Supplemental Information

Structural Analysis of dsRNA Binding
to Anti-viral Pattern Recognition Receptors

LGP2 and MDA5

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Supplemental Information

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Supplementary Figure Legends.

Figure S1 Sequence alignment of chicken and human LGP2 and MDA5 and duck and human RIG-I (excluding the CARDs). Related to Figures 1 and 4.

The secondary structure of chLGP2 and chMDA5 are at the top and bottom respectively. Outline boxes in green, yellow, cyan, red and orange delimit the Hel1, Hel2i, Hel2, Pincer and CTD domains. Helicase conserved motifs are indicated in black on the bottom and important loops such as the Hel2i, Hel2, end-binding and capping loops are highlighted with a green, yellow, cyan, magenta or orange background respectively. Figure made with ESPript (Gouet et al., 1999).

Figure S2. ATP analogue binding and ATP hydrolysis by chLGP2. Related to Figure 1.

(A) Diagram showing interaction network between chLGP2 and ADP:AlF$_4$·Mg$^{2+}$. Residue colours are as in Figure 1C with polar interactions (blue) and hydrophobic interactions (black).

(B) Ligand electron density for ADP:AlF$_4$·Mg$^{2+}$ in the chLGP2 5’p 10-mer dsRNA complex with Mg$^{2+}$ (magenta), Al (grey), F (pale blue) and coordinated water (marine). Final 2Fo-Fc electron density is contoured at 2.0 σ.
(C) Comparative RNA-dependent ATP hydrolysis activity by LGP2, RIG-I and MDA5 showing initial reaction velocities versus ATP concentration. The concentration of proteins and dsRNAs are 0.125 μM dRig-I and 0.5 μM dsRNA, 0.25 μM chLGP2 and 1 μM dsRNA, 0.5 μM chMDA5 and 2 μM dsRNA. Plotted values are mean ±SD (n=3).

(D) ATPase activity of RLHs as a function dsRNA length using 18-mer hairpin (5′-GGGCGGCUCUCCGCCGCCC-3′), 24-mer (same dsRNA as for crystallization), 32-mer hairpin (5′-pppGGGCGAGCGUGCUUCGCGCAGCGCUCGCCC-3′), or 38-mer (5′-GGGACGUAGCAUCCGAUGUAACAUCGGAUGCUACGCUCCC-3′). The graph compares the amount of hydrolysis after 16 minutes. The reaction conditions were 1 μM RLH, 4 μM dsRNA and 2 mM ATP. Poly(I:C) is polyinosinic-polycytidylic acid (Amersham Biosciences).

(E) Multiple sequence alignment of Motif I of RLHs

(F) Multiple sequence alignment of Motif VI of RLHs

Figure S3. Sequence alignment of LGP2 from representative vertebrates. Related to Figure 1.

Sequences aligned include those from birds (chicken, Muscovy duck), mammals (human, mouse), reptile (alligator), amphibians (frog) and fish (zebrafish). Annotation is similar to Figure S1.

Figure S4. 5′ tri-phosphate conformation and summary of RNAs used. Related to Figures 2, 3 and 5.

(A) Schematic diagram showing the interactions with the 5′ tri-phosphate in the chLGP2_10ppp structure.

(B) Schematic diagram showing the interactions with the 5′ tri-phosphate in the chLGP2_3′ovg structure.
(C) RNAs used in various experiments.

Figure S5. Comparative binding of RLHs to dsRNA. Related to Figure 2.

(A) Electron density showing residues from Hel2i helix α10 interacting directly or via water molecules with nucleotides from the 3’ (violet) and 5’ (yellow) strands in the chLGP2 5′p 10-mer dsRNA complex. Final 2Fo-Fc electron density is contoured at 1.5 σ.

(B) Representative curves of fluorescence anisotropy changes measured by titrating full-length hRIG-I (upper panel), chLGP2 (middle panel) or chMDA5ΔCARD (bottom panel) to FAM labelled 5′OH, 5′ppp, 3′ overhang and 5′ overhang 12-mer dsRNA with or without various nucleotides.

Figure S6. Comparison of helicase opening in known RLH crystal structures and comparison of the closed and semi-closed states of chLGP2. Related to all Figures.

(A) Disposition of helicase Hel1 (green) and Hel2 (cyan) domains for known RLH crystal structures (with PDB entry code indicated) after superposition of Hel1 of each structure onto that of the closed state of chLGP2, used as reference. ATP analogues (slate blue) and Mg²⁺ (magenta) are shown with spheres and the dsRNA back bone (orange). Other domains are coloured as in Fig 1A. According to the relative displacement of Hel1 and Hel2, the structures are classified closed, semi-closed, semi-open and opened (see Table S1 for quantification).

(B) Comparison of the structures of the closed and semi-closed states of chLGP2 after superposition of the Hel1 domain. The Hel1, Hel2, Hel2i and CTD domains are coloured green (pale green), cyan (pale cyan), yellow (sand) and orange (pale orange) for respectively and the dsRNA 5′ strand violet (pale violet) and 3′ strand yellow (sand) for the closed (semi-closed) states respectively.
(C) Schematic diagram comparing interactions made in the closed (left) and semi-closed (right) states of \textit{chLGP2} to the dsRNA illustrating the shift of Hel2 interactions (red box) one phosphate down the 3’ strand.

Figure S7. Co-operativity of LGP2 with MDA5 in human cells. Related to Figure 7.

(A) Expression of \textit{chLGP2}, \textit{chLGP2} variants and \textit{hLGP2} in chicken DF1 cells (top) and expression of \textit{hLGP2} and \textit{hLGP2} variants in human Huh7.5 cells (bottom). The protein expression of other MDA5 constructs used in this work has been previously determined and found to be comparable to that of their \textit{wt} counterpart (Louber et al., 2015).

(B) RIG-I and MDA5-mediated endogenous response to dsRNA in human HEK293 (in blue) and Huh7.5 (in red) cells. Activation of \textit{huIFNβ} promoter by RIG-I agonist 5’ppp-dsRNA (61mer), MDA5 agonist Φ6 bacteriophage 2.9, 4.1 and 6.4 kbp long dsRNA, and RIG-I/MDA5 agonist poly(I:C). Note the lack of detectable endogenous response observed in Huh7.5 cells and the neutral effect of the pCG-duF vector (noted “F”) used as a complement loading DNA to ensure identical amounts of DNA in the experiments with expression vectors as a source of exogenous MDA5 and/or LGP2. (Related to Figure 7B)

(C) Enhancing effect of exogenously supplied \textit{hLGP2} on endogenous response to poly(I:C) in human HEK293 cells with the unit 1 corresponding to 5.5 ng LGP2 DNA/well. (Related to Figure 7A).

(D) Lack of signalling ability of exogenous \textit{hLGP2} upon activation with poly(I:C) or 5’ ppp-dsRNA (left) and dose-response enhancement by \textit{hLGP2} of the activation of exogenous \textit{hMDA5} by poly(I:C) (right) in human Huh7.5 cells. Note the high constitutive activation by MDA5_1 (corresponding to 5.5 ng DNA/well) (as
previously reported see (Louber et al., 2015) and references therein, see also panel E below, upper left histogram, condition 0 RNA) that is neither enhanced by exogenously added hLGP2 (compare histograms with no RNA (0) nor upon transfection of RIG-I agonist $5'\text{ppp}dsRNA$ with or without exogenous hLGP2 (compare histograms with 0 and $5'\text{ppp}dsRNA$ conditions). (Related to Figure 7B).

(E) Enhancing effect of hLGP2 on exogenous hMDA5 is observed only when transfected in limited amount and/or activated by a sub-optimal amount poly(I:C) in human Huh7.5 cells. Note the loss of dose-dependent enhancing effect of hLGP2 (with the unit 1 corresponding to 5.5 ng LGP2 DNA/well) in the presence of 0.05 ng versus 0.005 ng of poly(I:C) in the presence of 0.55, 1.67 and 5.5 ng/well of hMDA5 DNA (MDA5_0.1, MDA5_0.3, MDA5_1 at the upper right, bottom left and bottom right, respectively) and the reduction of this enhancing effect with increasing amount of hMDA5 (compare luciferase signal gradation according to hLGP2 amounts for a same amount of poly(I:C) in the presence of hMDA5 (MDA5_0.1, MDA5_0.3, MDA5_1)).

Note also that the enhancing effect of hLGP2 on hMDA5 is restricted to poly(I:C) and is not observed with another MDA5 RNA agonist, the $\Phi6$ bacteriophage 2.9, 4.1 and 6.4 kbp long dsRNA, while hMDA5 do respond also to this agonist in the absence of huLGP2 (compare $\Phi6$ and the no (0) conditions in the upper left panel for each amount of transfected hMDA5). (Related to Figure 7B).

(F) RNA binding deficient hLGP2 mutants (K138E/R492E or hel°, K650E/K651E or CTD°, K138E/R492E/K650E/K651E or hel°CTD°) but not ATPase-deficient E132Q mutant (see Figure 1E for ATPase activity) no longer exhibit enhancing activity for hMDA5 activated by poly(I:C) in human Huh7.5 cells (Related to Figure 7B).

(G) RNA binding deficient hLGP2 mutants (K138E/R492E or hel°, K650E/K651E or CTD°, K138E/R492E/K650E/K651E or hel°CTD°) but not ATPase-deficient E132Q
mutant no longer exhibit enhancing activity for the homopolymerisation-defective
hMDA5 IE/KR mutant activated by poly(I:C) in human Huh7.5 cells. Cells were
transfected with a mixture of LGP2 (27.5 ng DNA/well) and MDA5 (1.67 ng
DNA/well), i.e. at 5:0.3 ratio (Related to Figure 7C).
Figure S2

A

B

C

D

E

F

Figure S2
| Anisotropy                        | RNA                                                                 |
|----------------------------------|----------------------------------------------------------------------|
| 12 dsRNA<sub>OH</sub>            | 5′-<sub>GGGCUCCACAUG</sub>-3′                                       |
|                                  | 3′-<sub>CCCGAGGUGUAC</sub>(FAM)-5′                                  |
| 12 dsRNA<sub>ppp</sub>           | 5′-<sub>GGGCUCCACAUG</sub>-3′                                       |
|                                  | 3′-<sub>CCCGAGGUGUAC</sub>(FAM)-5′                                  |
| 3′ overhang<sub>OH</sub>         | 5′-<sub>GGCCAGUGC</sub>GA-3′                                       |
|                                  | 3′-<sub>AACGGUCACGC</sub>(FAM)-5′                                  |
| 5′ overhang<sub>OH</sub>         | 5′-<sub>GGCCAGUCGCA</sub>A-3′                                       |
|                                  | 3′-<sub>GGCCAGUCGCA</sub>(FAM)-5′                                  |
| <i>chLGP2</i> crystallisation     |                                                                       |
| 10 dsRNA<sub>p</sub>             | 5′-<sub>pGGUACG</sub>UACC-3′                                       |
|                                  | 3′-<sub>CCAUGCAUGG</sub>p-5′                                       |
| 10 dsRNA<sub>ppp</sub>           | 5′-<sub>pGGUACG</sub>UACC-3′                                       |
|                                  | 3′-<sub>CCAUGCAUGG</sub>p<sub>ppp</sub>-5′                         |
| 12 dsRNA<sub>p</sub>             | 5′-<sub>pGGUACGC</sub>GUA-3′                                       |
|                                  | 3′-<sub>CCAUGCG</sub>GUA-5′                                       |
| 5′-ppp and 3′ two nucleotide (GG) | overhang hairpin RNA duplex                                         |
|                                  | 5′-<sub>pGGAGCGUGCCG</sub>Y-3′                                     |
|                                  | 3′-<sub>GGCCUCGCACGC</sub>GA                                       |
| <i>chMDA5</i> crystallisation     |                                                                       |
| 10 dsRNA<sub>p</sub>             | 5′-<sub>pGGUACG</sub>UACC-3′                                       |
|                                  | 3′-<sub>CCAUGCAUGG</sub>p-5′                                       |
| 24 dsRNA<sub>p</sub>             | 5′-<sub>GGGACG</sub>UUAUGCAUGCAUGCAUGUC-3′                         |
|                                  | 3′-<sub>CCCUGCAUGCAUGCAUGCAUGCAUGG</sub>p-5′                      |
| 26 dsRNA<sub>p</sub>             | 5′-<sub>GGGACG</sub>UUAUGCAUGCAUGCAUGCAUGUC-3′                     |
|                                  | 3′-<sub>CCCUGCAUGCAUGCAUGCAUGCAUGCAUGG</sub>p-5′                  |
| 27 dsRNA<sub>p</sub>             | 5′-<sub>GGGACG</sub>UUAUGCAUGCAUGCAUGCAUGCAUGUC-3′                 |
|                                  | 3′-<sub>CCCUGCAUGCAUGCAUGCAUGCAUGCAUGG</sub>p-5′                  |
Figure S5

A

B

\[ \triangle \text{Isotropy} \]

hRig-I concentration (µM)

\[ \triangle \text{Isotropy} \]

chLGP2 concentration (µM)

\[ \triangle \text{Isotropy} \]

chMDA5 concentration (µM)

| NTP    | RNA          | Protein | Hill coefficient |
|--------|--------------|---------|------------------|
| ADP:AIF4 | 12dsRNAOH   | wt      | 1.19 ± 0.098     |
| ATP     | 12dsRNAOH   | wt      | 1.14 ± 0.078     |
| ADP     | 12dsRNAOH   | wt      | 1.12 ± 0.168     |
| no NTP  | 12dsRNAOH   | wt      | 0.96 ± 0.104     |
| ATP     | 12dsRNAOH   | wt      | 1.35 ± 0.120     |
| no NTP  | 12dsRNAOH   | wt      | 1.18 ± 0.058     |
| no NTP  | 12dsRNAOH   | wt      | 0.96 ± 0.092     |
| no NTP  | 12dsRNAOH   | wt      | 0.97 ± 0.090     |
| no NTP  | 12dsRNAOH   | wt      | 0.68 ± 0.047     |
| no NTP  | 12dsRNAOH   | wt      | 1.38 ± 0.121     |
| no NTP  | 12dsRNAOH   | wt      | 1.47 ± 0.064     |
| no NTP  | 12dsRNAOH   | chMDA5:CARD | 1.50 ± 0.224    |
| no NTP  | 12dsRNAOH   | chMDA5:CARD | 1.50 ± 0.224    |
Figure S6

A

open

dRIG-I (4A2W)

semi-open

hRIG-I (4AY2)

semi-closed

hRIG-I (3TMI)

closed
dRIG-I (4A36)

chMDA5

chLGP2

hMDA5 (4GL2)

chLGP2

B

C

CTD

Hel1

Hel2

1*

2*

3*

4*

5*

6*

7*

G82 (lb)

F103 (lc)

V57 (la)

S404 (Ia)

G403 (Va)

T436 (V)

R375 (IV)

S404 (IVa)

G403 (IVa)

T436 (V)

R375 (IV)

P

P

P

P

P

P

P

P

P

P

P

P

P

P

P

P

P

P

P
Figure S7

A. Graph showing expression levels of chLGP2, helCTD, hLGP2, and hMDA5 in Chicken DF1 cells and Human Huh7.5 cells.

B. Graph showing IFNβ promoter activation in Human Huh7.5 cells under different conditions.

C. Bar graph showing hIFNβ promoter activation in Human HEK 293 cells.

D. Bar graph showing hIFNβ promoter activation in Human Huh7.5 cells.

E. Bar graph showing hIFNβ promoter activation in Human Huh7.5 cells.

F. Bar graph showing hIFNβ promoter activation in Human Huh7.5 cells.

G. Bar graph showing hIFNβ promoter activation in Human Huh7.5 cells.
Table S1. Quantification of the degree of opening (rotation angle and center of mass distance) of the Hel1 and Hel2 helicase domains in published RLH structures. Related to Figures 1, 4 and S6.

| RLH Structure | PDB ID | RNA | Bound Nucleotide | Angle (degree) | Distance* (Å) | State  |
|---------------|--------|-----|------------------|---------------|--------------|--------|
| chLGP2 (Reference) | 5JAJ | 10 base pair 5’p | ADP:AlF₄ | 0.0 | 0.0 | closed |
| dRIG-I | 4A36 | 19 base pair 5’OH | ADP:AlF₄ | 2.5 | 1.3 | closed |
| hMDA5 | 4GL2 | 12 base pair 5’OH | AMPPNP | 8.0 | 3.3 | semi-closed |
| chMDA5 | 5JC3 | 10 base pair 5’p | ADP | 10.1 | 3.9 | semi-closed |
| hRIG-I | 5E3H (3TMI) | 14 base pair 5’OH | ADP:BeF₃ | 12.1 | 5.3 | semi-closed |
| chLGP2 | 5JBJ | 12 base pair 5’p | - | 13.1 | 5.4 | semi-closed |
| hRIG-I | 4AY2 | 20-mer hairpin 5’ppp | ADP | 39.3 | 14.9 | semi-open |
| hRIG-I | 2YKG | 10 base pair 5’OH | SO₄ | 40.3 | 15.9 | open |
| dRIG-I | 4A2W(A) | no RNA | - | 51.8 | 18.5 | open |
| dRIG-I | 4A2W(B) | no RNA | - | 59.8 | 19.2 | open |
| mRIG-I | 3TBK | no RNA | AMPPNP | 68.2 | 24.4 | open |
Supplementary Experimental Procedures

Protein preparation

**chMDA5.** Chicken (*Gallus gallus*) MDA5 constructs (Genbank: NP_001180567) full-length *chMDA5* (residues 1-1001), *chMDA5 ΔCARD* (residues 298-994), *chMDA5ΔCARD-Q* (residues 298-994 with E436Q mutation) were cloned into pETM11 (EMBL) using a synthetic gene (GeneArt). All constructs were expressed in *E. coli* Rosetta 2 (Novagen) and cells were harvested and lysed by sonication in buffer containing 50 mM Tris pH 7.6, 500 mM NaCl, 10 % glycerol, 20 mM imidazole and 1 mM DTT. After centrifugation the lysate was applied to nickel-NTA-Superflow resin (QIAGEN). The protein was dialyzed against dialysis buffer (20 mM Hepes pH 7.5, 100 mM NaCl, 5% Glycerol, 1 mM DTT) at 4°C for 16 hours. The N-terminal 6-histidine-tag was cleaved with his-tagged Tobacco Etch Virus (TEV) protease during dialysis and cleaved protein repurified on nickel-NTA resin. *chMDA5* constructs were further purified with a cation exchange column (HiTrap SP, GE Healthcare) and size-exclusion chromatography (Superdex 200, GE Helthcare) in a buffer containing 20 mM Hepes pH 7.5, 150 mM NaCl and 2 mM DTT.

**chLGP2.** A synthetic gene (GeneArt) comprising the coding sequence of *chLGP2* (Genbank: AEK21509) with an added N-terminal GGGGS linker was cloned into pETM11SUMO (EMBL). Full-length *chLGP2* full length was expressed in *E.coli* Rosetta 2 as a Sumo fusion protein and cells were harvested and lysed by sonication in sonication buffer (50 mM Na₂HPO₄ pH 8, 300 mM NaCl, 5% glycerol, 50 mM arginine, 5 mM β-mercapto-ethanol). After centrifugation the lysate was applied to nickel-NTA-Superflow resin. The protein was dialyzed against sonication buffer at 4°C for 16 hours. The 6-histidine-tag and Sumo-tag was cleaved with TEV protease during dialysis. Cleaved protein was further purified with anion exchange column (HiTrap Q GE Healthcare) and size-exclusion chromatography (Superdex 200, GE Helthcare) in a buffer containing 20 mM Hepes pH 7.5, 200 mM NaCl and 250 µM TCEP (tris(2-carboxyethyl)phosphine).
**hLGP2.** Full-length hLGP2 (Genbank NM_024119.2) was cloned into the pETM11SUMO vector (EMBL) and expressed in *E.coli* and purified as for *chLGP2*. The chromatograph of a HiTrap Q purification shows two peaks, corresponding to hLGP2 monomers or dimers (the majority), as deduced from size-exclusion chromatography.

**RIG-I.** Full-length hRIG-I was cloned into pFastBAC and expressed in HiFive insect cells. Cells were harvested and lysed by sonication in buffer (50 mM Tris pH7.6, 500 mM NaCl, 10% glycerol, 20 mM Imidazole, 1 mM DTT). After centrifugation the lysate was applied to nickel-NTA-Superflow resin and further purified as for *chLGP2*. Full-length dRIG-I was purified from insect cells as described (Kowalinski et al., 2011).

**Crystallization**

For protein crystallization, 576 different conditions were initially screened in vapour diffusion sitting drop format using a Cartesian robot. After screening, the crystal conditions were optimized in 24-well hanging drop crystallization plates.

**chMDA5 1:1 complex:** Directly after size exclusion chromatography *chMDA5ΔCARD-Q* was mixed with 10 bp dsRNA in a 1:1 molar ratio and incubated for 30 minutes on ice. The complex was concentrated using an Amicon Ultra concentrator to around 10 mg/ml and 2 mM AMPPNP (adenosine 5'- (β,γ-imido)triphosphate lithium salt hydrate) was added. Sample and reservoir buffer (0.025 M Bis-Tris pH 6.5, 0.075 M succinic acid pH 7.0, 12-14% PEG 3350, 2% sucrose) were mixed in a 2:1 ratio. Three hours after setup, cover glasses with drops were transferred from a reservoir containing 12-14% PEG 3350 to one containing 8% PEG 3350. Crystals grew in one week at 20°C and were harvested in cryo-protectant solution (0.025 M Bis-Tris pH 6.5, 0.075 M succinic acid pH 7.0, 25% 3350, 10% ethylene glycol) before flash freezing with liquid nitrogen.

**chMDA5 2:1 complexes:** Directly after size exclusion chromatography *chMDA5ΔCARD-Q* was mixed with 24, 26 or 27 bp dsRNA in a 0.5:1 molar ratio and incubated for 30 minutes
on ice. The complexes were concentrated with an Amicon Ultra concentrator to around 10 mg/ml and 2 mM AMPPNP and 200 mM NDSB211 (Dimethyl(2-hydroxyethyl)ammonium propane sulfonate, Hampton Research) were added.

**chMDA5ΔCARD-Q 24-mer dsRNA-AMPPNP** was mixed with reservoir buffer (0.1 mM Hepes pH 7.5, 11-12% PEG 3350, 2-4% sucrose) in a 2:1 ratio. Crystals grew in three days at 20 degree and were harvested in cryo-protecting solution (0.1 mM Hepes pH 7.5, 25% 3350, 10% ethylene glycol) before flash freezing with liquid nitrogen.

**chMDA5ΔCARD-Q : 27-mer dsRNA-AMPPNP** was mixed with reservoir buffer (0.1 mM Hepes pH 7.5, 4-6% PEG 6000, 2-4% sucrose) in a 2:1 ratio. Crystals grew in three days at 20°C. The cover glasses of crystal-containing drops were transferred to a reservoir containing cryo-protecting solution (0.1 mM Hepes pH 7.5, 10% PEG 6000, 25% ethylene glycol) 12 hours before harvesting and flash freezing with liquid nitrogen.

**chLGP2**: chLGP2, directly after size exclusion chromatography, was mixed with dsRNA in a 1:1 ratio and incubated for 30 minutes on ice. The complexes were concentrated with an Amicon Ultra concentrator to around 10 mg/ml and then 2 mM ADP:AlF<sub>4</sub> (adenosine-5'-diphosphate: aluminium fluoride) and 2 mM MgCl<sub>2</sub> were added. All chLGP2 crystals were harvested in the reservoir buffer containing 25% ethylene glycol and flash frozen with liquid nitrogen.

**chLGP2 12-mer dsRNA-ADP:AlF<sub>4</sub>** complex was mixed with reservoir buffer (0.1 M Bis-Tris propane pH 6.5, 0.05-0.1 M magnesium formate) in a 2:1 ratio. Crystals grew in three days at 4°C.

**chLGP2 5' monophosphate 10-mer dsRNA-ADP:AlF<sub>4</sub>** complex was mixed with reservoir buffer (0.1 M tri-sodium citrate dihydrate pH 6.1, 0.04-0.08 M ammonium dihydrogen phosphate) in a 2:1 ratio. Crystals grew in one week at 4°C.

**chLGP2 5' triphosphate 10-mer dsRNA-ADP:AlF<sub>4</sub>** complex was mixed with reservoir
buffer (0.1 M Mg formate, 20% PEG3350M) in a 2:1 ratio. Crystals grew in one week at 4°C. **chLGP2 5' triphosphate, 3'-GG overhang hairpin RNA-ADP:AlF4** complex was mixed with reservoir buffer (0.1 M citric acid pH 5.0, 0.8 M ammonium sulphate) in a 1:1 ratio. Crystals grew in one day at 4°C from a sitting drop 96 well plate.

**Crystallography**

Diffraction data were collected at 100 K on beamlines ID23-1 or ID29 equipped with a Pilatus 6M detector or ID23-2 equipped with a Pilatus 2M-F (DECTRIS), at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Data were processed with the XDS suite and scaled with XSCE (Kabsch, 2010). All further analysis was done with the CCP4 suite (Winn et al., 2011) and refinement performed with REFMAC5 (Murshudov, 1997) or PHENIX (Adams et al., 2002). Models were built using COOT (Emsley and Cowtan, 2004), structure validation by MOLPROBITY (Chen et al., 2010) and structure figures drawn with PYMOL (DeLano, 2002). Buried surface are calculations performed with PISA (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (Krissinel and Henrick, 2007). Crystallographic and refinement statistics are given in Tables 1 and 2.

The *chMDA5* structure was initially determined by molecular replacement with PHASER (McCoy et al., 2007) using the *hMDA5* structure (PDB 4GL2). The *chLGP2* structure was initially determined with PHASER using the *hLGP2* CTD (PDB: 2W4R) and RIG-I helicase sub-domains as search models.

Crystals of MDA5 with 10- and 24-mer dsRNAs were of highly variable quality, requiring testing of many to obtain usable data. For the 10-mer complex, several related space-groups were obtained with two (*P2_1* or *P2_12_12_1*) or four (large *P2_1*) 1:1 complexes in the asymmetric unit. Both 10- and 24-mer crystals were generally twinned (probably lattice translocation disorder), this being manifested by weak but significant residual extra density after refinement that could be explained by the same model but referred to a different origin.
One 10-mer crystal in the $P2_1$ form was not twinned and gave a cleaner map and lower R-factors (Table 2).

**RNA preparation**

The palindromic 10 bp dsRNA with 5’ mono-phosphate or 5’ tri-phosphate (5’-pGGUACGUACC-3’ or 5’-pppGGUACGUACC-3’), 12 bp dsRNA with 5’ mono-phosphate (5’-pGGUAGCGCUACC-3’), 24 bp dsRNA with 5’ mono-phosphate (5’-pGGGACGUCAUGCGCAUGACGUCCC-3’) and 27 bp dsRNA with 5’ monophosphate (5’-pGGGCACGUGCAGGCACGCAGUCCC-3’) and 5’ triphosphate 3’-GG overhang 26-mer hairpin RNA (5’-pppGGAGCGUGCCGUACGGCACGCUCCGG-3’) were prepared by *in vitro* T7 transcription. Five times excess of GMP over GTP was added to the reaction for the 5’ mono-phosphate RNAs. RNA was purified by excising the band from denaturing urea-PAGE. A summary of RNAs used is shown in Figure S4.

**Electron microscopy**

Φ6 RNA (Thermo Scientific) is a mixture of 2948 bp (S segment), 4063 bp (M), and 6374 bp (L) dsRNAs. To form complexes, full-length chMDA5 or chLGP2 and Φ6 bacteriophage dsRNA were incubated during 5 min at 20°C in a buffer containing 20 mM Hepes pH 7.5, 150 mM NaCl, 2 mM ATP and 2 mM MgCl$_2$. Different protein:RNA ratios were used with a 1:1 ratio of MDA5/LGP2:dsRNA corresponding to one MDA5/LGP2 molecule for 15 bp of dsRNA. Where indicated, ATP was replaced by ADP, ADP:AlF$_4$ or no NTP analogues in order to test their influence.

For each condition tested, 4 μl of sample was applied to the clear side of carbon on a carbon-mica interface and stained with 1% (wt/vol) uranyl acetate. Images were recorded under low-dose conditions with a JEOL 1200 EX II microscope at 100 kV with a nominal magnification of 15,000×.

Cryo-EM grids were prepared by mixing 4 μl of MDA5 or LGP2 at 5 μM with
dsRNA in a 1:1 ratio. The sample was applied onto a glow-discharged quantifoil grid 400 mesh 3.5/1 (Quantifoil Micro Tools GmbH, Germany), the excess solution was blotted for 2 s with a Vitrobot (FEI) and the grid frozen in liquid ethane. Data collection was performed on a FEI Polara microscope operated at 300 kV. Fifty cryoEM micrographs were manually collected on a K2 summit electron direct detector (Gatan) at a magnification of 20,000×, giving a pixel size of 0.97 Å. Movies of 6 frames were collected with a total exposure time of 6 s and a total dose of 30e−/Å². The contrast transfer function (CTF) for each micrograph was determined with CTFFIND3 (Mindell and Grigorieff, 2003) and showed defocuses between 2.2 and 3.8 µm. A total of 7185 overlapping segments of MDA5 and 3153 overlapping segments of LGP2 (400 pixels long) were picked using EMAN boxer (Ludtke et al., 1999) with a shift of 64 pixels between adjacent segments. Corresponding segments were suggested to 2D classification in Relion1.3 (Scheres, 2012). Power spectra were calculated from masked 2D class averages.

**Fluorescence polarisation anisotropy**

dsRNAs used for anisotropy experiments were formed by annealing the following separate strands for 1 min at 95 °C and cooling down on ice in a buffer containing 20 mM Hepes, 2 mM EDTA at pH 6.8 (see also Figure S5 for RNA sequences):

- 12-mer dsRNA : 5′-FAMCAUGUGGAGCCC-3′ and 5′-OHGGGCUCCACAUG-3′,
- 12-mer dsRNA Appp : 5′-FAMCAUGUGGAGCCC-3′ and 5′-pppGGGCUCCACAUG-3′,
- 3′ overhang dsRNA : 5′-OHGGCCAGUGCGA-3′ and 5′-FAMCGCACUGGCGCA-3′,
- 5′ overhang dsRNA : 5′-OHAAAACCGGUCACGC-3′ and 5′-OHGAAGCGUGACCGGFAM-3′.

Anisotropy measurements were performed at room temperature using a multimodular fluorometer (Quantamaster QM4CW; Manufacturer: Photon Technology International). RNA concentration was adjusted to 2.5 nM (12-mer 5′ppp-dsRNA) or 5 nM (12-mer 5′OH-dsRNA, 3′ 2 nt overhang dsRNA and 5′ 2 nt overhang dsRNA for LGP2 measurements), 13
or 50 nM (12-mer 5’OH-dsRNA for MDA5 measurements) by mixing in the cuvette with buffer (20 mM Hepes pH 7.5 150 mM NaCl, 200 µM TCEP, 4 mM MgCl₂). Protein was titrated into the cuvette and incubated for 5 minutes before measurements. The total volume of titrated protein was less than 10% of the final volume. Anisotropy measurements were made with excitation wavelength 495 nm and emission wavelength 515 nm for 100 seconds. Experiments were performed more than three times per system. Binding data was analysed by Graph Pad Prism software and fitted with the following equation (eq. 1).

\[
y = \frac{(R+P^n+Kd^n) - \sqrt{(R+P^n+Kd^n)^2 - 4RP^n}}{2R} \times b + (m - b) \quad (\text{eq. 1})
\]

The experimental anisotropy (y), Initial anisotropy (b), the maximum anisotropy (m), the RNA concentration (R), and the protein concentration (P), equilibrium binding constant (Kd) and hill coefficient (n).Rig-I measurement curves are fitted with n=1 and the value of n are written in Figure S4. The error bars represent the S.D. value of at least three individual experiments.

**ATPase activity assays**

ATPase reactions were monitored using a Malachite green assay kit (Bioassays) over 0.5-30 min time courses. All proteins were pre-incubated with 4-fold molar excess of dsRNA for 10 minutes at 28 °C in ATP hydrolysis buffer (20 mM Hepes pH7.5, 100 mM NaCl, 1% glycerol, 2 mM DTT, 4 mM MgCl₂). The reaction was initiated by adding 2 mM ATP. For determination of kinetic parameters, the ATP concentration was varied from 0.125 to 10 mM. The reactions were quenched at 5 time points between 30 seconds to 4 minutes. The 10 µl reaction aliquots were quenched by mixing with 10 µl of quenching buffer (20 mM Hepes pH 7.5, 100 mM NaCl, 1% glycerol, 2 mM DTT, 100 mM EDTA). 80 µl of 5 times diluted Malachite green solution was added and developed for 30 minutes at room temperature. The absorbance at 622 nm was measured with a plate reader. For each ATP concentration, the
initial velocity was plotted and $K_m$ and $k_{cat}$ calculated using the Michaelis–Menten equation (eq. 2) in Graph Pad software Prism.

\[ Y = E_t \cdot k_{cat} \cdot \frac{X}{(K_m + X)} \]  (eq. 2)

$X$ is the substrate concentration, $Y$ is enzyme velocity and $E_t$ is the concentration of enzyme.

**Cellular assays.**

Myc-$h$MDA5 and Flag-$h$RIG-I constructs in pEF-BOS vector have been described previously (Kowalinski et al., 2011; Louber et al., 2015; Louber et al., 2014) and $ch$MDA5, $ch$LGP2, $h$LGP2 tagged at the N terminus with Flag peptide were sub-cloned into pCDNA3 (chicken) and pEF-BOS (human) vector as detailed elsewhere (Louber et al., 2015; Louber et al., 2014).

Chicken DF1 (Himly et al., 1998) and human Huh7.5 (Sumpter et al., 2005) cells were maintained in Dulbecco’s Minimum Essential Medium culture medium supplemented with 10% foetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 10 mg/ml gentamycin and 1% non-essential amino acids for Huh7.5 cells at 37°C and 5% CO2. Being of chicken origin, DF1 cells naturally lack endogenous RIG-I (Barber et al., 2010), but express functional endogenous MDA5, LGP2 and type I IFN system (Karpala et al., 2011; Liniger et al., 2012). Huh7.5 cell line is defective in MDA5 and IFN receptor expression and expresses inactive T55I RIG-I (Binder et al., 2011; Eguchi et al., 2000; Keskinen et al., 1999; Li et al., 2005; Sumpter et al., 2005), a phenotype verified experimentally by the inability of these cells to respond to both RIG-I and MDA5 RNA agonists (Figure S7 and our previous data (Louber et al., 2015)).

Protein expression was analysed after lysis of DF1 and Huh7.5 cells (6-well plate, 6x10^5 cells/well) transfected the day before with 2.5 µg DNA using TransIT®-LT1 Transfection Reagent (Mirus Bio) (7.5 µl/well), separation by polyacrylamide gel
electrophoresis and visualisation by western immunoblotting using anti-Flag (1:1,000; M2; Sigma, St. Louis, MO, USA), anti-C-Myc (1:50) 9E10, anti-GAPDH (1:2,000; Millipore, Billerica, MA, USA) monoclonal antibodies.

The human IFNβ promoter luciferase assay was performed essentially as described (Louber et al., 2015; Louber et al., 2014). Briefly, Huh7.5 cells seeded one day before (96 wells, 2 x104 cells/well) were co-transfected with MDA5 and LGP2 expressing vectors (33 ng of DNA/well to which was added 17 ng of DNA/well of pHRL-tk-Renilla-Luc (Promega)) together with the reporter pβIFN-fl-lucter (King and Goodbourn, 1994) (50 ng of DNA/well), using TransIT®-LT1 Transfection Reagent (Mirus Bio) (0.3 µl /well), followed 24 hours later by transfection of either poly(I:C) (Amersham Biosciences), Φ6 dsRNA (Thermo Scientific) or T7-transcribed 61-mer 5′ppp-dsRNA (Marq et al., 2011) in complex with Oligofectamine™ Transfection Reagent (Invitrogen) (0.6 µl/well). The chicken IFNβ promoter luciferase assay was performed following exactly the same procedure, but using chicken adapted material, i.e. DF1 cells, pCDNA3 based expression vectors for MDA5 and LGP2, pHRL-CMV-Renilla-Luc (Promega) instead of pHRL-tk-Renilla-Luc, the reporter pGL3-P_chIFNβ-Luc (Liniger et al., 2012). For both human and chicken assay, control experiments were included by transfecting the same amount of a neutral plasmid (pCG-duF) (Cathomen et al., 1995). This plasmid was also used to complement transfected DNA so as to reach total DNA amount of 100 ng per well. Data were expressed as mean +/-SD of normalized luciferase activity. Statistical analysis (Student t test) was done on data from three independent experiments, each done in independent triplicates.
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