1 A/Vietnam/1203/04 (VN1203), whereas chickens appear to lack RIG-I. However, we demonstrate that transfection of duck RIG-I into a chicken embryonic fibroblast cell line can restore recognition of RIG-I ligand and initiate an antiviral IFN response.

Results

RIG-I Is Present in Ducks and Apparently Absent in Chickens. Given its role in antiviral defense in mammals, we searched for avian homologues of RIG-I. We identified a duck (Anas platyrhynchos) RIG-I homologue with 53% amino acid identity to human and 78% identity to zebra finch RIG-I (Fig. 1). Remarkably, we are unable to identify a chicken homologue of RIG-I, even though we used a variety of approaches to identify one. Searches of the chicken (Gallus gallus) genome (14) with the duck or finch RIG-I sequence do not reveal a match. In addition, a phylogenetic analysis of RIG-like receptors also noted that RIG-I was absent in chickens (15). However, the related melanoma differentiation associated gene-5 (MDA5) is present in the chicken genome (16). RIG-I and MDA5 initiate signaling cascades that converge on the same pathway at IPS-1 and lead to induction of IFN-β and expression of downstream IFN-stimulated antiviral genes (Fig. S1). MDA5 is a detector of long double-stranded RNA, polyinosinic–polycytidylic acid [poly (I:C)], and picornaviruses (13). To ensure that the helicase we isolated was RIG-I and distinct from MDA5, we amplified a large fragment of duck MDA5. The fragment of MDA5 shared 91% amino acid identity with chicken MDA5 (Fig. S2), yet only 33% identity with duck RIG-I.

We identified the RIG-I syntenic region on the chicken Z chromosome where acontinase I is encoded; this is a gene flanking the mammalian RIG-I homologue. A local BLAST search of the adjacent 4 Mb reveals no match to RIG-I, although there are sequence ambiguities in this region. Confirming the syntenic region is conserved in other birds, we identified a RIG-I homologue in the recently released draft genome for zebra finch (Tae-niopterygia guttata) on chromosome Z and flanked by acontinase I. A search of the finch expressed sequence tag (EST) database revealed two RIG-I transcripts among the 92,000 sequences. In contrast, no RIG-I sequences are present among the 600,000 ESTs.

www.pnas.org/cgi/doi/10.1073/pnas.1001755107

PNAS March 30, 2010 vol. 107 no. 13 5913–5918


The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU363349 and GU36432).

This article contains supporting information online at www.pnas.org/cgi/content/full/1001755107/DCSupplemental.
from chicken. Notably, \textit{MDA5} transcripts are present. Thus, \textit{RIG-I} appears to be absent from the chicken genome sequence derived from the Red Jungle Fowl, which resembles the ancestral chicken, as well as the modern chicken lines represented in the EST sequences. Combined, these data suggest that chickens may have lost \textit{RIG-I} before their domestication.

Avian \textit{RIG-I} proteins have features in common with mammalian \textit{RIG-I}. Duck \textit{RIG-I} is 933 amino acids, and zebra finch \textit{RIG-I} is 927 amino acids. Domain prediction reveals the expected tandem N-terminal \textit{CARD} domains, a helicase domain and a \textit{DeXD/H box} helicase domain, consistent with the mammalian structure (10, 17). \textit{RIG-I} is a ligand-dependent ATPase, and the Walker A ATP-binding motif is conserved. The hydrophobic core and the four lysine residues implicated in ligand-binding, K858/861/888/907 (17), are completely conserved within the C-terminal regulatory domain. However, residues T55 and K172, critical for interaction and polyubiquitination by \textit{TRIM25} needed for \textit{IPS-1} binding and signal induction (18, 19), are not conserved, suggesting this pathway does not function or involves different residues in birds.

To provide further evidence for the absence of \textit{RIG-I} in chickens, we hybridized a Southern blot of genomic DNA from ducks and chickens with a probe amplified from the helicase region of duck \textit{RIG-I} (Fig. 2A). Although we observed a polymorphic pattern of hybridization to duck DNA, we did not detect cross-hybridization to chicken DNA. However, the duck probe cross-hybridizes with \textit{RIG-I} from the more phylogenetically distant pigeon (Fig. 2B), providing evidence that the probe can recognize \textit{RIG-I} from other avian species. The duck \textit{RIG-I} probe hybridized to pigeon DNA in only the PstI digestion, although this may be because the NdeI and SpeI digestions produced hybridizing fragments of DNA too small to be visualized. Hybridization of our duck probe to pigeon DNA was observed on several other blots, whereas hybridization to chicken DNA was never observed. It is noteworthy that pigeons are remarkably resistant to influenza viruses, including HPAI H5N1 (20). In comparison, strong cross-hybridization of a duck \textit{MDA5} probe with DNA from pigeon and chicken (Fig. 2C) suggests it has diverged considerably less than \textit{RIG-I} and is presumably under less selective pressure in avian species. Demonstrating that our duck \textit{RIG-I} probe hybridizes to pigeon but not to chicken genomic DNA further supports the absence of \textit{RIG-I} in chickens. However, we cannot rule out that chicken \textit{RIG-I} has diverged to an extent that it is not detectable through bioinformatics or hybridization approaches, which may preclude function in any case.

\textbf{Duck \textit{RIG-I} Detects in Vitro Transcribed RNA and Activates the Chicken IFN-\textit{\beta} Promoter.} The apparent absence of \textit{RIG-I} in chickens led us to investigate whether the DF-1 chicken cell line (chicken embryonic fibroblasts) can respond to a \textit{RIG-I} ligand. \textit{RIG-I} signaling is activated by 5\textsuperscript{′} triphosphate RNA (5\textsuperscript{′} ppp RNA) containing short regions with double-stranded conformation, such as that derived from viral RNA with panhandle structures (21, 22), and from in vitro transcribed products (23). With this in mind, we challenged...
DF-1 cells respond to poly(I:C) mostly through MDA5, demonstrating that they possess the downstream components of the shared RIG-I/MDA5 pathway.

We next investigated whether duck RIG-I could confer recognition of a RIG-I ligand and signal through these downstream components. DF-1 cells were transfected with duck RIG-I before stimulation with 5′ppp RNA. DF-1 cells expressing duck RIG-I responded to 5′ppp RNA with a twofold induction of the IFN-β promoter compared with mock-transfected cells. However, phosphatase removal of the 5′ triphosphate abrogated the response, similar to the previous report in mammalian cells (23). Thus duck RIG-I is functional and can induce IFN-β promoter activity in the DF-1 chicken cell line. Although the twofold induction of the IFN-β promoter is modest, this is consistent with the up-regulation by poly (I:C) stimulation downstream of chicken MDA5. Given the estimated 90 My of divergence between chicken and duck (25), it is remarkable that the duck RIG-I even binds to chicken IPS-I to connect to downstream signaling components.

Transfected Duck RIG-I Detects Influenza and Induces an Antiviral Response in Chicken Cells. To determine if duck RIG-I could detect influenza virus and induce an antiviral response in the chicken DF-1 cells, we transfected the cells with duck RIG-I or vector only, followed by infection with influenza viruses. We chose H5N2 A/ma- lard/British Columbia/500/2005 (BC500), a low pathogenic avian influenza (LPAI) isolated from wild ducks that causes no pathology in its natural host and VN1203, an HPAI isolated from a fatal human infection and known to be lethal to ducks and chickens (7).

After a 15 h infection with BC500, there was increased expression of IFN-β as well as the antiviral IFN-stimulated genes Mx1 and PKR, known to be RIG-I responsive in mouse fibroblasts (11) (Fig. 3B). Although IFN-β and PKR were only slightly up-regulated, the IFN-stimulated gene Mx1 was induced approximately 30 fold. Influenza A matrix gene expression was significantly reduced in RIG-I-transfected DF-1 cells compared with vector-transfected control cells (Fig. 3B). Furthermore, the IFN response initiated by duck RIG-I resulted in significant reduction in viral titer for both BC500 (Fig. 3C) and VN1203 (Fig. 3D) infection, indicating that

![Image](Image)

**Fig. 2.** RIG-I is present in ducks and pigeons, but apparently absent in chickens. (A) Hybridization of a multiple exon duck RIG-I probe to HindIII and XbaI-digested genomic DNA from four White Pekin ducks and two White Leghorn chickens. (B) Hybridization of a single exon duck RIG-I probe to PstI, NdeI, and SacI digested genomic DNA from duck, chicken, and pigeon. (C) Hybridization of a single exon duck MDA5 probe to same blot.

**Fig. 3.** Duck RIG-I rescues detection of 5′ppp RNA and induces an antiviral response in DF-1 chicken embryonic fibroblast cells. (A) IFN-β promoter activity in RIG-I or empty vector–transfected DF-1 cells following 15 h of ligand stimulation compared to mock-treated cells, shown as mean fold induction (±SD). Results are representative of three independent experiments and were analyzed using a single-factor ANOVA and Tukey post hoc test (different letters, P < 0.05). (B) RIG-I–transfected DF-1 cells respond to BC500 infection (MOI, 1) with increased expression of chicken IFN-β and the IFN-stimulated genes Mx1 and PKR, and decreased influenza matrix gene expression, relative to empty vector–transfected cells. RNA was extracted from cells for qRT-PCR 15 h PI, and fold difference in gene expression calculated for RIG-I and vector-only–transfected DF-1 cells. Results are representative of three independent experiments and error bars show RDn/MinMax at a 95% confidence level and represent SE (n = 3). (C and D) RIG-I–transfected DF-1 cells had significantly lower influenza virus titers compared with empty vector–transfected cells for BC500 (C) or VN1203 (D). Both infections were performed 24 h after transfection at an MOI of 1. After 15 h, titer was determined by plaque assay from triplicate wells and results were analyzed with the two-tailed Student’s t test (n = 3; P = 0.002).
duck RIG-I is capable of reducing influenza replication in chicken cells.

RIG-I Is Highly Up-Regulated in Ducks Infected with VN1203. To determine whether RIG-I contributes to the antiviral response to influenza infection in ducks, we measured the expression of RIG-I in 6-week-old White Pekin duck tissues following infections with BC500 (H5N2) or VN1203 (H5N1). Infection with VN1203 induced significant up-regulation of RIG-I gene expression in the infected lung (Fig. 4A). RIG-I expression was induced more than 200-fold by d 1 postinfection (PI), whereas by d 3 PI, RIG-I was only modestly expressed, suggesting the induction is early and transient (Fig. 4B). In comparison, infection with BC500 induced only slight up-regulation of RIG-I in lung tissue. Because LPAI gene expression in the RIG-I pathway prevents activation of IFN-β (9), embryonic fibroblasts from a RIG-I-KO mouse fail to induce IFN-β and a subset of genes involved in innate immunity following influenza infection (11). siRNA knockdown of RIG-I (33) or introduction of a dominant-negative RIG-I (34) has been demonstrated to significantly reduce the influenza-induced IFN-β production in human cell lines. Additionally, IFN-β-KO mice show reduced survival and enhanced influenza viral titers in the lung (35). Thus, IFN-β appears to be protective during an influenza infection and cannot be compensated for by IFN-α. Chicken embryonic fibroblasts infected with influenza produce IFN and inhibit an IFN-sensitive GFP-tagged vesicular stomatitis virus (27); however, this response is 80% IFN-α (36). Injection of primary chicken embryo fibroblasts with H5N1 strains induced low Mx1 and IFN-α gene expression, whereas IFN-β was not significantly induced (37). Similar to influenza infection, Newcastle Disease Virus, also detected by RIG-I, causes substantially more pathology in chickens than ducks. In accord with our findings, others have found that Newcastle Disease Virus infection does not induce IFN-β promoter activity in chicken embryonic fibroblasts (38), also consistent with lack of RIG-I.

Here we provide evidence for the antiviral function of RIG-I in ducks, and its apparent absence in chickens. RIG-I independent pathways, such as TLR7, also contribute to influenza detection and IFN production in chickens (39) and ducks (40). However, chickens lack a key regulator of the antiviral response, so it is not surprising that they suffer remarkable pathology from HPAI infection compared with ducks. Chickens lacking RIG-I would be without a first line of defense at the lung epithelial cell layer during influenza infection, and antiviral genes downstream of RIG-I may not be expressed (11). Considering that RIG-I may also inhibit influenza replication directly and independent of IFN (10), loss of this undefined pathway may also contribute to unchecked virus replication in chickens and development of influenza-induced pathology in ducks.

Discussion

This study establishes the presence of RIG-I in ducks—the natural reservoir of influenza viruses—but its apparent absence in chickens. This may reflect the differential susceptibility of ducks and chickens to influenza-induced pathology. We found that RIG-I is expressed during the innate immune response to influenza infection in ducks, providing evidence for the antiviral relevance of RIG-I in the natural host of the virus. RIG-I is highly up-regulated early in an innate immune response to VN1203 infection in ducks, likely contributing to antiviral defense against this potentially lethal virus. In contrast, RIG-I appears to be absent in chickens and a chicken embryonic fibroblast cell line fails to respond to 5′ppp RNA, a function that can be conferred to the cells by transfection with duck RIG-I. Expression of duck RIG-I augments the antiviral IFN response and reduces influenza replication in chicken cells.
replication in chickens. RIG-I loss-of-function mutants in humans (41, 42) may also be predicted to cause increased viral pathogenesis. The presence of a functional RIG-I in ducks eliciting an early antiviral response may contribute to survival of what otherwise may be a lethal influenza infection.

Materials and Methods

Identification and Cloning of Duck RIG-I. A PCR fragment of RIG-I was obtained from duck (A. platyrhynchos) splenic cDNA using primers 5′-GAT CCC ACC AAT GAG GAT AAT CAA CT-3′ and 5′-CAA TG TCA CGT CCC TCT CAG C-3′ based on a conserved region of the human RIG-I sequence. The complete cDNA sequence was obtained via 5′ and 3′ RACE using the SMART RACE cDNA Amplification Kit (Clontech). The complete coding region of duck RIG-I was amplified using the primers 5′-UTR S′-GGG GCA GGG GTA GGTATA CAG-3′ and 3′-UTR S′-GTG TAG GAG AAT AGA AGA TCT A-3′ using Phusion High-Fidelity DNA Polymerase (New England Biolabs), cloned into pcR 2.1-TOPO (Invitrogen) and completely sequenced.

Plasmids. pcDNA-RIG was obtained by cloning duck RIG-I into the mammalian expression vector pcDNA 3.1 hygro+ (Invitrogen). Duck RIG-I was digested out of pcR 2.1-TOPO (Invitrogen) using Spel and Notl. RIG-1 was then inserted between Nhel and Notl sites of pcDNA 3.1 hygro+ (Invitrogen). The chicken IFN-β promoter luciferase reporter (pGL3-chIFN-β′) was used as a positive control (Ambion). The promoter fragment was then inserted between Nhel and MluI sites of the pGL3-basic luciferase reporter vector (Promega).

In Vivo Transcription and RNAs. The 21-mer RNA, 5′-GGG GGC UCC CAC AGG CAC CAG TAT AGT GAT TTA TTT-3′ was used as a template and cloned into the mammalian expression vector, pRRLS-A (Invitrogen). The 21-mer RNA was obtained as a recombinant DNA oligonucleotides 5′-TAA TAC GAC TCA TTA GG-3′ and 5′-GGG AAC TCG AGC GGC CTC CCT AGT AGT GAC TGC TG-3′ containing T7 promoter. A 21-mer RNA was then transcribed using mT7, transcription buffer (Promega) at 30 °C for 3 h. The transcription was terminated with 20 μl of 10× Stop Mix (Promega). The complete 21-mer RNA was purified by phenol:chloroform extraction and precipitation. Poly (I:C) (25 μg/ml) was obtained from Invivogen.

Cell Culture, Infections, and Transfections. UMSAH/Df-1, a spontaneously immortalized chicken embryonic fibroblast cell line derived from East Lansing strain eggs (43), was maintained in DMEM plus 10% FBS. Cells (1.25 × 105) were seeded overnight in 24-well plates. Cells were cotransfected with 150 ng of pcDNA-RIG or empty pcDNA, 150 ng of pGL3-chIFN-β′, and 10 ng of the constitutive renilla luciferase reporter plasmid (Promega). The infection of transfected Df-1 cells was carried out in 96-well plates. Cells were transfected with 1 μg of pdRNA-RIG or empty pcDNA. Twenty-four hours after transfection, cells were infected at a multiplicity of infection (MOI) of 1 with H1N1 A/Avian/BC/500/05 or HSN1 A/Avian/12/Ohio/2004 virus. L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TAEK) treated (Promega) The complete coding region of duck RIG-I was amplified using the primers 5′-UTR S′-GGG GCA GGG GTA GGTATA CAG-3′ and 3′-UTR S′-GTG TAG GAG AAT AGA AGA TCT A-3′ using Phusion High-Fidelity DNA Polymerase (New England Biolabs), cloned into pcR 2.1-TOPO (Invitrogen) and completely sequenced.

Viruses and Duck Infections. The HSN1 A/Avian/12/Ohio/2004 HPAI was generated by reverse genetics (45) and HSN2 A/Avian/BC/500/05 LPAI was isolated by screening of environmental samples. The viruses were propagated in 10-d-old embryonated chicken eggs and handled at St. Jude Children’s Research Hospital, with VN1203 handled in biosafety level 3+ facilities approved by the United States Department of Agriculture and Centers for Disease Control and Prevention. Outbred White Pekin ducks (A. platyrhynchos) were purchased from Ideal Poultry or Metzer Farms, and all animal experiments were approved by the Animal Care and Use Committee of St. Jude Research Hospital and performed in compliance with relevant institutional policies, National Institutes of Health regulations, and the Animal Welfare Act. A total of 105 of 30% egg infectious dose of BC500 and VN1203 were used to inoculate 6-week-old mallards via the natural route, in naries, eyes, and trachea. Mock infection was PBS only. Ducks were killed and tissues collected at 1 and 3 PI (n = 3, except for BC500 d 3 samples, n = 2). Tracheal and cloacal swabs were collected to monitor viral shedding.

Southern Hybridization. Genomic DNA was extracted from blood of White Pekin ducks (A. platyrhynchos), White Leghorn chickens (G. gallus), and pigeon (Columba livia). Genomic DNA (10 μg) was digested to completion, separated on 0.8% agarose, and blotted to Nytran Supercharge (Schleicher & Schuell). DNA was immobilized by UV cross-linking and baking for 3 h at 80 °C. A multiple-exon 307-bp RIG-I probe in the helicase domain was amplified using the primers 5′-ACA GTG ATG ACC CTC CCA AGA CAG-3′ and 5′-CAG ATT TCT GGA TCA TCT AAG CAG-3′ from a defined duck RIG-I clone. A 171-bp probe predicted to contain a single exon was amplified from the same template using 5′-GTA TGA CCC TCA TCA GGA AGA AGG-3′ and 5′-CTC TGA CTT GGA TTT TGG TCA CG-3′. A 150-bp duck MDAS probe was amplified with the following primers: 5′-CAC AAG GAA GTC ATT GAT AAA TTC C-3′ and 5′-GGC CTG CCA CAT AGC AAT TTC-3′ from a duck MDAS clone. All probes were radiolabeled with 32P by random priming (Promega Random Primer Labeling Kit; Stratagene). Blots were hybridized overnight at 42 °C in 50% formamide, 5× Denhardt solution, 4× SSPE, 5% dextran sulfate, 1% SDS, and 100 μg/ml salmon sperm DNA. Washes were carried out at low stringency in 1× sodium chloridesodium phosphate/EDTA and 0.1% SDS at 52 °C. Film was exposed for 3 d.

Real-Time qRT-PCR. RNA was extracted using TRIzol (Invitrogen), followed by purification from the final aqueous phase using the RNeasy Mini Kit (Qiagen). First strand cDNA synthesis was performed using the SuperScript III kit (Invitrogen) using Oligo(dT) (Invitrogen) and random primers (Invitrogen). To quantify RIG-I gene expression from duck tissues, 50 ng of cDNA was amplified in a 10-μl reaction using the Applied Biosystems 7500 real-time PCR system (Applied Biosystems). Duck primers and probes for Mx1 were designed according to the touchdown primer and probe mixes were obtained from Applied Biosystems and used with FastStart Universal Probe Master Real (Roche Applied Science). Duck GAPDH was used as an endogenous control. Primer and probe sequences were as follows: duck RIG-I primers 5′-GTG TAT GGA GGA AAA CCC TAT TTC TTA ACT-3′ and 5′-GGAA GTG ATG TAC TTG TCT GAT-3′, and probes 5′-TCC GCC CCA TCA A-3′. Duck GAPDH primers were 5′-GCC TCT TGC ACC AACT-3′ and 5′-GGAT GAC GTG CAT AAC AGC-3′ and probe 5′-CACC AAT GGC AAA GGT-3′. Changes in gene expression were expressed as a ratio of the level observed in a mock-infected animal. RT-PCR was performed for RIG-I and GAPDH in a single-plex format, with the following cycling conditions: 95 °C for 10 min for activation, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Quadruplicate cycle threshold (Ct) values were analyzed with SDS software (Applied Biosystems) using the comparative Ct (ΔΔCt) method.

To quantify gene expression from transfected DF-1 cells, 20 ng of cDNA was amplified in a 10-μl reaction using the Applied Biosystems 7500 real-time PCR system (Applied Biosystems). Chicken 28S RNA (endogenous control) and IFN-β probes and primer (46) were obtained from Applied Biosystems and used with TaqMan Fast Universal PCR Master Mix (Applied Biosystems). For influenza A matrix gene expression, primers were designed according to CDC recommendations. The primers for Mx1 were designed and validated. Influenza forward primer was 5′-GAC CRA TGT CCA ACC GCT CCA C-3′; Influenza reverse was 5′-AGG GCA TTT TGG ACA AAK CTT CTA C-3′; and Influenza probe 5′-TGC AGT CCT GCC TCG ATG GGC AAG-3′. For influenza B matrix gene expression, primers were designed according to CDC recommendations. The primers for Mx1 were 5′-GTG TTT GAC TAT GGA GGT AGG AA-3′ and 5′-GGC TAT GAC TTT AAC TAT TTG GGC-3′ (UPL probe 80). The primers for PKR were 5′-TGT TGC TGT AGG GAA GCT ACT-3′ and 5′-TCA GTC AAG AAT AAA CCA CTT GGT-3′ (UPL probe 29). Cycling conditions were 95 °C for 20 s for activation, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Changes in gene expression were expressed as a ratio of the level observed with cells transfected with RIG-I or vector only.

ACKNOWLEDGMENTS. We thank Patrick Seiler for excellent technical assistance in BL3, John Pasick for the BC500 influenza isolate, Rick Wood-Sammon for provision of avian blood samples, Troy Locke and John Franks for qRT-PCR technical assistance, Savita Shrivastava for bioinformatics help, and Brad Magill for helpful comments on the research manuscript. This work was supported by the Canadian Institute for Health Research (K.E.M.) and Natural Sciences and Engineering Research Council of Canada (NSERC) (K.E.M.); Contract HHSN266200700005C from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services; and American Lebanese Syrian Associated Charities (R.G.W.). M.R.W.B. is supported by an NSERC Postgraduate Scholarship and a Canadian Poultry Research Council Postgraduate Scholarship Supplement.
Supporting Information

Barber et al. 10.1073/pnas.1001755107

Legend continued on following page
**Fig. S1.** RIG-I detection of influenza viral RNA triggers the IFN response in duck epithelial cells, whereas chickens apparently lack RIG-I. MDAS and RIG-I, pattern-recognition receptors, initiate signaling pathways that converge at the activation of the transcription factors IFN-regulatory factor 3 (IRF3), IRF7, and/or nuclear factor-κB; this leads to the expression of IFN-β. (A) Hypothetically, in duck epithelial cells, influenza infection stimulates IFN-β production and an antiviral program that reduces viral replication. (B) In chicken epithelial cells lacking RIG-I, there is a delayed or weak antiviral program and influenza rapidly replicates to cause a lethal infection. PPP, 5’ triphosphate.

**Fig. S2.** Duck and chicken MDAS are highly conserved. Alignment of partial duck MDAS (accession no. GU936632) and chicken MDAS (accession no. XM_422031) was performed using the ClustalW program and edited with Boxshade. Black shading indicates amino acid identity and gray shading indicates similarity (50% threshold). A partial duck MDAS clone was amplified using primers based on the chicken sequence: forward mda5 5′-AGT GGC AAA ACC AGA GTG GCT GTT TA-3′ and reverse mda5 5′-CAT CAG CTC GAG CTC GAC CC-3′.