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Duck *TRIM27-L* enhances MAVS signalling and is absent in chickens and turkeys

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Abstract

Wild waterfowl, including mallard ducks, are the natural reservoir of avian influenza A virus and they are resistant to strains that would cause fatal infection in chickens. Here we investigate potential involvement of TRIM proteins in the differential response of ducks and chickens to influenza. We examine a cluster of *TRIM* genes located on a single scaffold in the duck genome, which is a conserved synteny group with a TRIM cluster located in the extended MHC region in chickens and turkeys. We note a *TRIM27-like* gene is present in ducks, and absent in chickens and turkeys. Orthologous genes are predicted in many birds and reptiles, suggesting the gene has been lost in chickens and turkeys. Using quantitative real-time PCR (qPCR) we show that *TRIM27-L*, and the related *TRIM27.1*, are upregulated 5- and 9-fold at 1 day post-infection with highly pathogenic A/Vietnam/1203/2004. To assess whether TRIM27.1 or TRIM27-L are involved in modulation of antiviral gene expression, we overexpressed them in DF1 chicken cells, and neither show any direct effect on innate immune gene expression. However, when co-transfected with duck RIG-I-N (d2CARD) to constitutively activate the MAVS pathway, TRIM27.1 weakly decreases, while TRIM27-L strongly activates innate immune signalling leading to increased transcription of antiviral genes *MX1* and *IFN-β*. Furthermore, when both are co-expressed, the activation of the MAVS signalling pathway by TRIM27-L over-rides the inhibition by TRIM27.1. Thus ducks have an activating TRIM27-L to augment MAVS signalling following RIG-I detection, while chickens lack both TRIM27-L and RIG-I itself.

Keywords

Influenza A; Tripartite-motif family; TRIM27; avian immunology; Innate immune modulation; MAVS signaling pathway; evolution

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1. Introduction

Tripartite motif (TRIM) proteins are implicated in a number of innate immune functions, including antiviral activity (Rajsbaum *et al.* 2008). Their name derives from the conserved three part N-terminal domain architecture (Reymond *et al.* 2001) or tripartite-motif (RBCC motif) composed of RING-, B-box and coiled-coil domains (Nisole *et al.* 2005; Ozato 2008). The RING-domain encodes the E3-ubiquitin ligase function, while B-box and coiled-coil domains determine protein-protein interactions and complex formation. The *TRIM* family appears to be rapidly evolving (Sawyer *et al.* 2007; Sardiello *et al.* 2008) and the repertoire of TRIM genes varies among species (Boudinot *et al.* 2011; Marin 2012). While TRIM proteins have not been extensively explored in birds, a cluster of TRIM genes was identified in the chicken Major Histocompatibility (MHC) B-locus, suggesting their importance in immune functions (Ruby *et al.* 2005). TRIM proteins have diverse roles in modulating intracellular immune responses often involving their function as E3-ubiquitin ligases (Kawai & Akira 2011; Uchil *et al.* 2013; Versteeg *et al.* 2013). Several members of the tripartite-motif (*TRIM*) gene family are induced by interferon during influenza A virus infection (Rajsbaum *et al.* 2008; Carthagen *et al.* 2009). Recently, the immunomodulatory function of the entire human TRIM repertoire was systematically examined and almost half of the TRIM proteins modulate the innate signalling pathway at various points downstream of pattern recognition receptors (Uchil *et al.* 2013; Versteeg *et al.* 2013).

A subset of TRIM proteins shows evidence of being under positive selection suggesting they interact directly with pathogens, including human TRIM5 and TRIM22 (Sawyer *et al.* 2005; Sawyer *et al.* 2007). Examples of species-specific viral restriction include HIV by TRIM5 α in old world monkeys (Stremlau *et al.* 2004; Stremlau *et al.* 2005) and tick borne encephalitis virus by TRIM79 α in mice (Taylor *et al.* 2011). The TRIMs involved in viral restriction are elegant examples of co-evolution of viruses and innate defenses of the host. Since ducks are the natural host and reservoir of influenza viruses, we are investigating TRIM genes in ducks in comparison to chickens, for potential contributions to their resistance to influenza A virus (IAV).

With the availability of the duck genome (Huang *et al.* 2013), we examined the TRIM repertoire in the duck for notable differences between ducks and chickens. We focussed initially on genes within the *TRIM* cluster that is a conserved synteny group shared with the MHC B-locus of the chicken (Kaufman *et al.* 1999; Shiina 2007) and turkey (Chaves *et al.* 2009). One gene within this region, *TRIM27-L*, is unique in ducks and absent in chickens and turkeys. Since the MHC-linked gene cluster is evolutionarily dynamic, we wondered whether this was due to selective pressure by pathogens. The function of avian MHC-linked TRIMs is unknown, and while they share limited homology to human TRIM27, we were intrigued by the innate immune modulation by human TRIM27 protein (also known as Ret-finger protein, RFP). Human TRIM27 (huTRIM27) was originally identified as the C-terminal half of an oncogenic fusion protein; the RBCC motif of the huTRIM27 fused to Ret-tyrosine kinase causes dysregulation in cell growth (Takahashi *et al.* 1988). Through the interactions with IKK family members, huTRIM27 represses NF- κ B activation and subsequent nuclear translocation, and inhibits *IFN*- β promoter activity (Zha *et al.* 2006). Because many TRIM proteins were recently shown to modulate the RIG-I pathway

(Versteeg *et al.* 2013) we investigated whether these proteins could modulate innate immune signalling. Here, we show that duck *TRIM27.1* and *TRIM27-L* are upregulated during IAV infection, and the expressed recombinant proteins have opposite immunomodulatory effects on the antiviral program downstream of RIG-I signalling.

2. Materials and Methods

2.1. Annotation of the TRIM genes in duck genome sequence

The draft sequence (preEnsembl release 66, July 2012) of the White Pekin duck *Anas platyrhynchos* was used to predict *TRIM* genes. Several *TRIM* genes were identified on a single scaffold, 618, and their order and orientation were similar to genes within the chicken and turkey extended MHC region.

2.2. Viruses, Infections and RNA extractions

Tissues were obtained from ducks infected in a previous study (Barber *et al.* 2010). Briefly, six week old White Pekin ducks were infected by the natural route in eyes, nares and trachea, with 10^6 EID₅₀ doses of highly pathogenic, A/Vietnam/1203/2004 (H5N1) (VN1203) produced by reverse engineering (Salomon *et al.* 2007), and a low pathogenic duck isolate obtained through environmental surveillance, A/mallard/British Columbia/500/2005 (H5N2) (BC500). At 1, 2 and 3 dpi, total RNA was isolated from the tissues, as previously described (Barber *et al.* 2010). Infection was monitored by tracheal and cloacal swabs, and reported previously (Vandervan *et al.* 2012). Briefly, tracheal swabs taken from animals at 3 dpi with VN1203 ranged from 10^2 to 10^4 , while cloacal swabs for BC500 at 3 dpi were between 10^4 and 10^7 .

2.3. Expression analysis by qPCR

Total RNA was quantified and used for cDNA synthesis using 500 ng RNA/sample. RNA was DNase treated prior to synthesis and cDNA was prepared according to manufacturer's instructions (Invitrogen). Q-PCR probes (PrimeTime Probes (6-FAM/ZEN/IBFQ), IDT Technologies) were designed using standard conditions on Primer Express v3.0 (Applied Biosystems) or an online RealTime PCR design tool (IDT). Quantification of gene expression level was accomplished with FastStart TaqMan[®] master mix (Roche) in the Prism 7500 Real Time PCR machine (Applied Biosystems) with gene specific probes and primers (Table 1). Analysis of gene expression uses a relative expression assay ($-C_t$) based on expression of an endogenous control (Glyceraldehyde 3-phosphate dehydrogenase, GAPDH) and the gene of interest. Absolute quantification of transcript amounts used a pCR2.1-TOPO clone of each target gene, chicken *myxovirus resistance gene 1* (*MX1*) and chicken *interferon beta* (*IFN- β*) in a standard curve analysis.

2.4. Amplification and cloning

Amplification of *TRIM27.1* and *TRIM27-L* from duck lung cDNA infected with highly pathogenic IAV (VN1203) (AmpliTaq[®] Gold PCR Master Mix, Invitrogen) was done using gene specific primers with 3'- and 5'-modifications for directional cloning and inclusion of an epitope tag (C-terminal-V5, amino acid sequence GKPIPPLLGLDST) to confirm protein expression in transiently transfected cells. A 1574 bp PCR fragment of *TRIM27.1*

with C-terminal V5 epitope and a 1493 bp PCR fragment of *TRIM27-L* with C-terminal V5 epitope were cloned into pCR2.1-TOPO first, then cloned using restriction sites NotI/NheI into pcDNA3.1/Hygro+ expression vector (Invitrogen). For analysis of immunomodulation of the MAVS pathway we used two additional constructs, one which contains the glutathione-S-transferase (GST) gene fused to the two tandem CARD domains from duck RIG-I (d2CARD) and one containing the GST-I protein only as control (Miranzo-Navarro & Magor 2014).

2.5. Cell culture, transfection, luciferase and RNA extraction

The spontaneously immortalized chicken embryonic fibroblast cell line (DF1) (Schaefer-Klein *et al.* 1998) was cultured in DMEM with 10% fetal bovine serum and seeded at a density of 4×10^5 cells/ml. Seeded cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen) as described previously (Miranzo-Navarro & Magor 2014). Luciferase expression experiments with a dual-luciferase reporter assay (Promega®) were carried out as described previously (Sick *et al.* 1998; Childs *et al.* 2007; Barber *et al.* 2010). Briefly, DF1 cells were transfected with fixed amounts of pGL3-chIFN β (150 ng), the synthetic *Renilla* luciferase reporter construct phRTK as internal control (10 ng) and d2CARD or GST (5 ng). Cells were also transfected with increasing amounts of duck *TRIM27.1* or *TRIM27-L* construct (between 25 ng and 500 ng) and of pcDNA3.1 (Hygro+) (Invitrogen) to transfect equivalent amounts of DNA. TRIzol® reagent (Invitrogen) was used to lyse and extract RNA from the adherent cells according to the manufacturer's instructions. We used chicken DF-1 cells for these transfections because no duck cell line is available, and expression of endogenous chicken TRIM27.1 is low, and is not upregulated in our microarray data by signalling through RIG-I upon infection with either BC500 (0.54-fold change) or VN1203 viral infection (0.83-fold change) (Barber *et al.* 2013).

2.6. Immunoblotting for protein expression

SDS-PAGE gels of 8% acrylamide were run and blotted to Trans-Blot Nitrocellulose membranes (BioRad). Membrane was blocked overnight at 4°C in PBS +5% (w/v) skim milk. Mouse primary anti-V5 (1:5000) (Invitrogen) antibodies were bound for 2 h, and goat anti-mouse HRP-conjugated antibody (1:5000) (BioRad) for 1 hr at 24°C. Imaging membranes was through chemiluminescence substrate cleavage, using Amersham Enhanced Chemiluminescence (ECL) solution (GE healthcare).

2.7. Statistical analysis

Statistical analysis was compiled using GraphPad Prism version 5.0 using repeated measures ANOVA and a Tukey's post-hoc test.

2.8. Phylogenetic analysis

From a blastp query using the amino acid sequences of both duck TRIM27.1 and TRIM27-L we selected 35 unique hits from Genbank, 4 human TRIMs (huTRIM27, huTRIM39, huTRIM7 and huTRIM10) and duck TRIM7.2 and aligned the protein sequences with TRIM27.1, TRIM27.2, and TRIM39.2 from duck, chicken and turkey. We aligned them using the MUSCLE algorithm in the MEGA6.02 program (Tamura *et al.* 2011). We then

constructed a maximum-likelihood tree and conducted evolutionary analysis using partial deletion, site coverage cut-off of 95% and the Jones-Taylor-Thornton model (JTT+G+I) model of maximum-likelihood for amino acid substitutions, which was deemed to have the lowest Bayesian information criterion value of all the available models (Jones *et al.* 1992; Tamura *et al.* 2011; Hall 2013). We ran 1000 trials for bootstrapping analysis.

3. Results

3.1. The MHC-linked TRIM cluster has an additional gene in ducks, absent in chickens and turkeys

We identified a cluster of *TRIM* genes in the genome sequence of the White Pekin duck (Huang *et al.* 2013), which has the characteristics of a conserved synteny group with the chicken and turkey extended MHC region (Ruby *et al.* 2005; Shiina 2007; Chaves *et al.* 2009) (Fig. 1). The MHC-linked TRIM cluster of the duck encodes *TRIM7.2*, *TRIM7.1*, *TRIM39.2*, *TRIM39-like/B30.2-related (TRIM39-L/BR)*, *TRIM27.2*, *TRIM27.1*, a unique gene we named *TRIM27-L* and *TRIM41*. Interestingly, the *TRIM27-L* gene is absent from the chicken and turkey TRIM clusters.

To determine paralogy and orthology relationships of TRIM27 sequences, we generated a protein sequence alignment and phylogenetic tree, and TRIM27-L is distantly related to TRIM27.1, TRIM39.2 and TRIM27.2 (Suppl. Fig. 1). We determined percent identity of TRIM27-L to the other TRIM proteins in the locus (Suppl. Table. 1). TRIM27-L shares similar percent identity with TRIM27.1 (67% identity in the RING domain and 51% identity overall) and TRIM39.2 (62% in RING domain and 52% overall). The functional domain architecture between TRIM27.1, TRIM27-L and TRIM39.2 are similar (Suppl. Fig. 2). Thus, we named the gene TRIM27-L based on the position in the scaffold, adjacent to TRIM27.1, and having the highest percent identity of the RING-domain (responsible for E3-ubiquitin ligase activity) with TRIM27.1. However, TRIM27-L is only distantly related to other TRIM genes in the locus, suggesting it did not arise from a recent duplication.

To determine whether TRIM27-L is unique to ducks, and if the gene has been lost in the galliformes, we searched for homologues of TRIM27.1 and TRIM27-L in other species and aligned them and generated a neighbour joining tree in MEGA6.02 (Fig. 2). As predicted by synteny, TRIM27.1 clusters with the chicken and turkey TRIM27.1 and more distantly with RFP-L (Ret-finger protein-like or TRIM27-like) proteins. However, duck TRIM27-L segregates with predicted TRIM7-L proteins from 6 different avian species, and predicted avian RFP-L proteins from the little egret and hoatzin, and more distantly with RFP-L proteins from turtles and alligators. Duck TRIM7.1 and TRIM7.2 do not group with these sequences, but do group with human TRIM7, so we do not favor the annotation of these predicted sequences as TRIM7-like. We propose they should be annotated as TRIM27-L, as they are most similar to avian TRIM27 genes. We cannot easily predict the relationship of duck TRIM27 to the human TRIM genes since they each share approximately 40% identity, and the duck sequences do not group with them in the phylogenetic tree. We compared TRIM27-L to each mammalian and duck sequence across all domains (Supplementary Table 1). The most similar human TRIMs to duck TRIM27-L are TRIM39 and TRIM7. However, duck TRIM27-L is most similar to duck TRIM27.1 (and the related TRIM39.2) within the

RING domain. We conclude from our comparison of related TRIM genes, that a homologue of duck *TRIM27-L* is shared by many birds and reptiles, thus has been lost in the galliformes.

Because these genes are located in the extended MHC region, we anticipated that they might be polymorphic. Therefore, we cloned and sequenced allelic variants from 3 ducks for each *TRIM27.1* and *TRIM27-L*. Alignment of these sequences revealed polymorphisms, with 27 SNPs in *TRIM27.1* and 10 SNPs in *TRIM27-L*. Only a few of these SNPs alter the coding sequence. In *TRIM27.1* we noted amino acid substitutions within the B box (M116T), coiled-coil (T312S) and the PRY domains (T365I). In *TRIM27-L* we noted only two changes to the protein, one in the RING domain, I18T, and one in the PRY domain (V320A). For all functional experiments, we used the dominant allele of *TRIM27.1* and *TRIM27-L*. While *TRIM27.1* and *TRIM27-L* are polymorphic, amino acid substitutions are conservative, thus we do not expect functionally different alleles.

3.2. *TRIM27.1* and *TRIM27-L* are upregulated in influenza infected ducks

Using reverse transcription PCR, we examined expression of *TRIM27.1* and *TRIM27-L* in tissues relevant to influenza infection, including lung, spleen and intestine and demonstrate both transcripts are present at low level in these tissues, compared to *GAPDH*. Transcript abundance was increased for both *TRIM27.1* and *TRIM27-L* in influenza infected duck tissues (data not shown). To quantitatively examine upregulation of duck MHC *TRIM27* genes, we assessed the relative expression of each transcript with qPCR in lung tissues at 1, 2 and 3 days post-infection (dpi) with low pathogenic avian influenza A/British Columbia/500/2005 (H5N2) or highly pathogenic A/Vietnam/1203/2004 (H5N1) compared to the level of expression in mock infected duck lungs. Expression of *TRIM27.1* is upregulated in lung tissue at 1dpi 6-fold by BC500 and 34-fold by VN1203 relative to a mock animal (Fig. 3A). One mock animal was selected as the calibrator for samples for each day, and expression compared to this for all genes and all tissues. Since the mock sample selected for duck lung tissue showed lower expression than other mock animals for *TRIM27.1* only, we note that the mean values for mock and infected indicates upregulation is less than 2-fold for BC500 infection and 9-fold for VN1203. Upregulation is very short lived, and by 3 dpi, expression in lung tissue is consistent between infected and uninfected animals. Expression of *TRIM27-L* is also upregulated in lung tissues at 1 dpi with highly pathogenic VN1203 (Fig. 3B), although to a lesser extent than *TRIM27.1*. Upregulation of *TRIM27-L* is 4-fold higher than the average expression level of mock-infected ducks. Upregulation of *TRIM27-L* is more prolonged compared to *TRIM27.1* as it still has an average of 3-fold higher expression at 2 dpi, but is not elevated at 3dpi compared to the mock-infected animals. *TRIM27.1* was also slightly upregulated in intestine following infection with BC500, and *TRIM27-L* is slightly elevated in spleen at 1dpi with VN1203 (Suppl. Fig. 3). In contrast, *TRIM41* was not detectable by qPCR after 40 cycles, and *TRIM27.2* was detectable only at 39 cycles. *TRIM39.2* was elevated 5-fold relative to a mock-infected animal in spleen at 1 dpi with highly pathogenic IAV VN1203 (Suppl. Fig. 4), and *TRIM7.2* was upregulated at 1dpi with VN1203 by 4-fold relative to a mock-infected animal (Suppl. Fig. 5). The increased expression of both *TRIM27.1* and *TRIM27-L* following infection suggests they are potentially involved in the innate immune response to influenza.

3.3. TRIM27.1 and TRIM27-L have opposite effects on the RIG-I signalling pathway

We expressed the two V5-epitope tagged duck TRIM27.1 proteins in chicken DF-1 embryonic fibroblasts, and determined their relative protein expression level by Western blot. Both proteins were abundantly expressed at 24 hr post-transfection, but TRIM27.1 was expressed at a higher level than TRIM27-L (Suppl. Fig. 6). We assessed immune activation using absolute transcript qPCR of two genes downstream of the RIG-I pathway, *interferon-beta* (*IFN-β*) and *myxovirus resistance gene 1* (*MX1*) (Barber *et al.* 2013). Expression of duck *TRIM27.1* or *TRIM27-L* alone had no effect on the relative expression of *IFN-β* or *MX1* in comparison to a control vector expressing glutathione-S-transferase (GST) (Fig. 4).

Human TRIM27 interacts with members of the IKK-family (Zha *et al.* 2006), which are signalling components in the MAVS pathway downstream of RIG-I detection. We activated the MAVS signalling pathway in DF1 cells using a constitutively active duck RIG-I N-terminal d2CARD-GST construct (Miranzo-Navarro & Magor 2014), and examined the expression of *IFN-β* and *MX1* when each duck *TRIM27* gene was coexpressed. Co-expression of *TRIM27.1* reproducibly decreased transcription of *IFN-β* in four independent experiments (Fig. 4A) and *MX1* in two replicates (Fig. 4B), in comparison to d2CARD alone. In contrast, *TRIM27-L* greatly increased transcription of *IFN-β* in comparison to the d2CARD expressing constitutively activated cells (Fig. 4C). Upregulation of *MX1* transcript was also observed in comparison to constitutively activated cells expressing d2CARD (Fig. 4D). Thus, duck TRIM27.1 inhibits innate signalling, while TRIM27-L augments induction of *IFN-β* and *MX1* in chicken cells.

To examine the effects of *TRIM27.1* and *TRIM27-L* on the activity of the RIG-I pathway in a dose-dependent manner, we measured activation of the *IFN-β* promoter (Sick *et al.* 1998) using a dual-luciferase reporter assay, as we previously used to demonstrate activation of the MAVS signalling pathway downstream of RIG-I (Barber *et al.* 2010; Miranzo-Navarro & Magor 2014). Adding increasing amounts of *TRIM27.1* (from 25 ng to 500 ng) resulted in a trend of decreased activation of the *IFN-β* promoter driven by the constitutively active d2CARD (Fig. 5A). In contrast, co-expression of *TRIM27-L* reproducibly increased the level of *IFN-β* promoter activity downstream of the constitutively active d2CARD (Fig. 5B). To determine if the immunostimulatory effect of *TRIM27-L* was stronger than the suppressive effect of *TRIM27.1*, we co-expressed equivalent amounts of *TRIM27.1* and *TRIM27-L* (250 ng) with d2CARD to activate the MAVS pathway (Fig. 5B). Co-expression of *TRIM27.1* and *TRIM27-L* together reproducibly increased the activity of the *IFN-β* promoter compared to constitutively activated cells expressing d2CARD.

Because both *TRIM27.1* and *TRIM27-L* are upregulated during innate immune responses we sought to determine at what concentration the enhancing effect of *TRIM27-L* overrides the suppressive effect of *TRIM27.1* on the MAVS signalling pathway. We transiently transfected DF-1 cells with d2CARD (5 ng) and the inhibitory *TRIM27.1* (250 ng), and added increasing amounts of *TRIM27-L* and measured the *IFN-β* promoter activity. *TRIM27.1* decreased the signalling through MAVS compared to d2CARD alone, while co-expression of the immunostimulatory *TRIM27-L* enhanced expression of the *IFN-β* promoter compared to the d2CARD alone (Fig. 5C). Expression of the immunostimulatory *TRIM27-L*

(from 25 ng to 250 ng) along with the suppressive *TRIM27.1* (transfected with 250 ng of vector, which was previously observed to suppress the production of both *IFN- β* and *MX1*) resulted in enhanced *IFN- β* promoter activity downstream of RIG-I signalling. Immunosuppression with *TRIM27.1* was observed in two of four independent experiments, while immunostimulation with *TRIM27-L* was reproducible in 4 replicate experiments, although there is variability in the magnitude of change. Overall, these results demonstrate that the immunostimulatory *TRIM27-L* over-rides the *TRIM27.1*-dependent inhibition of the MAVS signalling pathway.

4. Discussion

We compared the TRIM gene cluster located adjacent to the MHC between chickens, turkeys and ducks, and identified an additional *TRIM27* gene in ducks. The duck *TRIM27-L* most closely resembled *TRIM27.1* and *TRIM39.2*, albeit it showed considerable divergence and shared only 51% and 52% amino acid sequence identity with these, suggesting they all arose from ancient duplication. We showed that both *TRIM27.1* and *TRIM27-L* are upregulated in lung and intestine tissue of influenza-infected ducks, particularly by highly pathogenic influenza A virus VN1203, suggesting that both gene products could be involved in an innate immune response to influenza. We cloned each gene and expressed it in chicken DF-1 embryonic fibroblasts. Neither *TRIM27* had any discernable effect on the innate immune signalling of these cells when expressed alone. Because human *TRIM27* has an inhibitory effect on innate immune signalling by interaction with IKK family members, and many TRIM proteins are immunomodulatory, we co-expressed *TRIM27.1* and *TRIM27-L* genes together with the constitutively active duck RIG-I N terminal 2CARD domain to drive transcription of the interferon-beta pathway. *TRIM27.1* inhibited innate immune signalling, while duck *TRIM27-L* augmented signalling through the MAVS pathway. Since both would be expressed together in a natural infection, it is also notable that the immunostimulatory *TRIM27-L* always over-rides the inhibitory *TRIM27.1*.

Our reverse transcription data show that both *TRIM27.1* and *TRIM27-L* are present at low transcript abundance in tissues relevant to influenza infection prior to infection, but are upregulated upon infection. Similarly, the transcriptome of ducks infected with highly pathogenic H5N1 viruses showed that both duck *TRIM27.1* and *TRIM27-L* are upregulated upon infection, and the expression level of *TRIM27-L* is higher than *TRIM27.1* (Huang *et al.* 2013). *TRIM27-L* activation would enable ducks to elicit a greatly amplified interferon response downstream of RIG-I detection of RNA viruses, including influenza A viruses and Newcastle disease virus. Furthermore, since influenza is known to interfere in the RIG-I pathway by targeting activation by TRIM25 (Gack *et al.* 2009), a mechanism to augment signalling downstream would be highly advantageous. Chickens and turkeys lack *TRIM27-L*, and also lack RIG-I, while ducks upregulate and use RIG-I during an innate immune response to influenza (Barber *et al.* 2010). While chickens do have MDA5, which elicits signalling through the MAVS pathway and can partially compensate for the loss of RIG-I (Karpala *et al.* 2011; Liniger *et al.* 2012), the absence of *TRIM27-L* would also decrease their ability to elicit *IFN- β* in response to influenza A, in comparison to ducks. While the loss of *TRIM27-L* and *RIG-I* genes in chickens are undoubtedly due to two independent events, since they are on the MHC microchromosome (chr. 16) and the Z chromosome,

respectively, both events were presumably disadvantageous. However, we cannot rule out the possibility that the loss of either or both were advantageous in a scenario in which aberrant and excessive interferon responses were elicited through MAVS signalling, by chronic inflammatory disease or pathogen subversion.

The TRIM cluster in ducks is arranged as a conserved synteny group with those of chickens and turkeys, which are within the extended MHC. In addition, a related family of TRIM genes is also located in the MHC in humans (Meyer *et al.* 2003) and fish (Boudinot *et al.* 2011). MHC linkage is significant for two reasons. First, these sequences may accumulate functional polymorphism, and secondly, the genes in the cluster have the potential to be co-ordinately regulated. The polymorphism we see between individuals can be attributed to MHC linkage, as changes are likely to hitchhike along with polymorphisms selected for in the neighbouring MHC region. While we noted 27 SNPs in *TRIM27.1* between 3 sequenced animals, only 3 changed the protein sequence, and since these were conservative changes these were unlikely to alter protein function. We noted 10 SNPs in *TRIM27-L*, and two altered the protein sequence. Although possible, there is little evidence to suggest that genes in the cluster are co-ordinately regulated. Our analysis of expression of other genes in the duck TRIM cluster, showed no expression of *TRIM41* and *TRIM27.2*, while *TRIM39.2* is elevated in spleen. Only *TRIM7.2* (with 43% sequence identity) showed expression similar to *TRIM27-L*. Thus the MHC cluster genes do not appear to be co-ordinately regulated. Similarly, Rajsbaum also noted that human MHC linked genes TRIM26 and TRIM39 show no similarity in their patterns of gene expression in leukocytes or response to influenza or TLR ligands (Rajsbaum *et al.* 2008).

Duck *TRIM27.1* and *TRIM27-L* genes are both upregulated in lung tissues from influenza-infected ducks. This is in contrast to TRIM27 from humans, which is not significantly upregulated by influenza infection of macrophages or dendritic cells, nor upregulated by Type I or Type II interferons (Rajsbaum *et al.* 2008; Carthagen *et al.* 2009). Mouse *TRIM27* was downregulated in macrophages (Martinez *et al.* 2006), while human *TRIM27* is constitutively expressed in pDC (Rajsbaum *et al.* 2008). Mouse *TRIM27* is highly expressed in testis, and had low levels of expression in spleen and PBMCs (Tezel *et al.* 1999). Chicken TRIM27 is expressed in the testis, bursa of Fabricius and thymus, but not the spleen (Ruby *et al.* 2005). The avian TRIM genes within the MHC cluster are more similar to each other than to the human TRIM27 homologue, although they are distantly related (Sardiello *et al.* 2008).

We showed that duck TRIM27.1 down-modulates the innate immune signalling downstream of RIG-I in chicken cells, suggesting that TRIM27.1 inhibits signalling through interaction with components downstream in the pathway. The down-modulation by TRIM27.1 is modest, which may reflect poor conservation of its target in chicken cells, or interference by the endogenous chicken homologue. It is not known which proteins that are downstream of MAVS that TRIM27.1 interacts with. However, human TRIM27 was identified in a yeast two-hybrid strategy to identify proteins interacting with IKK ϵ (Zha *et al.* 2006). Subsequently they demonstrated that huTRIM27 binds to other IKK proteins and TBK, and further inhibits translocation of IRF3 to the nucleus for signalling. Human TRIM27 has been shown to have E3-ubiquitin ligase activity (Napolitano *et al.* 2011) and SUMOylating E3-

ligase activity (Chu & Yang 2011). Human TRIM27 functions as an E3-ligase in the addition of K27 linked polyubiquitin to itself and to PTEN, an inhibitor of the AKT pathway, targeting it for degradation (Lee *et al.* 2013). Also, huTRIM27 adds K48-linked polyubiquitin to NOD2 leading to proteosomal degradation (Zurek *et al.* 2012). Duck TRIM27.1 has residues necessary for E3 ubiquitin ligase activity, thus it is most likely that avian TRIM27.1 modifies proteins downstream of MAVS to modulate innate immune signalling.

In contrast, duck TRIM27-L enhances innate immune signalling downstream of RIG-I. TRIM27-L does not appear to interact directly with RIG-I 2CARD (unpublished results), and does not work alone to enhance immune signalling, thus it is likely that duck TRIM27-L also modifies a protein downstream in the MAVS signalling pathway. We should point out that since TRIM27.1 and TRIM27-L share only 51% amino acid identity, they might have completely different cellular targets. The *TRIM27-L* gene appears to have been lost from the chicken and turkey genomes, which is notable since galliformes also lack RIG-I. Remarkably, the interaction of duck TRIM27-L protein occurs with chicken pathway components, considering the loss of TRIM27-L in chickens and turkeys, suggesting the components and interaction domains are conserved. Almost half of the human TRIM proteins can augment innate immune signalling at various points in the MAVS signalling pathway downstream of pattern recognition detection (Versteeg *et al.* 2013). Ducks, but not chickens, have a TRIM27-L that augments MAVS signalling.

Our comparison of ducks, chickens and turkeys suggests that the avian MHC linked TRIM cluster is evolutionarily dynamic. The MHC TRIM proteins carry the PRY/SPRY domain or B30.2 motif (Rhodes *et al.* 2005), associated with the direct restriction of retroviruses, and innate immune modulation. While we have not yet investigated whether TRIM27.1 and TRIM27-L can directly restrict any viruses, the presence of multiple TRIM27 genes and potential decoy or TRIM-like receptors lacking functional components, such as the partial TRIM gene annotated as *TRIM39/BR* in birds (Ruby *et al.* 2005), also hints at co-evolution with viruses. The B30.2 TRIM genes appear to have undergone expansion in many vertebrate genomes, and many genes are specific to each species (Sardiello *et al.* 2008). The evolutionary pressure leading to the loss of the immunostimulatory TRIM27-L in galliform birds is unknown. However, the possession of an immunostimulatory TRIM27 gene, capable of augmenting innate immunity downstream of MAVS signalling, would be expected to be advantageous to ducks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- > The TRIM cluster in the avian MHC region is evolutionarily dynamic
- > A TRIM27-like gene is present in ducks, but not chickens or turkeys
- > Both TRIM27.1 and TRIM27-L are upregulated by influenza infection
- > TRIM27.1 weakly inhibits MAVS signalling
- > TRIM27-L strongly activates MAVS signalling

Duck TRIM Cluster

Fig. 1.

Organization of the TRIM gene cluster adjacent to avian MHC regions. The gene organization implies the locus is a conserved synteny group of duck *TRIM* genes compared to the chicken and turkey MHC adjacent TRIM clusters (Ruby *et al.* 2005; Chaves *et al.* 2009). The duck genes are on Ensembl scaffold #KB742989.1.

Fig. 2.

Phylogenetic tree of *TRIM27* related genes. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [1]. The tree with the highest log likelihood (-17555.5723) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.0142)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 1.4941% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 57 amino acid sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 394 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.02 (release # 6140226) (Tamura *et al.* 2011).

Fig. 3.

Transcript abundance for duck *TRIM27.1* and *TRIM27-L* is increased in lung tissue following infection with highly pathogenic VN1203. Total RNA was extracted from duck tissues harvested at 1, 2 and 3 dpi with BC500 and VN1203, cDNA was synthesized and analyzed with qPCR using gene specific probe and primers for targets and endogenous control (GAPDH). Fold expression of *TRIM27.1* (A) and *TRIM27-L* (B) in duck lung tissues is shown relative to a mock-infected animal on each day. Dots represent individual ducks (n = 3) and the values are the mean fold change relative to expression level for a mock animal each day (***) is p < 0.001).

Fig. 4.

TRIM27.1 and TRIM27-L expression have opposite effects on transcription of antiviral genes downstream of MAVS signalling. Duck TRIM27.1 reduced expression of (A) *IFN-β* and (B) *MXI* in chicken DF-1 cells activated with the RIG-I N-terminal domain (d2CARD), which constitutively activates MAVS signalling. TRIM27-L increased (C) *IFN-β* and (D) *MXI* expression in cells activated with d2CARD. Each unique symbol represents data from four independent transfection experiments, made with triplicate samples. GST-I is an irrelevant protein control for d2CARD-GST.

Fig. 5. TRIM27.1 suppresses and TRIM27-L enhances MAVS signalling. Four replicate dual-luciferase reporter co-transfection experiments with the *IFN- β* promoter, d2CARD and increasing concentrations of plasmids corresponding to (A) *TRIM27.1* or (B) *TRIM27-L* or (C) increasing concentration of *TRIM27-L* co-transfected with *TRIM27.1* were done in DF-1 cells. A dose-dependent increase in *IFN- β* promoter activity is observed with increasing

amounts of *TRIM27-L*, even in presence of *TRIM27.I*. Different symbols indicate results from independent transfection experiments.

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Table 1

Primer and probe sequences used for qPCR

Gene	Primer and probe sequence (5'→3')	
<i>TRIM27.1</i>	F	CCCATCTGGAAAGAAGACTTGAG
	R	GGTTGGCTCTTGCAATTTAAACA
	Probe	CCTCAGCGTGCATCTCACGAGGAC
<i>TRIM27-L</i>	F	GGGTGTTTCGTCCCATCTCA
	R	GGGCAAACGTGACTCTGGAT
	Probe	CAGACACAGCAAACCCCCACCTTGTC
<i>Chicken GAPDH</i>	F	GGTGCTAAGCGTGTATCATCTCA
	R	CATGGTTGACACCCATCACAA
	Probe	CTCCCTCAGCTGATGCCCCCATG
<i>Chicken IFN-β</i>	F	TCCAACACCTCTTCAACATGCT
	R	TGGCGTGTGCGGTCAAT
	Probe	AGCAGCCCACACACTCCAAAACACTG
<i>Chicken MX1</i>	F	GGACTTCTGCAACGAATTG
	R	TCCCACAAGTTCATCTGTAAG
	Probe	CTTACCTCCGCAATCCAGCAAGA