



HAL
open science

Salmonid Double-stranded RNA–Dependent Protein Kinase Activates Apoptosis and Inhibits Protein Synthesis

Lise Chaumont, Mathilde Peruzzi, Pierre Boudinot, Bertrand Collet, François Huetz, Claudine Raffy, Jérôme Le Hir, Jules Minke

► **To cite this version:**

Lise Chaumont, Mathilde Peruzzi, Pierre Boudinot, Bertrand Collet, François Huetz, et al.. Salmonid Double-stranded RNA–Dependent Protein Kinase Activates Apoptosis and Inhibits Protein Synthesis. 2024. hal-04724637

HAL Id: hal-04724637

<https://hal.science/hal-04724637v1>

Preprint submitted on 12 Dec 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Salmonid double-stranded RNA-dependent protein kinase (PKR) activates apoptosis and**
2 **inhibits protein synthesis**

3 **Running title: Loss and gain of PKR function in salmonids**

4 Lise Chaumont*, Mathilde Peruzzi*, François Huetz†, Claudine Raffy‡, Jérôme Le Hir‡, Jules
5 Minke‡, Pierre Boudinot*, Bertrand Collet*

6 * Université Paris-Saclay, INRAE, UVSQ, VIM, 78350 Jouy-en-Josas, France

7 † Unit of Antibodies in Therapy and Pathology, UMR 1222 INSERM, Institut Pasteur, 75015 Paris,
8 France

9 ‡ VIRBAC S.A., 06510 Carros, France

10 Corresponding author:

11 bertrand.collet@inrae.fr

12 This project was funded in part by the European Union through AQUAEXCEL3.0 (Grant Agreement
13 871108) and AQUAFAANG (Grant Agreement 817923) and by the Research Council of Norway
14 through the project PMCV (Project 301083). LC was a recipient of PhD funded by Virbac and the
15 French Association for Research and Technology (ANRT) [Convention CIFRE #2020/0646] in
16 collaboration with the Fish Infection and Immunity laboratory (INRAE, VIM, Jouy-en-Josas,
17 France).

18 **Abstract (250 words)**

19 The dsRNA-dependent protein kinase (PKR) is a key factor of innate immunity. It is involved in
20 translation inhibition, apoptosis and enhancement of the proinflammatory and interferon responses.
21 However, how these antiviral functions are conserved during evolution remains largely unknown.
22 Overexpression and knockout studies in a Chinook salmon (*Oncorhynchus tshawytscha*) cell line
23 were conducted to assess the role of salmonid PKR in the antiviral response. Three distinct mRNA
24 isoforms from a unique *pkr* gene, named *pkr-fl* (full length), *pkr-ml* (medium length) and *pkr-sl* (short
25 length), were cloned and a *pkr*^{-/-} clonal fish cell line was developed using CRISPR/Cas9 genome
26 editing. PKR-FL includes an N-terminal dsRNA-binding domain and a C-terminal kinase domain,
27 while PKR-ML and PKR-SL display a truncated or absent kinase domain, respectively. PKR-FL is
28 induced during IFNA2 stimulation but not during viral hemorrhagic septicemia virus (VHSV)
29 infection. Overexpression experiments showed that only PKR-FL possesses antiviral functions
30 including activation of apoptosis and inhibition of *de novo* protein synthesis. Knockout experiments
31 confirmed that PKR is involved in apoptosis activation during late stage of VHSV infection.
32 Endogenous PKR also plays a critical role in translation inhibition upon poly(I:C) transfection after
33 IFNA2 treatment. It is, however, not involved in translational arrest during VHSV infection. Extra-
34 and intracellular titrations showed that endogenous PKR does not directly inhibit viral replication but
35 apparently favors virion release into the supernatant, likely by triggering late apoptosis. Altogether,
36 our data confirm that salmonid PKR has conserved molecular functions, that VHSV appears to bypass
37 with subversion strategies.

38 **Key points (85 characters including spaces/sentence)**

- 39
- 40 • Three isoforms of Chinook salmon PKR are present in CHSE-EC cells
 - 41 • Full-length PKR triggers apoptosis and inhibits *de novo* protein synthesis
 - 42 • Endogenous PKR is not involved in translational arrest during VHSV infection
 - 43 • Endogenous PKR favors virion release into the supernatant at a late infection stage

43 Keywords: PKR, EIF2AK2, Chinook salmon, *Oncorhynchus tshawytscha*, interferon response,
44 apoptosis, translational arrest, antiviral activity

45 **Introduction**

46 The host innate immunity is the first line of defense against viral infections. Rapid and efficient
47 detection of viruses is critical to mount an immune response capable of limiting virus replication and
48 propagation to neighboring cells. It is well established that pathogen-associated molecular patterns
49 (PAMPs) are recognized by sensors called pattern recognition receptors (PRRs). Activation of PRRs
50 triggers signaling cascades, which subsequently leads to the production of host defense molecules,
51 including type I interferons (IFNs), proinflammatory cytokines and chemokines. Type I IFNs induce
52 the transcription of hundreds of IFN-stimulated genes (*ISGs*), including effector genes, which can
53 have direct antiviral actions or modulate cell physiology to inhibit viral infection, replication and
54 propagation ¹.

55 Eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2), better known as dsRNA-
56 dependent protein kinase (PKR), is one of the most studied proteins encoded by an *ISG*. It is
57 recognized as a multifunctional key factor of innate immunity, as it acts both as a sensor and an
58 effector in response to viral infections. Mammalian PKR is constitutively and ubiquitously expressed
59 at low levels in all tissues and as all *ISG* products, its expression is induced by a variety of stress
60 associated responses, including type I IFNs, LPS stimulation and viral infections ^{2,3}. Structurally,
61 PKR contains an N-terminal regulatory region with two dsRNA-binding motifs (dsRBMs) and a C-
62 terminal kinase domain ^{2,4}. PKR requires an activation step to be fully catalytically functional ^{5,6}. This
63 activation is primarily mediated by binding to dsRNA, which is produced during the replication cycle
64 of RNA viruses ⁶. Interactions with dsRNA occur through its dsRBMs, leading to homodimer
65 formation mediated by the N-terminal lobe of the kinase domain ^{4,7}. PKR dimerization induces
66 conformational changes that allow the *trans* autophosphorylation of the activation loop at critical
67 conserved threonine residues ⁷. Once activated, PKR phosphorylates the α -subunit of the eIF2
68 complex, which is required for the initiation of mRNA translation. Phosphorylation of eIF2 α blocks
69 the recycling of inactive eIF2-GDP complex by the GTP exchange factor eIF2B, resulting in the
70 inhibition of the cell translation machinery ⁸.

71 Although PKR is best described for its effect on protein translation, it is also involved in many other
72 antiviral mechanisms. Overexpression of mammalian PKR is known for promoting apoptosis in
73 transfected cells via both the intrinsic mitochondrial and extrinsic death receptor pathways ⁹⁻¹² while
74 *Pkr*^{-/-} mouse embryonic fibroblasts (MEFs) or *PKR*^{-/-} HeLa cells displayed resistance to apoptosis in
75 response to dsRNA, TNF- α , or LPS ^{13,14}. The molecular mechanisms underlying PKR-mediated
76 apoptosis involve eIF2 α phosphorylation-dependent induction of specific stress response genes ¹⁴⁻¹⁸

77 and activation of transcription factors NF- κ B and p53¹⁹⁻²¹. PKR was also reported to modulate the
78 inflammatory and the type I IFN responses, by activating mitogen-activated protein kinases
79 (MAPKs)-dependent²²⁻²⁴ and NF- κ B signaling pathways^{20,25} and enhancing type I IFN production
80 upon infection with some but not all viruses^{21,26-29}. In most cases, the precise role of PKR in the
81 activation of these different pathways remains elusive and whether PKR acts directly or indirectly
82 has not been fully clarified. The importance of PKR role in the antiviral response is further
83 emphasized by the numerous subversion strategies developed by viruses to antagonize PKR-mediated
84 antiviral mechanisms, as recently reviewed by Cesaro and Michiels³⁰.

85 Orthologs of mammalian *PKR* have been found in fish genomes, in cartilaginous fish as well as bony
86 fish³¹. Some teleost fish families, including cyprinids, salmonids and clupeids, also possess a fish-
87 specific paralog of *pkr*, called *pkz*, which encodes a Z-DNA-dependent protein kinase³². *pkz* genes
88 in fish were isolated before fish *pkr* genes, in Crucian carp³³, zebrafish³⁴ and Atlantic salmon³⁵,
89 among other species. Although most studies on fish PKR did not dissect the structure-function
90 relationship as extensively as in mammals, they indicate that most antiviral functions attributed to
91 mammalian PKR are overall conserved in these organisms. Briefly, a few studies have reported that
92 fish PKR (and PKZ) are able to phosphorylate eIF2 α and inhibit *de novo* protein synthesis³⁶⁻⁴⁰, trigger
93 apoptosis^{38,41,42}, modulate the NF- κ B signaling pathway^{36,43} and the production of type I IFN^{44,45},
94 although the molecular mechanisms have not been investigated. Of note, in fish, most studies were
95 performed using overexpression approaches, which generally lead to unnaturally high levels of the
96 protein of interest. A few other studies have also used knock-down approaches based on morpholino
97 oligonucleotides⁴⁶, short interfering RNAs⁴⁰ or chemical inhibition^{44,47} but to the best of our
98 knowledge, no knockout *in cellulo* fish models have been developed so far.

99 In this study, we identified and cloned three different isoforms of *pkr* in a salmonid cell line from
100 Chinook salmon (*Oncorhynchus tshawytscha*). To investigate their respective antiviral functions, we
101 used both overexpression and knockout approaches. For this purpose, we developed the first *pkr*
102 knockout clonal fish cell line using CRISPR/Cas9 genome editing. We showed that PKR is involved
103 in apoptosis activation and plays a major role in translational arrest upon poly(I:C) stimulation but
104 not during VHSV infection. Our data further suggest that PKR favors the release of VHSV virions
105 into the supernatant at a late infectious stage, likely by triggering apoptosis. Taken together, our
106 results indicate that salmonid PKR exhibits conserved molecular functions but the endogenous
107 protein does not have a major antiviral effect against VHSV, likely due to evasion strategies evolved
108 by the virus.

109 **Material and methods**

110 **1. Cell lines, culture conditions and viruses**

111 The *Epithelioma papulosum cyprinid* (EPC) cell line was grown in Leibovitz's L-15 medium (Gibco)
112 supplemented with 10% fetal bovine serum (FBS, Eurobio) and penicillin (100 U/mL)-streptomycin
113 (100 µg/mL) (P/S) (BioValley). The Chinook salmon (*Oncorhynchus tshawytscha*) embryo (CHSE-
114 214) cell line was maintained in Glasgow's modified Eagle's medium (GMEM) containing 25 mM
115 HEPES (Biosera) supplemented with 10% FBS, 2 mM L-glutamine (Eurobio), and penicillin
116 (100 U/mL)-streptomycin (100 µg/mL). The CHSE-EC cell line, that was previously genetically
117 modified to stably express a monomeric enhanced green fluorescence protein (mEGFP)⁴⁸, as well as
118 its derivatives were grown in L-15 medium supplemented with 10% FBS, penicillin (100 U/mL)-
119 streptomycin (100 µg/mL), 500 µg/mL G418 (Invivogen), 30 µg/mL hygromycin B Gold
120 (Invivogen). All cell lines were maintained at 20°C without CO₂.

121 Recombinant viral hemorrhagic septicemia virus expressing tomato red fluorescent protein (rVHSV-
122 Tomato) was a kind gift from Dr. Stéphane Biacchesi (Université Paris-Saclay, INRAE, UVSQ, VIM,
123 Jouy-en-Josas, France)⁴⁹. rVHSV-Tomato was propagated in EPC cells (multiplicity of infection
124 (MOI) of 1): briefly, the virus was adsorbed onto the cells for 1h at 14°C with regular gentle shaking;
125 L-15+2% heat-inactivated FBS was added afterwards and the supernatants were collected at 5 days
126 post-infection, 0.2 µm-filtered, aliquoted and stored at -80°C. Golden shiner virus (GSV) was
127 propagated in CHSE-214 (MOI 1.5) at 22°C in GMEM+2% FBS, as described above for rVHSV-
128 Tomato. At 10 days post-infection, the remaining cells were scraped and the cell suspension was bath-
129 sonicated (4 x 1 min at ~40 kHz) (EMAG AG), clarified at 400 g for 5 min, 0.2 µm-filtered, aliquoted
130 and stored at -80°C. rVHSV-Tomato and GSV titers were determined by plaque assay (as described
131 in section 11).

132 **2. Development and validation of a *pkr*^{-/-} cell line**

133 The previously established CHSE-EC cell line (hereafter referred to as EC) was used to develop a
134 *pkr*^{-/-} cell line. Four single guide RNAs (sgRNAs) were designed within the first and second coding
135 exons of the *pkr* gene (LOC112253229, LG24), using CRISPOR v5.01 web tool (Table I)⁵⁰. To
136 ensure the specificity of the sgRNAs, care was taken that no off-target genes with more than 3
137 mismatches in the first 12 bp adjacent to the PAM (most likely off-targets) were identified in the
138 Chinook salmon genome (Otsh_v2.0, NCBI RefSeq assembly GCF_018296145.1). A sgRNA
139 targeting the *mEGFP* gene was also used as previously designed⁴⁸.

140 The sgRNAs were synthesized using the T7 RiboMAX™ Express Large Scale RNA Production
141 System kit (Promega) using 0.5 µg of each primer, incubated with 1µL of RQ1 DNase (Promega) for
142 1h at 37°C and purified using TRIzol™ reagent (Invitrogen), according the manufacturers'
143 instructions. The sgRNAs were resuspended in RNase- and DNase-free water and quantified using a
144 Nanodrop spectrophotometer. The purity of the sgRNAs was checked on a 2% agarose-EtBr gel
145 before or after a 30 min treatment with RNase A (Qiagen) at room temperature. Each sgRNA was
146 mixed with recombinant TrueCut™ Cas9 Protein v2 (Invitrogen) at a 1:1 molar ratio (0.2 µg sgRNA
147 and 1 µg rCas9 ie. 6.1 pmol each in 2 µL) and incubated at room temperature for 20 min. The sgRNA-
148 mEGFP/Cas9 complex was mixed with each sgRNA-PKR/Cas9 complex at 1:1 volume ratio in
149 resuspension buffer R (Neon™ Transfection System kit, Invitrogen) (1 µL of each complex in a final
150 volume of 5 µL). The mix was transfected into EC cells using the Neon™ Transfection System
151 (Invitrogen): EC cells were prepared as described in section 4 and 5 µL of cell suspension at 2×10^7
152 cells/mL was mixed with 5 µL of sgRNA/Cas9 complex (ie. 1×10^5 cells, 6.1 pmol of Cas9 and 6.1
153 pmol of sgRNA per 10 µL of transfection reaction). The cells were transfected using the same
154 conditions established for plasmids, as described in section 4. All transfected cells ($\sim 5 \times 10^5$ cells)
155 were mixed in 5 mL L-15+10%FBS+P/S in a 25 cm² flask (Sarstedt) and incubated at 20°C for 3-4
156 weeks.

157 Once the cell population reached confluency, the transfected cells were passaged (surface ratio 1:4),
158 and $\sim 2 \times 10^6$ cells were used for genomic DNA extraction using NucleoSpin Tissue Mini kit
159 (Macherey-Nagel), according to the manufacturer's instructions. Genomic DNA segments containing
160 the targeted sites were amplified by PCR using GoTaq® G2 Flexi DNA Polymerase (Promega) using
161 the following genotyping primers: PKR-gen1-F/PKR-gen1-R and PKR-gen2-F/PKR-gen2-R for *pkc*
162 coding exon 1 and exon 2, respectively (Table I). The PCR cycling program was performed in a
163 thermal cycler (Eppendorf) and was as follows: 94°C for 3 min then 35 cycles of 94°C for 15 s, 58°C
164 for 15 sec, 72°C for 40 sec, and a final extension of 72°C for 5 min. The PCR products were purified
165 using NucleoSpin Gel and PCR Clean-up Mini kit (Macherey-Nagel) and directly sequenced using
166 the same amplification primers (Sanger sequencing service, Eurofins) (Table I). Sequences were
167 analyzed using Synthego ICE analysis tool v3 (Synthego)⁵¹ to assess the percentage of mutated cells
168 in the transfected cell populations (bulks). sgRNA-PKR2-4 failed to show any genome editing at the
169 targeted sites so only the bulks transfected with sgRNA-PKR1 were further used for clonal isolation.

170 Cells from two independent cell populations transfected with sgRNA-PKR1 were either manually
171 isolated or sorted by FACS, respectively. For manual isolation, $\sim 1 \times 10^5$ cells were seeded in a 6-well
172 plate and serially diluted (6-fold dilutions) in duplicates. Three to four weeks post-seeding, clonal

173 cell patches were marked, analyzed under a fluorescent Axio Observer Z1 microscope (Zeiss,
174 Oberkochen, Germany) and ~50 mEGFP-deficient clones were selected, detached mechanically by
175 scraping with a pipette tip and sub-cultured into 48-well plates. After 3-4 weeks, 22 clones were sub-
176 cultured and propagated in 25 cm² flasks and their genotype was characterized as described above.
177 For FACS-sorted clones, cells were detached by trypsin-EDTA action and mEGFP-deficient single
178 cells at a density of ~4 x 10⁶ cells/mL were individualized by a BD FACSAria™ Fusion Flow
179 Cytometer (Institut Pasteur, Paris, France) using a 100 µm nozzle into a 96-well plate (Sarstedt) in L-
180 15 supplemented with 10% FBS, penicillin (200 U/mL)-streptomycin (200 µg/mL), 500 µg/mL G418
181 and 30 µg/mL hygromycin B Gold. Seven weeks later, 5 clones were sub-cultured and propagated in
182 25 cm² flasks and their genotype was characterized as described above.

183 Two manually isolated clones, EC-PKR-C19 and EC-PKR-C28, presenting a 1-nt insertion
184 (29_30insT resulting in V11fsX22, KO) and a 6-nt deletion (30_35delGTGCGA, resulting in
185 C11_E12del, WT-like) at the sgRNA targeted site respectively, and two FACS-sorted clones, EC-
186 PKR-C3 and EC-PKR-C5, presenting the same 1-nt insertion (29_30insT resulting in V11fsX22, KO)
187 at the targeted site, were kept for knock-out validation by western blot. For this purpose, these clones
188 and the WT EC cell line were seeded into 6-well plates at a density of 1.2 x 10⁶ cells/well in L-
189 15+2%FBS+P/S. The next day, cells were stimulated in triplicates with *Salmo salar* IFNA2
190 supernatant (produced as described in section 5) diluted to 1:10 in L-15+2%FBS+P/S or left untreated
191 and incubated at 20°C. At 72h post-stimulation, medium was removed, cells were washed once with
192 ice-cold DPBS, scraped in 1 mL ice-cold DPBS supplemented with 2.5 mM EDTA and centrifuged
193 at 1500 g at 4°C for 5 min. The cell pellets were drained, resuspended in 100 µL NP-40 lysis buffer
194 (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% NP40, 1 mM DTT, 10% glycerol,
195 cOmplete™ protease inhibitor (Merck), PhosSTOP™ phosphatase inhibitor (Merck)) and lysed for
196 45 min at 4°C under gentle shaking. The cell lysates were clarified by centrifugation at 5000 g at 4°C
197 for 5 min and stored at -80°C until use for western blot analysis as described in section 7.

198 **3. Plasmid constructions**

199 Chinook salmon *pkc* open reading frame (ORF) sequences were identified *in silico* using NCBI
200 Reference Otsh_v2.0 Primary Assembly and predicted transcripts XM_042305680.1 and
201 XM_042305681.1. Total RNA from 1.5 x 10⁶ EC cells infected with rVHSV-Tomato (MOI 1) at 48
202 and 72hpi in triplicates was extracted using the QiaShredder and RNeasy mini kits (Qiagen) according
203 to the manufacturer's instructions. RNA (1 µg) was used as template for reverse transcription and
204 generation of cDNA using the QuantiTect Reverse Transcription kit (Qiagen) with random primers.

205 The cDNA was diluted two-fold with RNase-free water and used as template to amplify the *pkr* ORF
206 sequence. Nested PCR amplifications were performed using Q5 2X High-Fidelity mastermix (New
207 England Biolabs) and 2 sets of specific primers according to the manufacturer's instructions: OtPKR-
208 R0-F/ OtPKR-R0-R and OtPKR-R0bis-F/OtPKR-R0bis-R were used for the first PCR round while
209 OtPKR-P2A-F and OtPKR-HindIII-R were used for the second PCR round (Table I). The PCR
210 cycling programs were performed in a thermal cycler (Eppendorf) and were as follows: 98°C for 30
211 sec followed by 35 cycles of 98°C for 15 s, 66°C (1st round) or 60°C (2nd round) for 15 sec, 72°C for
212 90 sec, and a final extension of 72°C for 2 min. PCR products were purified with the NucleoSpin Gel
213 and PCR Clean-up Mini kit (Macherey-Nagel) and sent to sequencing. Purified PCR products were
214 digested with HindIII enzymes (ThermoFisher), cloned into HindIII/EcoRV-digested pcDNA3.1-Zeo-
215 BFP vector using T4 DNA ligase (New England Biolabs) according to the manufacturer's instructions
216 and fully sequenced. The pcDNA3.1-Zeo-BFP vector was initially obtained by subcloning the BFP
217 gene amplified from pCite-P-BFP⁵² into a pcDNA3.1(-)-Zeo backbone (Invitrogen) using BFP-
218 F/BFP-R primers (Table I) followed by XhoI/HindIII digestion. The pCite-PBFP plasmid was a kind
219 gift from Dr. Hortense Decool (Université Paris-Saclay, INRAE, UVSQ, VIM, France). All plasmids
220 were produced in Stellar™ Competent Cells (Takara) and were purified using NucleoBond Xtra Maxi
221 EF (Macherey-Nagel) according to the manufacturer's instructions.

222 **4. Transfections**

223 Transfections were performed by electroporation using the Neon™ Transfection System (Invitrogen)
224 as described previously⁴⁸. Briefly, the cells were washed in DPBS (Sigma-Aldrich), detached by
225 trypsin-EDTA action, resuspended in L-15+10%FBS+P/S and centrifuged at 400 g for 5 min. The
226 cell pellet was drained, resuspended in L-15 without Phenol Red (Gibco), centrifuged at 13 000g for
227 30 sec, and resuspended again in L-15 without Phenol Red. The cell concentration was adjusted to
228 2×10^7 cells/mL. Depending on the experiment, the cell suspension was mixed with various plasmid
229 constructs (Table II) to reach a final concentration of $0.5 \mu\text{g}/1 \times 10^5$ cells in 10 μL of transfection
230 reaction. A fluorescent vector (mEGFP or RFP-KDEL) was added to verify and/or normalize
231 transfection efficiency between each condition.

232 The cells were then transfected using the same conditions established for plasmids for CHSE-214
233 cells or EPC cells⁵³. Briefly, transfections were carried out in an electroporator MPK5000 (Neon™
234 Transfection System, Invitrogen) using either a 10 μL or a 100 μL transfection kit (Neon™
235 Transfection System, Invitrogen) set to two pulses for 20 ms at 1300 V (CHSE-214 and derivatives)

236 or 1400 V (EPC). All transfected cells were mixed in L-15+10%FBS+P/S, split into flasks or plates
237 (depending on the experiment) and incubated at 20°C for a time determined for each experiment.

238 **5. Production of *Salmo salar* IFNA2 supernatant**

239 In order to produce IFNA2-containing supernatant, 44 x 10⁶ EPC cells were transfected with
240 pcDNA3.1-Zeo-ssIFNA2⁵⁴ or pcDNA3.1-Zeo-mEGFP (control) using the conditions described in
241 section 3. The transfected cells were pooled in 24 mL of L15+10%FBS+P/S, split into two 75 cm²
242 flasks and incubated at 20°C. At 48h post-transfection, supernatants were collected and clarified by
243 centrifugation 400 g, 5 min, 0.2 µm filtered, aliquoted and stored at -80°C until use. The RTG-P1 cell
244 line (ATCC CRL-2829), which constitutively expresses the firefly luciferase gene under the promoter
245 of the IFN-induced *mx1* gene⁵⁵, was used to determine the IFN activity of the IFNA2 supernatants
246 produced. Briefly, 1:4 serial dilutions in L-15 of the harvested supernatants were applied onto 4.5 x
247 10⁴ RTG-P1 cells in 96-well plates in quadruplicates and cells were incubated at 20°C. At 30h post-
248 stimulation, medium was removed and 75 µL of Steady-Glo® Luciferase Assay System (Promega)
249 was added in each well and the cell lysates were processed as described in section 8.

250 **6. Real-time quantitative PCR**

251 EC (WT) cells were seeded in 6-well plates to a final density of 1.5 x 10⁶ cells/well in L-
252 15+2%FBS+P/S. The next day, cells in triplicates or quadruplicates were infected with rVHSV-
253 Tomato (MOI 1) at 14°C for 8 or 24h, stimulated with recombinant *Salmo salar* IFNA2 supernatant
254 (produced as described in section 5) diluted to 1:10 in L-15+2%FBS+P/S for 72h or left untreated.

255 Total RNA was extracted from cells in individual P6 wells in triplicates or quadruplicates using
256 QiaShredder and RNeasy mini kits (Qiagen) in accordance with the manufacturer's instructions.
257 Quality control of the samples was determined using a Nanodrop spectrophotometer. The cDNA was
258 generated from 1.5 µg of total RNA using the iScript™ Advanced cDNA Synthesis Kit for RT-qPCR
259 (BioRad) and the synthesis was performed in a thermal cycler (Eppendorf) as recommended by the
260 manufacturer. cDNA was diluted to 1:3 in DNase- and RNase-free water and stored at -20°C until
261 use. "No RT" control reactions were made by omitting the reverse transcriptase. The cDNA was
262 mixed with TB Green qPCR Premix Ex Taq (Tli RNaseH Plus) (Takara) along with forward and
263 reverse primers (Table III) at a final concentration of 210 nM each in Twin.tec® real-time PCR plates
264 (Eppendorf). Amplification was performed using a CFX Connect cycler (BioRad) using the following
265 cycling program: initial denaturation at 95°C for 30 sec followed by 40 cycles of 10 sec at 95°C and
266 30 sec at 60°C. For each biological replicate, mean Cq values of target genes were calculated based

267 on technical duplicate reactions and then normalized using the geometric mean of Cq values of 3
268 housekeeping genes (*otelf1a*, *otrps29*, *otgapdh3*). The relative expression of each target gene (*otpk-*
269 *fl*, *otpk-ml*, *otpk-fl* and *otmx123*) was expressed as $2^{-\Delta Cq}$, which was then used to calculate their
270 respective fold change in comparison to non-stimulated cells.

271 For each set of primers, the efficiency was calculated by linear regression obtained by using ten-fold
272 serial dilutions of plasmid containing the target sequence (target genes) or of a pool of cDNA
273 (housekeeping genes) and the qPCR products were validated by gel migration and sequencing. For
274 the primers targeting each PKR isoform, the specificity of each primer set was validated by checking
275 their cross-reactivity using plasmid constructs developed in this study.

276 **7. Immunoblotting**

277 Aliquots of 60 μ L of cell lysates (obtained as described in sections 2 and 8) were mixed with 30 μ L
278 Laemmli buffer (45 mM Tris, 345 mM glycine, 38% glycerol, 4.8% SDS, 20% β -mercaptoethanol,
279 0.04% bromophenol blue) and incubated at 100°C for 5 min.

280 A volume of 8 μ L of cell lysates was loaded on 10% or 12% polyacrylamide gels and protein samples
281 were separated by electrophoresis in Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8,3).
282 Proteins were then transferred onto a nitrocellulose membrane (BioRad) using the mixed molecular
283 weight program from the Trans-Blot® Turbo™ Transfer System (BioRad). The blots were blocked
284 either with 5% non-fat milk or 5% BSA in TBST (10 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween
285 20) for 1h at room temperature and then incubated with primary antibodies overnight at 4°C. The
286 following primary antibodies were used with the dilution factors and buffers indicated in Table VI:
287 rabbit anti-PKR antiserum, mouse monoclonal anti-GFP, rabbit polyclonal anti-eIF2 α -P, mouse
288 monoclonal anti-puromycin, mouse monoclonal anti-VHSV N, rabbit anti-GSV antiserum, rabbit
289 anti-Mx123 antiserum. The custom-made anti-PKR antibody, initially raised against *Salmo salar* PKR
290 ⁴⁷, was a generous gift from Pr. Øystein Evensen (NMBU, Oslo, Norway). The anti-VHSV N
291 antibody was a kind gift from Dr. Stéphane Biacchesi (Université Paris-Saclay, INRAE, UVSQ, VIM,
292 Jouy-en-Josas, France). The anti-Mx123 antibody, raised against rainbow trout Mx3 ⁵⁶, was a
293 generous gift from Dr. Marta Alonso-Hearn (Department of Animal Health, NEIKER-Basque
294 Institute for Agricultural Research and Development, Basque Research and Technology Alliance,
295 Derio, Spain) and Pr. Jorunn Jorgensen (UiT The Arctic University of Norway, Tromsø, Norway).
296 The blots were washed 5 times in TBST, incubated with horseradish peroxidase (HRP)-conjugated
297 anti-mouse or anti-rabbit (1:4000) secondary antibodies (SeraCare), washed 4 times in TBST and

298 once in PBS. Western blots were developed using Clarity™ Western ECL substrate (BioRad) and
299 detected using ChemiDoc Touch Imaging System (BioRad). After the first detection, the membrane
300 was washed twice with TBST, stripped for 15 min at 37°C using Restore™ Plus buffer (Thermo
301 Scientific), washed twice in TBST, saturated with TBST-5% non-fat milk for 1h and re-probed with
302 a new primary antibody for 2.5h-3h and developed as described above. The following primary
303 antibodies were used for the second round of detection: mouse monoclonal anti- α -tubulin antibody,
304 rabbit polyclonal anti-actin, mouse monoclonal anti-eIF2 α (Table IV). Densitometric analysis of the
305 blots was performed using Image Lab software (v 6.1.0, Biorad).

306 **8. Assays for assessing translation inhibition activity of Chinook salmon PKR**

307 The translation inhibition activity of Chinook salmon PKR was assessed by luciferase assay and
308 immunoblotting. Luciferase assay is a method commonly used for detecting protein synthesis
309 inhibition activity of PKR in mammalian models and other vertebrates^{36-38,40,46,57,58} and was adapted
310 to quantify the translation inhibition activity of PKR isoforms. For this purpose, EC-PKR-C19 cells
311 were co-transfected with pcDNA3.1-Hyg-RFP-KDEL (0.2 μ g/10 μ L), pcDNA3-Neo-Luc
312 (0.25 μ g/10 μ L) and either pcDNA3.1-Zeo-BFP or pcDNA3.1-Zeo-BFP-P2A-PKR-FL/ML/SL
313 (0.25 μ g/10 μ L) or mock transfected, split into 96-well plates ($\sim 1 \times 10^4$ cells/well in 16 wells per
314 condition) and incubated at 20°C. At 24h post-transfection, medium was removed, 100 μ L DPBS was
315 added into each well and RFP fluorescence was measured using a fluorometer (Tecan Infinite
316 M200PRO) with excitation and emission wavelengths of 544 and 603 nm, respectively. Then, DPBS
317 was removed, 75 μ L of Steady-Glo® Luciferase Assay System (Promega) was added in each well
318 and the cell lysates were incubated in the dark at room temperature for 5 min. Cell lysates were
319 transferred into a flat-bottom white-walled plate (Greiner Bio-One) and luminescence was measured
320 using a Tecan Infinite M200 Pro luminometer over an integration period of 1000 ms (Tecan,
321 Männedorf, Switzerland). The luminescence value from each well was corrected by subtracting the
322 mean values obtained from the wells containing medium (blank), normalized to the corrected
323 fluorescence value from each well and graphed as fold change relative to the BFP transfected cells.

324 In a similar fashion, the protein synthesis inhibition activity of PKR isoforms was assessed by western
325 blot using mEGFP as a reporter system and puromycin as a marker of de novo protein synthesis. For
326 this purpose, EC-PKR-C19 cells were co-transfected with pcDNA3-Hyg-mEGFP (0.2 μ g/10 μ L) and
327 either pcDNA3.1-Zeo-BFP or pcDNA3.1-Zeo-BFP-P2A-PKR-FL/ML/SL (0.5 μ g/10 μ L) or mock
328 transfected, split into 6-well plates ($\sim 1.5 \times 10^6$ cells/well in quadruplicates) and incubated at 20°C for
329 30h. In parallel, cells were either treated with cycloheximide (CHX, 50 μ g/mL, Thermofisher) for

330 24h or treated with thapsigargin (Tg, 2 μ M, Merck) for 30 min. Both drugs are well-established
331 translation inhibitors were used as positive controls for translational inhibition^{59,60}. At 30h post-
332 transfection, cells were pulsed with 5 μ g/mL puromycin (Gibco) diluted in growth medium for 15
333 min at 20°C. The cells were then processed as described in section 2, with a lysis step performed in
334 75 μ L NP-40 lysis buffer, and cell lysates were stored at -80°C until use for western blot analysis as
335 described in section 7.

336 For knockout experiments, poly(I:C) treatment and virus infections were carried out. Poly(I:C)
337 transfections and not simple incubation were performed, as CHSE-214 are known for not responding
338 to extracellular poly(I:C)⁶¹. Non-stimulated or IFN-pretreated EC, EC-PKR-C28 and EC-PKR-C19
339 were transfected with high molecular weight poly(I:C)-rhodamine (Invivogen) at a final
340 concentration of 0.15 μ g per 1 x 10⁵ cells per 10 μ L of transfection reaction. Transfected cells were
341 split into 12-well plates (~3.3 x 10⁵ cells/well in triplicates), incubated at 20°C for 24h. For virus
342 infections, EC, EC-PKR-C28 and EC-PKR-C19 were seeded in 6-well plates to a final density of
343 1.5x10⁶ cells/well in L-15+2%FBS+P/S. The next day, cells were infected in triplicates with rVHSV-
344 Tomato or GSV at MOI 1 or left uninfected and incubated at 14°C or 22°C, respectively. At 24h post-
345 transfection or 16, 24 and 40h post-infection, cells were pulsed with puromycin and processed as
346 described above until use for western blot analysis.

347 **9. Apoptosis assay**

348 Apoptosis was assessed by genomic DNA (gDNA) fragmentation and by measuring enzymatic
349 activity of caspase 3/7, caspase 8 and caspase 9.

350 For the gDNA fragmentation assay, EC-PKR-C19 cells were co-transfected with pcDNA3.1-Hyg-
351 RFP-KDEL (2 μ g/100 μ L) and either pcDNA3.1-Zeo-BFP or pcDNA3.1-Zeo-BFP-P2A-PKR-
352 FL/ML/SL (5 μ g/100 μ L). Around 4 x 10⁶ transfected cells were seeded in 25 cm² flasks incubated
353 at 20°C. At 6hpt, growth medium was removed, cells were washed three times in PBS to eliminate
354 dead cells and new growth medium was added into each flask. At 72 hpt, growth medium was
355 collected, pooled with trypsinized cells and centrifuged at 400 g for 5 min. The cell pellets were
356 drained and used for genomic DNA extraction using NucleoSpin Tissue Mini kit (Macherey-Nagel),
357 according to the manufacturer's instructions. For each condition, 3 μ g of gDNA was loaded onto a
358 1.5% agarose-EtBr gel for each condition.

359 The enzymatic activity of caspase 3/7, caspase 8 and caspase 9 was measured by using the Caspase-
360 Glo[®] 3/7 assay kit (Promega), in accordance with the manufacturer's instructions. For overexpression

361 experiments, EC-PKR-C19 cells were co-transfected with pcDNA3.1-Hyg-RFP-KDEL
362 (0.2 µg/10 µL) and either pcDNA3.1-Zeo-BFP or pcDNA3.1-Zeo-BFP-P2A-PKR-FL/ML/SL
363 (0.5 µg/10 µL) or mock transfected with water instead of plasmid. A volume of 75 µL of transfected
364 cells was seeded in 96-well plates (Sarstedt) to a final density of 1×10^5 cells/well and incubated at
365 20°C. In parallel, EC-PKR-C19 cells were stimulated with staurosporine (STS, 1µM, Santa Cruz) or
366 infected with GSV (MOI 1) and incubated at 22°C for 24h. At 72h post-transfection or 24h post-
367 treatment (STS or GSV), 75 µL of either Caspase-Glo[®] 3/7, Caspase-Glo[®] 8 or Caspase-Glo[®] 9
368 reagent was added to each well (8 and 4 technical replicates per condition for transfection and
369 treatment conditions, respectively). The plates were gently shaken on a plate shaker for 30 sec and
370 incubated in the dark at room temperature for 30 min. The luminescence levels in the cell lysates
371 were measured as described above in section 8. For knockout experiments, EC, EC-PKR-C28, EC-
372 PKR-C19, EC-PKR-C3 and EC-PKR-C5 cells were seeded in 96-well plates to a final density of $5 \times$
373 10^4 cells/well in L-15+2%FBS+P/S. The next day, the growth medium was removed and the cells
374 were infected in quadruplicates with 75 µL of rVHSV-Tomato suspension at MOI 10, MOI 1 or MOI
375 0.1 or left uninfected and incubated at 14°C. At 24, 48 and 72 hpi, cells were lysed with 50 µL of
376 Caspase Glo[®] 3/7 reagent and the same protocol described above was applied for luminescence
377 measurement.

378 **10. rVHSV-Tomato fluorescence monitoring**

379 The replication of rVHSV-Tomato in cells expressing PKR isoforms or in knockout cell lines was
380 monitored by sequential fluorescence measurement. For overexpression experiments, EC-PKR-C19
381 cells were co-transfected with pcDNA3.1-Pur-mEGFP (0.2 µg/10 µL) and either pcDNA3.1-Zeo-
382 BFP or pcDNA3.1-Zeo-BFP-P2A-PKR-FL/ML/SL (0.5 µg/10 µL) or mock transfected and split in
383 quadruplicates in 96-well plates and incubated at 20°C for 24h. For knockout experiments, EC, EC-
384 PKR-C28, EC-PKR-C19, EC-PKR-C3 and EC-PKR-C5 cells were seeded in 96-well plates to a final
385 density of 5×10^4 cells/well in L-15+2%FBS+P/S. The next day, the medium was removed and cells
386 were infected in quadruplicates (overexpression experiment) or octuplicates (KO experiment) with
387 rVHSV-Tomato at MOI 0.1, 1 or 10 in L-15 without Phenol Red (Gibco)+2%FBS+P/S or left
388 uninfected. At 24, 32, 48, 56, 72, 80 and 96h post-infection, the tomato red fluorescence was
389 measured using a fluorometer (Tecan Infinite M200PRO) with excitation and emission wavelengths
390 of 548 and 593 nm, respectively. The fluorescence values were corrected by subtracting the mean
391 values obtained from the non-infected wells.

392 **11. Virus titration in extracellular supernatants and intracellular sonicates**

393 EC, EC-PKR-C28, EC-PKR-C19 cells were seeded in 25 cm² flasks to a final density of 4x10⁶
394 cells/flask in L-15+2%FBS+P/S. The next day, cells were infected in quadruplicates with rVHSV-
395 Tomato at MOI 1 incubated at 14°C. At 96 h post-infection, the supernatants (4 mL) were collected
396 and kept on ice. The remaining cells were washed 3 times in cold DPBS, scrapped in 2 mL of new
397 growth medium and bath-sonicated (4 x 1 min~40 kHz) (EMAG AG). Supernatants and sonicates
398 were clarified by centrifugation at 400 g for 5 min and virus titers were determined by plaque assay
399 on EPC cells under a carboxymethylcellulose overlay (0.75% in MEM (Eurobio) supplemented with
400 25 mM HEPES, 2 mM L-glutamine, 350 mg/L NaHCO₃, 2.5% FBS and P/S. At 3 to 4 dpi, cell
401 monolayers were fixed with 3.7% formol for 1h at room temperature, stained with 0.5% crystal violet
402 and plaque forming units (PFU) were counted.

403 **12. Statistical analysis**

404 Results shown in each figure were derived from at least two independent experiments; the data
405 presented are means ± standard deviation (SD). Statistical tests used are indicated in the legend of
406 each. All statistical analyses were performed using GraphPad Prism software version 9.5.1.

407

408 **Results**

409 *Diversity of pkr mRNA isoforms in salmonid EC cells*

410 In addition to the two rounds of whole genome duplication (WGD) events that occurred during the
411 early evolution of chordates and vertebrates, the genomes of salmonid fish have undergone two
412 supplementary WGD rounds, including a teleost-specific third WGD that dates back to 225-333
413 million years ago and a salmonid-specific WGD ~80-100 million years ago⁶²⁻⁶⁴. As a consequence,
414 for each single-copy gene in tetrapods, up to four copies can be found in distinct loci in salmonids, if
415 all have been retained. In reality, following WGD events, duplicated genes can either be lost,
416 pseudogenized, sub- or neo-functionalized, with frequent additional tandem duplication events⁶⁵. In
417 mammals and birds, the *pkr* gene is typically unique^{31,32}. In contrast, two or three *pkr* paralogs have
418 been reported in amphibians (e.g. *Xenopus laevis* and *X. tropicalis*)³². Similarly, several fish genomes
419 also comprise two or more *pkr* paralogs and several ancient paralogs including *pkz* (Ensembl release
420 110). In salmonids, tBlastn analysis revealed a unique *pkr* gene in species belonging to genus *Salmo*
421 and *Oncorhynchus*. In the genome of Chinook salmon (*Oncorhynchus tshawytscha*), the *pkr* gene
422 was found at the locus LOC112253229 on LG24. The sequences of predicted isoforms
423 XP_042161614.1 and XP_042161615.1, which result from alternative splicing, contain 729 and 728
424 amino acids, and share 34.5%, 33.6% and 35.2% identity with human PKR (NP_001129123.1),
425 chicken PKR (NP_989818.3) and amphibian PKR (NP_001091256.1), respectively.

426 We first analyzed the diversity of *pkr* mRNAs in CHSE-EC cells (hereafter referred to as EC)⁴⁸.
427 Amplification of the full-length CDS using cDNA of rVHSV-Tomato-infected EC cells resulted in 3
428 distinct products of 2187 bp 1122 bp and 464 bp, respectively (Figure 1A, Table V). In the following
429 paragraphs, these three products will be referred to as *pkr-fl* (full length), *pkr-ml* (medium length) and
430 *pkr-sl* (short length). The *pkr-fl* product matched the predicted *pkr* transcript (XM_042305681.1) with
431 99.95% identity. It spans 19 exons, which corresponds to NCBI's predicted model, and encodes a
432 728 aa polypeptide matching the predicted PKR protein (XP_042161615.1) with 99.86% identity. In
433 contrast, the two shorter 1122 pb (*pkr-ml*) and 464 pb (*pkr-sl*) products covered 51% and 21% of the
434 sequence, respectively, and comprise 9 exons and 6 exons only. Of note, missing exons from *pkr-ml*
435 were also absent in *pkr-sl* and were all located in the middle part of the ORF. *pkr-ml* and *pkr-sl* are
436 likely products of alternative splicing and encode polypeptides of 373 aa and 107 aa, respectively.

437 Using SMART (Simple Modular Architecture Research Tool)^{66,67} and InterProScan database, three
438 double-stranded RNA binding motifs (dsRBM1-3) and a kinase domain were identified in the N-
439 terminal and C-terminal domains of PKR-FL, respectively (Figure 1C). This organization of

440 functional domains is generally shared by PKR of other vertebrates. Indeed, although only two
441 dsRBMs are found in mammalian and amphibian PKR, the numbers of dsRBMs present in fish PKR
442 actually varies from one to three³². Importantly, PKR-ML only contains one complete dsRBM and
443 the N-terminal portion of a second dsRBM fused to a truncated kinase domain, while PKR-SL only
444 comprises one dsRBM and no kinase domain.

445 Several important structural features known in mammalian PKR are conserved in Chinook salmon
446 PKR. The dsRBM(s) of all PKR isoforms are highly conserved, as shown by the alignment with
447 human PKR dsRBMs and consensus motif reported by Masliah et al.⁶⁸. In particular, the three motifs
448 of PKR-FL contain most of the residues involved in the canonical α_1 - β_1 - β_2 - β_3 - α_2 fold and/or dsRNA
449 binding (Figure 1D). In addition, dsRBM1 matches dsRBM consensus more closely than dsRBM2
450 and dsRBM3, as also observed for dsRBM1 and dsRBM2 in human PKR⁶⁹.

451 The primary structure of PKR-FL kinase domain contains the 11 conserved kinase subdomains
452 described by Hanks et al.⁷⁰, as mammalian PKR and other protein kinases (Figure 1E). In particular,
453 ATP binding motifs can be found, including the canonical Gly-X-Gly-X-X-Gly in subdomain I, Asp-
454 Leu-Lys-Pro-Ser-Asn in subdomain VI and Asp-Phe-Gln in subdomain VII, which are predicted to
455 interact with ATP through Mg²⁺ salt bridges². Subdomain II contains at position 461 the invariant
456 lysine residue that is directly involved in the transfer of the phosphate from ATP to its substrate².
457 Equivalents of the two threonine residues Thr446 Thr451 in human PKR⁷¹, which are key
458 autophosphorylation sites involved in PKR activation, namely, are also present in the so-called
459 activation loop of PKR-FL (Thr616 and Thr621). Finally, PKR-FL also contains features specific to
460 eIF2 α kinases, including an acidic kinase insert, that spans subdomain V and continues in the inter-
461 region between subdomains V and VI, and a conserved eIF2 α kinase motif located a few amino acids
462 away from the kinase insert, that is required for PKR kinase activity^{32,57}. On the other hand, PKR-
463 ML is missing the first 78 amino acids of a full-length kinase domain, which comprise domains I to
464 V. The invariant lysine residue of subdomain II is also absent from PKR-ML kinase domain. This
465 residue is required for mammalian PKR kinase activity^{71,72}. Therefore, the catalytic activity of PKR-
466 ML is expected to be limited or absent. PKR-SL lacks the entire kinase catalytic domain and is
467 expected to be catalytically inactive.

468 *PKR-FL is induced following IFN stimulation but not during VHSV or GSV infections*

469 The expression profiles of PKR isoforms in response to different stimuli were characterized by
470 western blot and RT-qPCR on EC cells stimulated with recombinant *Salmo salar* IFNA2 or infected

471 with two different viruses: viral hemorrhagic septicemia virus (VHSV), an enveloped negative-sense
472 single-stranded RNA virus belonging to the genus *novirhabdovirus*, and golden shiner virus (GSV),
473 a naked, double-stranded RNA virus belonging to the genus *aquareovirus*. A recombinant VHSV
474 encoding the fluorescent protein tdTomato, rVHSV-Tomato, was used to facilitate the monitoring of
475 the infection before each sampling timepoint⁴⁹. For both viruses, CPE appearance was also visually
476 checked and the progression of the infection was verified by western blot using specific antibodies
477 (data not shown).

478 At the protein level, PKR-FL was constitutively expressed at low levels in non-treated EC cells and
479 its expression was significantly induced in IFNA2-stimulated cells at the protein level from 48h post-
480 stimulation (~1.7-fold increase) to 72h post-stimulation (~2.4-fold increase) (Figure 2A,B). However,
481 no induction of PKR-FL was detected during viral infections with either rVHSV-Tomato or GSV at
482 any of the time points examined (16-40h post-infection). These results correlate with *pkr-fl* expression
483 at the transcript level: a strong induction of *pkr-fl* mRNA expression was observed after at 72h post-
484 stimulation compared to non-stimulated cells (~31-fold increase) (Figure 2D) but no significant
485 induction was detected in rVHSV-Tomato-infected cells at 8 or 24hpi. Remarkably, a similar
486 response was observed with transcripts of *mx1/2/3*, which are well-known IFN stimulated genes both
487 in mammals and fish^{73,74}. A ~5 log-fold increase in relative expression was observed upon IFNA2
488 stimulation, while no induction was detected in rVHSV-Tomato infected cells.

489 Although recombinant PKR-ML was detected by western blot using the same polyclonal anti-PKR
490 serum used to detect PKR-FL, endogenous PKR-ML could not be detected at the protein level in EC
491 cells in response to IFNA2 stimulation or virus infection (Figure 2A). However, *pkr-ml* mRNA was
492 amplified by RT-qPCR. Although its expression was lower than *pkr-fl* at the steady state (Figure 2C),
493 it displayed a modest but significant induction at 24h post-infection with rVHSV-Tomato and upon
494 IFNA2 stimulation (5-fold and 3.8-fold increase, respectively) (Figure 2D).

495 Endogenous PKR-SL expression could not be assessed by western blot, as recombinant PKR-SL was
496 not detected by our polyclonal anti-PKR antibody. RT-qPCR data revealed a very low expression of
497 *pkr-sl* compared to *pkr-ml* or *pkr-fl* (Figure 2C) and no induction was observed upon rVHSV-Tomato
498 infection or IFNA2 treatment.

499 Overall, our observations show that PKR-FL expression is predominant at both transcript and protein
500 levels and is strongly induced by type I IFN but not viral infection in EC cells. On the other hand,
501 *pkr-ml* mRNA expression is slightly induced by both type I IFN and viral infection, contrary to *pkr-*
502 *sl*, which is not induced in all contexts.

503 *CRISPR/Cas9-based edition of the pkr gene leads to null mutation and abolishes PKR expression in*
504 *EC cells*

505 To further characterize Chinook salmon PKR function in the antiviral response, we disrupted the
506 unique *pkr* gene in EC cells, using CRISPR/Cas9 technology. In the following study, four isolated
507 clones were further characterized. Three of them, namely EC-PKR-C19; EC-PKR-C3 and EC-PKR-
508 C5 presented a 1-nt insertion at the targeted cut site leading to a frameshift resulting in the
509 introduction of a premature codon at position 22 (29_30insT resulting in V11fsX22) (Figure 3A). A
510 fourth clone, EC-PKR-C28, exhibited a 6-nt deletion leading to the deletion of two amino acids,
511 Cys11 and Glu12 (30_35delGTGCGA, resulting in C11_E12del). This clone was considered “WT-
512 like” and it was used as an additional positive control for further experiments. As previous
513 experiments showed that IFNA2 was a fast and potent inducer of PKR-FL in EC cells, the PKR
514 expression status in each clone was validated by western blot using IFNA2 stimulated cells. Our
515 results confirm that PKR-FL was strongly expressed in WT EC cells and WT-like EC-PKR-C28 cells
516 stimulated with IFNA2 for 72h, while no induction of PKR-FL was observed in *pkr*^{-/-} EC-PKR-C19
517 and -C3 and -C5 clones (Figure 3B,C).

518 *Chinook salmon PKR triggers apoptosis during viral infection*

519 The functional role of mammalian PKR in apoptosis has been widely described in the literature^{9,11,13}
520 and has also been reported in fish^{38,39,41}. However, the contribution of PKR to apoptotic reaction
521 during viral infection remains poorly defined especially in non-mammalian models. We therefore
522 characterized the activation of the caspase cascade in EC cells by gain and loss-of-function
523 experiments after viral infection. For this purpose, we performed a genomic DNA laddering test,
524 which is a hallmark of apoptosis, and used a luminescence-based enzymatic assay to quantify the
525 catalytic activity of specific caspases, including Caspase 3/7, Caspase 8 and Caspase 9. Caspase3/7
526 is an executioner caspase that is activated downstream of the caspase cascade upon apoptosis
527 signaling. It can either be activated by (1) the extrinsic pathway via extracellular signals or (2) the
528 intrinsic pathway aka. mitochondrial pathway upon intracellular signals and stresses. Caspase 8 is
529 known for being activated by the extrinsic pathway while caspase 9 is mainly activated by the intrinsic
530 pathway⁷⁵.

531 **Overexpression of PKR-FL induces apoptosis.** To avoid inadvertent activation of endogenous PKR
532 in transfected cells, the ability of PKR isoforms to trigger apoptosis was assessed in *pkr*^{-/-} EC-PKR-
533 C19 cells transfected with the corresponding expression plasmids. Cells stimulated with staurosporine
534 (STS), a well-known activator of apoptosis^{76,77} or infected with GSV, a lytic virus known for

535 inducing apoptosis (unpublished data), were included as positive controls. Electrophoresis results
536 show gDNA laddering in GSV-infected cells and, to a lesser extent, in STS-stimulated cells,
537 suggesting that apoptosis was triggered by both treatments (Figure 4A, right panel). These qualitative
538 results were confirmed by measuring caspase enzymatic activity: indeed, caspase3/7 was activated
539 upon stimulation with STS or GSV (3.6-fold and 6.5-fold increase, respectively), thereby validating
540 the assay (Figure 4B). For cells transfected with PKR-FL but not with PKR-ML and PKR-SL, a faint
541 gDNA fragmentation was visible, suggesting the presence of apoptotic cells following overexpression
542 of PKR-FL (Figure 4A, left panel). Similarly, transfection of PKR-FL but not PKR-ML or PKR-SL
543 triggered the activation of caspase3/7 in *pkr*^{-/-} EC-PKR-C19 cells, with a 2.6-fold increase of activity
544 compared to BFP transfected cells (Figure 4B).

545 To decipher if both extrinsic and intrinsic pathways are involved in PKR-FL-mediated Caspase3/7
546 activation, the activation of Caspase 8 and Caspase 9 was assessed after overexpression of PKR
547 isoforms (Figure 4C,D). Our data show that both caspase 8 and caspase 9 were activated upon
548 overexpression of PKR-FL but not with PKR-ML or PKR-SL, although caspase 8 activation was less
549 pronounced (1.2-fold increase for caspase 8 compared to 1.6 fold-increase for caspase 9). Of note,
550 caspase 9 but not caspase 8 was activated in EC cells treated with STS, which is known to primarily
551 trigger apoptosis *via* the intrinsic pathway^{78,79}.

552 **Loss of function experiment in *pkr*^{-/-} cells show that endogenous PKR triggers apoptosis during**
553 **VHSV infection.** Although PKR-FL was not induced during rVHSV-Tomato infection (Figure
554 2A,B), constitutively expressed endogenous PKR could play a role in apoptosis activation during
555 viral infection. To test this hypothesis, WT EC cells, WT-like EC-PKR-C28 cells and *pkr*^{-/-} EC-PKR-
556 C19, -C3 and -C5 clones were infected with rVHSV-Tomato at two different MOI (MOI 1 or MOI
557 0.1) and caspase3/7 activity was measured at 24h, 48h and 72h post-infection.

558 Our results show that the caspase 3/7 activity observed in WT EC and WT-like EC-PKR-C28 was
559 almost fully abolished in all *pkr*^{-/-} clones starting from 48h post-infection at MOI 1 (Figure 5A). A
560 similar but delayed response was observed at MOI 0.1, with a significantly higher caspase3/7 signal
561 in WT EC and WT-like EC-PKR-C28 cells compared to *pkr*^{-/-} clones that occurred at 72h post-
562 infection (Figure 5B). Interestingly, caspase 3/7 signal was significantly lower in WT-like EC-PKR-
563 C28 compared to WT EC cells at 48h post-infection at MOI 1 (Figure 5A) and this difference was
564 even more marked at MOI 10 (data not shown). Two hypotheses can explain this discrepancy: (1) the
565 6-nt deletion resulting in a 2-aa deletion (Cys11 and Glu12) might alter PKR ability to induce
566 apoptosis; (2) as shown in Figure 3C, PKR-FL is slightly but significantly less induced in WT-like

567 EC-PKR-C28 upon IFNA2, suggesting that endogenous PKR-FL expression level is somewhat lower
568 in this clone for a reason independent from the 2-aa deletion (clonal effect) and as a consequence,
569 apoptosis might be slightly less activated. The fact that PKR-FL-C11_E12del isolated from EC-PKR-
570 C28 exhibits a similar translation inhibition activity that WT PKR-FL supports the second hypothesis
571 (Figure S2).

572 Our data illustrate that PKR-FL induces apoptosis *via* the caspase 8 and caspase 9 pathways and that
573 it plays a key role in the activation of apoptosis during rVHSV-Tomato infection.

574 *Chinook salmon PKR is involved in host translational arrest*

575 PKR is also known for its role in host translation inhibition in virus infected cells *via* phosphorylation
576 of eIF2 α . The role of PKR in protein synthesis inhibition in EC cells was investigated by western blot
577 using two different approaches: overexpression of PKR isoforms and activation of endogenous PKR
578 with poly(I:C).

579 **Overexpression of PKR-FL induces a cellular shutoff of host protein translation.** To examine
580 the role of Chinook salmon PKR isoforms on host protein translation, *pkrr*^{-/-} EC-PKR-C19 cells were
581 co-transfected with expression vectors encoding PKR isoforms and with a plasmid featuring a
582 reporter gene encoding either mEGFP or firefly luciferase. Protein synthesis was assessed by
583 quantification of mEGFP expression by western blot or by luciferase luminescence assay,
584 respectively. Importantly, in contrast to WT EC cells, *pkrr*^{-/-} EC-PKR-C19 cells do not constitutively
585 express mEGFP, as the disruption of the transgene is our screening criterion for successful gene
586 edition (see Material and Methods, data not shown). Therefore, the GFP signal only corresponds to
587 the mEGFP encoded by the transiently transfected plasmid. Co-transfections with plasmids encoding
588 PKR-FL but not PKR-ML or PKR-SL resulted in a drastic reduction of GFP signal intensity (Figure
589 6A,B). Similar results were obtained with luciferase as a reporter gene (Figure 6D). A similar assay
590 was performed to compare luciferase activity upon transfection with WT PKR-FL and mutated PKR-
591 FL-C11_E12del isolated from WT-like EC-PKR-C28 (Figure S2). Our results showed that co-
592 transfection with PKR-FL-C11_E12del expression plasmid resulted in a severe decrease in luciferase
593 activity, as WT PKR-FL overexpression, indicating that they exhibit similar translation inhibition.
594 These observations further suggest that the loss of Cys11 and Glu12 in WT-like EC-PKR-C28 does
595 not affect drastically the protein functions.

596 Since these assays only assess the expression of exogenous reporter genes and not on endogenous
597 cellular proteins, we also evaluated the impact of each PKR isoform on the translation of endogenous

598 proteins. For this purpose, transfected cells were pulsed with puromycin, an aminonucleoside which
599 is incorporated into nascent peptides, leading to premature chain termination and spontaneous
600 dissociation from the ribosome^{80,81}. Puromycylated nascent chains can be detected by western blot
601 using anti-puromycin antibodies. Puromycin can therefore replace radioactive tracers such as S³⁵
602 methionine and be used as a metabolic probe to measure *de novo* protein synthesis⁸⁰. Cycloheximide
603 and thapsigargin were used as positive controls, since these drugs are well-established translation
604 inhibitors^{59,60}. Consistent with reporter gene experiments, western blot analysis showed that the
605 levels of puromycin incorporated into newly synthesized proteins decreased significantly with PKR-
606 FL but not with PKR-ML or PKR-SL overexpression (Figure 6A,C). These results suggest that PKR-
607 FL overexpression is able to inhibit host protein translation in the absence of any PKR activator.

608 **Translation shutoff induced by type I IFN and poly(I:C) is abolished in *pkr*^{-/-} cells.** In order to
609 confirm the role of endogenous PKR in host translation inhibition, we compared the response of WT
610 and WT-like cells (EC and EC-PKR-C28, respectively) and *pkr*^{-/-} EC-PKR-C19 cells upon
611 transfection with poly(I:C), a synthetic analog of dsRNA. Poly(I:C) was used as a viral dsRNA mimic
612 to activate PKR, while simultaneously avoiding the potential viral subversion mechanisms that can
613 occur during viral infections. Before transfection, all cells were either pretreated with IFNA2 for 48h
614 in order to induce PKR expression or left untreated. Western blot analysis showed no differences in
615 puromycin signal intensity after poly(I:C) transfection between non-pretreated cell lines (Figure
616 7A,B, left panel). In contrast, when cells were pretreated with IFNA2, the levels of puromycin
617 incorporated into newly synthesized proteins significantly decreased upon poly(I:C) transfection in
618 WT EC and WT-like EC-PKR-C28 cells (37% and 48% reduction, respectively) but not in *pkr*^{-/-} EC-
619 PKR-C19 cells (Figure 7A and 7B, right panel). Taken together, these results show that endogenous
620 PKR expression is required for the inhibition of host translation induced by poly(I:C).

621 *Endogenous PKR is not the main driver of protein synthesis shutoff induced by rVHSV-Tomato*
622 *infection*

623 To assess the role of endogenous PKR in translation inhibition in a context of viral infection, which
624 is intrinsically more complex than poly(I:C) stimulation, we compared the response of WT EC and
625 WT-like EC-PKR-C28 cells with that of *pkr*^{-/-} EC-PKR-C19 cells during rVHSV-Tomato infection.
626 In all cell lines, the puromycin signal intensity decreased as early as 24h post-infection and drastically
627 dropped at 40h post-infection (Figure 8A and 8D). These data negatively correlate with eIF2 α
628 phosphorylation, which increased at 24h and peaked at 40h post-infection (Figure 8A and 8C).
629 Interestingly, VHSV N was expressed as early as 16h post-infection and expression levels increased

630 over time (24-40h post-infection), despite the decrease in host *de novo* protein synthesis (Figure
631 8A,B). However, no significant differences between WT, WT-like and *pkr*^{-/-} cell lines were detected
632 at all infection time points examined. These results indicate that VHSV-induced phosphorylation of
633 eIF2 α closely correlates with a decrease in host translation, while viral protein synthesis continued to
634 increase during the shutoff of host protein synthesis. The absence of differences between cell lines
635 shows this phenomenon involves a PKR-independent mechanism.

636 To further verify those results, all cell lines were pretreated with IFNA2 before rVHSV-Tomato
637 infection. However, there were, once again, no differences between cell lines despite high levels of
638 PKR expression (Figure S3). These findings further confirm the hypothesis that phosphorylation of
639 eIF2 α and host translation inhibition is not mediated by PKR during rVHSV-Tomato infection.
640 Similar results were obtained with GSV, a dsRNA virus. Indeed, although GSV infection led to
641 decreased levels of puromycin incorporated into proteins and increased phosphorylation of eIF2 α at
642 24h post-infection, no differences between cell lines could be observed (Figure S3).

643 *Chinook salmon PKR can play antagonistic roles in virus replication*

644 Immunoblots showing a similar expression of VHSV N protein in WT, WT-like and *pkr*^{-/-} cell lines
645 were indicative that PKR had little to no effect on VHSV replication at an early stage of infection. To
646 investigate further PKR role in virus replication and production, we used rVHSV-Tomato, in which
647 the expression cassette encoding tdTomato was inserted in the N-P intergenic region of VHSV
648 genome and is therefore expressed only during its viral replication cycle⁴⁹. The use of this virus
649 enabled us to monitor viral replication using fluorescence as a non-invasive proxy for viral
650 replication.

651 **Overexpression of PKR-FL inhibits rVHSV-Tomato replication.** To assess whether Chinook
652 salmon PKR is involved in the inhibition of viral replication, *pkr*^{-/-} EC-PKR-C19 cells were
653 transfected with plasmids encoding different PKR isoforms and subsequently infected with rVHSV-
654 Tomato. The fluorescence of rVHSV-Tomato was monitored from 24h to 96h post-infection. A
655 modest but constant reduction in measured fluorescence was observed in PKR-FL transfected cells
656 from 48h post-transfection to 96h post-transfection (Figure 9A). Once normalized to the mean
657 fluorescence measured in BFP-transfected cells at each time point, this represents a decrease of
658 around 30% (Figure 9B). Similarly, overexpression of PKR-FL but not PKR-ML or PKR-SL resulted
659 in a limited but significant decrease in viral titer in the supernatants at 96h post-infection (Figure 9C).
660 Consistent with previous fluorescence results, this constitutes a drop of ~25% (calculated on the basis

661 of non-log transformed titers) compared to BFP-transfected cells. Taken together, these results
662 suggest that overexpression of PKR-FL but not other isoforms inhibits rVHSV-Tomato replication.

663 **Endogenous PKR favors the release of virus into the supernatants.** The role of endogenous PKR
664 during rVHSV-Tomato infection was investigated by comparing the evolution of the red fluorescence
665 in WT EC, WT-like EC-PKR-C28 and *pkcr*^{-/-} EC-PKR-C19 cell lines from 24h to 96h post-infection.
666 The fluorescence signal was significantly higher in *pkcr*^{-/-} EC-PKR-C19 compared to the WT and WT-
667 like cell lines and this difference was more pronounced at a high MOI: significant differences between
668 *pkcr*^{-/-} and WT cell lines started to appear as early as 48h, 72h and 96h post-infection at MOI 10, MOI
669 1 and MOI 0.1, respectively (Figure 10A-C). Importantly, a similar trend was observed in other *pkcr*^{-/-}
670 ^{-/-} clones (EC-PKR-C3 and EC-PKR-C5), excluding the hypothesis of a clone-specific effect (Figure
671 S4). Because tdTomato is not present in newly formed virions, supernatants of infected cells were
672 titrated by plaque assay. Surprisingly, viral titers in the supernatants were slightly but significantly
673 lower in *pkcr*^{-/-} EC-PKR-C19 cells compared to WT EC and WT-like EC-PKR-C28 cells (Figure 10D),
674 which was inconsistent with our fluorescence data, indicative of the intracellular level of tdTomato.
675 Because our previous results showed that PKR was involved in apoptosis at a late stage of viral
676 infection, we hypothesized that absence of PKR could inhibit virion release. If this were true, virions
677 would accumulate in the infected cells and a higher intracellular viral titer could be expected. To
678 clarify this point, after supernatant collection, remaining infected cells were washed three times,
679 sonicated and the sonicates were titrated by plaque assay. Our results show that intracellular viral
680 titers were weakly but significantly higher in *pkcr*^{-/-} EC-PKR-C19 cells compared to WT EC cells
681 (Figure 10E). However, there was no difference between *pkcr*^{-/-} EC-PKR-C19 and WT-like EC-PKR-
682 C28 cells. As mentioned earlier, this discrepancy may be explained by the fact that PKR is less
683 expressed in WT-like EC-PKR-C28 compared to WT EC.

684 Whilst there are differences in extracellular and intracellular titers between cell lines, the “total titer”,
685 ie. the total amount of extracellular and intracellular infectious virions produced by each cell line,
686 was not significantly modified by *pkcr* disruption. These results suggest that endogenous PKR does
687 not affect viral replication *per se*, but rather favors the release of viral particles into the supernatant.

688 Discussion

689 In this study, we have identified and cloned three isoforms of a unique Chinook salmon *pkc* gene,
690 named *pkc-fl*, *pkc-ml* and *pkc-sl*, the last two lacking important catalytic regions. Using
691 complementary *in cellulo* approaches based on overexpression and knockout studies, we showed that
692 salmonid PKC is involved in apoptosis activation and plays a major role in translational arrest upon
693 poly(I:C) stimulation but not during VHSV infection. Our data further indicate that PKC favors the
694 release of VHSV virions into the supernatant at a late infectious stage, likely by triggering apoptosis.
695 Taken together, our results demonstrate that salmonid PKC has conserved molecular functions
696 compared to their mammalian counterparts. However, the endogenous protein did not have a major
697 antiviral effect against VHSV, likely due to subversion strategies evolved by the virus.

698 In line with previous studies on mammalian and fish cell lines^{2,47} both RT-qPCR and western blot
699 analysis revealed a constitutive but modest expression of PKC-FL in EC cells, which was strongly
700 induced after IFNA2 treatment. In contrast, neither *pkc-fl* nor *mx1/2/3* expression was induced during
701 infection with either rVHSV-Tomato or GSV, both at transcript and protein levels. Comparable
702 results were previously obtained in the rainbow trout RTG-P1 cell line, which expresses the firefly
703 luciferase gene under the control of the *mx1* gene promoter⁵⁵. These observations suggest that the
704 IFN response was limited in the epithelial-like salmonid EC cells during rVHSV-Tomato infection,
705 due to virus-mediated IFN suppression mechanisms, possibly *via* its NV protein⁸²⁻⁸⁵.

706 Although endogenous PKC was not induced in EC cells during rVHSV-Tomato infection, we still
707 investigated the potential antiviral activity of PKC *via* overexpression and loss-of-function
708 approaches. In line with other overexpression studies in fish models^{37,45,46}, we observed that
709 overexpression of PKC-FL but not PKC-ML or PKC-SL resulted in a limited but significant decrease
710 in rVHSV-Tomato titers in the supernatant. Using a knockout approach, we further showed that
711 VHSV extracellular titers were slightly but significantly lower, while intracellular titers were higher
712 in the *pkc*^{-/-} cell line, compared to the WT cell line. Our results therefore suggest that endogenous
713 PKC does not affect viral replication and virion assembly *per se*, but favors the release of viral
714 particles into the supernatant. In contrast, it was recently reported that chemical inhibition of PKC in
715 rainbow trout RTG2 and RTGill cells lines did not have any impact on extracellular VHSV titers in
716 comparison to untreated cells⁴⁴. Differences between our results and this study may arise from
717 remanent PKC activity following chemical inhibition, which may downplay the full effect observed
718 in our study.

719 Enzymatic assays also showed that PKR plays a preponderant role in the activation of apoptosis and
720 triggers it at a relatively late stage of VHSV infection. Based on previous titration results, we propose
721 that *pkr* knockout could reduce virion release by limiting apoptosis activation at a late infection stage.
722 In contrast, PKR-FL antiviral activity upon overexpression would mainly occur through early
723 activation of apoptosis, when it is detrimental to efficient virus replication. Interestingly, the kinetics
724 of caspase 3 activation correlated with apoptotic kinetics described in the literature for
725 novirhabdoviruses⁸⁶. This relatively late activation of apoptosis has been attributed to VHSV NV
726 protein, which inhibits apoptosis at the early stage of viral infection and prevents infected cells from
727 undergoing cell death before mature viral particles are produced⁸⁶. On the other hand, apoptosis can
728 be advantageous at the late stage of viral replication to facilitate viral release and dissemination^{87,88}.
729 Precisely, while VHSV NV protein has an “early” anti-apoptotic function, the matrix (M) protein of
730 novirhabdoviruses is known for its pro-apoptotic properties⁸⁹. At a late stage of infection, it has been
731 suggested that abundant proapoptotic M protein takes over and triggers a late apoptosis activation,
732 which facilitates viral release and spread⁸⁶. It is therefore tempting to speculate that VHSV has
733 evolved a NV-driven strategy which takes advantage of PKR-mediated apoptosis.

734 Besides apoptosis, a key antiviral mechanism of PKR is the inhibition of the translation machinery
735 *via* phosphorylation of eIF2 α . By using reporter genes and quantifying puromycin incorporation into
736 newly synthesized proteins, we further demonstrated that both overexpressed PKR-FL and
737 endogenous poly(I:C)-activated PKR were able to inhibit host *de novo* protein synthesis. These results
738 are in line with several studies on mammalian and fish PKR, as recently reviewed by Chaumont et al.
739³¹. In a context of viral infection, we showed that VHSV-induced phosphorylation of eIF2 α closely
740 correlates with a decrease in host protein translation. However, no differences were observed between
741 WT and *pkr*^{-/-} cell lines, revealing that this phenomenon is PKR-independent. Importantly, PKR is
742 not the only kinase that can trigger host translational arrest; eIF2 α can be phosphorylated by four
743 other kinases: PERK (PKR-like ER kinase), GCN2 (general control non-derepressible 2), HRI (heme-
744 regulated inhibitor) and PKZ (Z-DNA-dependent protein kinase)^{34,90}. Although each eIF2 α kinase
745 primarily responds to specific stresses, several studies provide evidence that they may have
746 cooperative functions. For instance, eIF2 α phosphorylation can be mediated by PKR but also PERK
747 and GCN2 during viral infections^{91,92}. A recent study using chemical inhibitors showed that eIF2 α
748 phosphorylation and shutoff of host translation was mediated by PERK and not PKR during VHSV
749 infection^{44,93}. Using *pkr*^{-/-} cell lines instead of chemical inhibitors, our results confirm that PKR is
750 not to be the main driver of host translational arrest during VHSV infection. These results suggest

751 that VHSV is able to bypass PKR-mediated translation inhibition but not an alternative host signaling
752 pathway activated by another eIF2 α kinase.

753 Besides PKR-FL, we have also identified and cloned two other isoforms expressed in EC cells during
754 VHSV infection, namely PKR-ML and PKR-SL, which are splice variants of PKR-FL. We
755 demonstrated that PKR-ML and PKR-SL do not mediate apoptosis and inhibition of translation, most
756 likely because that they both lack important catalytic regions and are defective in kinase activity. The
757 fact that these two isoforms exist but do not retain any of the typical antiviral functions established
758 for PKR-FL raises the question of their physiological role(s) in the cell. Interestingly, weakly
759 expressed splice variants have been reported for mammalian PKR⁹⁴⁻⁹⁶ as well as for zebrafish PKZ
760^{34,97}. Li and Koromilas reported a splice variant (PKR Δ E7), in which exon 7 was spliced out, resulting
761 in a truncated 174-aa protein containing only dsRNA binding domains. Interestingly, PKR Δ E7
762 exhibited a dominant negative function, as co-expression of both isoforms relieved PKR-mediated
763 translation inhibition⁹⁵. Because PKR-SL also contains dsRBM1 and a truncated dsRBM2, it might
764 have a similar dominant negative activity as human PKR Δ E7. Abraham et al. also reported that
765 murine lymphocytic leukemia cells expressed a *Pkr* transcript presenting an in-frame deletion of 579
766 bp. The resulting truncated protein is strikingly similar to PKR-ML: in both cases, the in-frame
767 deletion occurred within dsRBM2 and in the C-terminal portion of the acidic kinase insert of the
768 kinase domain. Abraham et al. showed that this truncated protein was able to form dimers with
769 endogenous PKR and to bind dsRNA⁹⁶. Whether PKR-ML possesses similar characteristics remains
770 to be determined.

771 In conclusion, our results establish that salmonid PKR has conserved molecular functions, including
772 apoptosis activation and inhibition of protein synthesis. However, endogenous PKR does not play a
773 major antiviral role during VHSV infection. It seems that VHSV has evolved a strategy to subvert
774 PKR antiviral action, by limiting early PKR induction and evading PKR-mediated translational arrest.
775 VHSV also appeared to take advantage of PKR-mediated apoptosis to favor viral spread at a late
776 stage of infection.

777 **Acknowledgements**

778 We thank Pr. Øystein Evensen and Dr. Amr Gamil (NMBU, Oslo, Norway) for their kind gift of anti-
779 PKR Ab. We thank Marta Alonso-Hearn (Department of Animal Health, NEIKER-Basque Institute
780 for Agricultural Research and Development, Basque Research and Technology Alliance, Derio,
781 Spain) and Pr. Jorunn Jorgensen (UiT The Arctic University of Norway, Tromsø, Norway) for their

782 generous gift of anti-Mx Ab. We are grateful to Dr. Stéphane Biacchesi and Emilie Mérour (INRAE,
783 Jouy-en-Josas, France) for providing rVHSV-Tomato and anti-VHSV N Ab as well as for helpful
784 discussions. We thank Dr. Hortense Decool (INRAE, Jouy-en-Josas, France) for providing pCite-P-
785 BFP plasmid. We thank Dr. Dean Porter (INRAE, Jouy-en-Josas, France) for careful reading of the
786 manuscript and Dr. Irene Salinas (CETI, Department of Biology, University of New Mexico,
787 Albuquerque, USA) for insightful exchanges on apoptosis.

788

- 790 (1) Flajnik, M. *Paul's Fundamental Immunology*; Lippincott Williams & Wilkins, 2022.
- 791 (2) Meurs, E.; Chong, K.; Galabru, J.; Thomas, N. S.; Kerr, I. M.; Williams, B. R.; Hovanessian,
792 A. G. Molecular Cloning and Characterization of the Human Double-Stranded RNA-Activated
793 Protein Kinase Induced by Interferon. *Cell* **1990**, *62* (2), 379–390.
794 [https://doi.org/10.1016/0092-8674\(90\)90374-n](https://doi.org/10.1016/0092-8674(90)90374-n).
- 795 (3) Thomis, D. C.; Doohan, J. P.; Samuel, C. E. Mechanism of Interferon Action: cDNA Structure,
796 Expression, and Regulation of the Interferon-Induced, RNA-Dependent P1/eIF-2 Alpha
797 Protein Kinase from Human Cells. *Virology* **1992**, *188* (1), 33–46.
798 [https://doi.org/10.1016/0042-6822\(92\)90732-5](https://doi.org/10.1016/0042-6822(92)90732-5).
- 799 (4) Dar, A. C.; Dever, T. E.; Sicheri, F. Higher-Order Substrate Recognition of eIF2 α by the RNA-
800 Dependent Protein Kinase PKR. *Cell* **2005**, *122* (6), 887–900.
801 <https://doi.org/10.1016/j.cell.2005.06.044>.
- 802 (5) Lemaire, P. A.; Lary, J.; Cole, J. L. Mechanism of PKR Activation: Dimerization and Kinase
803 Activation in the Absence of Double-Stranded RNA. *J Mol Biol* **2005**, *345* (1), 81–90.
804 <https://doi.org/10.1016/j.jmb.2004.10.031>.
- 805 (6) Lemaire, P. A.; Anderson, E.; Lary, J.; Cole, J. L. Mechanism of PKR Activation by dsRNA.
806 *J Mol Biol* **2008**, *381* (2), 351–360. <https://doi.org/10.1016/j.jmb.2008.05.056>.
- 807 (7) Mayo, C. B.; Erlandsen, H.; Mouser, D. J.; Feinstein, A. G.; Robinson, V. L.; May, E. R.; Cole,
808 J. L. Structural Basis of Protein Kinase R Autophosphorylation. *Biochemistry* **2019**, *58* (27),
809 2967–2977. <https://doi.org/10.1021/acs.biochem.9b00161>.
- 810 (8) García, M. A.; Gil, J.; Ventoso, I.; Guerra, S.; Domingo, E.; Rivas, C.; Esteban, M. Impact of
811 Protein Kinase PKR in Cell Biology: From Antiviral to Antiproliferative Action. *Microbiol*
812 *Mol Biol Rev* **2006**, *70* (4), 1032–1060. <https://doi.org/10.1128/MMBR.00027-06>.
- 813 (9) Gil, J.; Esteban, M. The Interferon-Induced Protein Kinase (PKR), Triggers Apoptosis through
814 FADD-Mediated Activation of Caspase 8 in a Manner Independent of Fas and TNF- α
815 Receptors. *Oncogene* **2000**, *19* (32), 3665–3674. <https://doi.org/10.1038/sj.onc.1203710>.
- 816 (10) Lee, S. B.; Esteban, M. The Interferon-Induced Double-Stranded RNA-Activated Protein
817 Kinase Induces Apoptosis. *Virology* **1994**, *199* (2), 491–496.
818 <https://doi.org/10.1006/viro.1994.1151>.
- 819 (11) Srivastava, S. P.; Kumar, K. U.; Kaufman, R. J. Phosphorylation of Eukaryotic Translation
820 Initiation Factor 2 Mediates Apoptosis in Response to Activation of the Double-Stranded
821 RNA-Dependent Protein Kinase *. *Journal of Biological Chemistry* **1998**, *273* (4), 2416–2423.
822 <https://doi.org/10.1074/jbc.273.4.2416>.
- 823 (12) Yeung, M. C.; Liu, J.; Lau, A. S. An Essential Role for the Interferon-Inducible, Double-
824 Stranded RNA-Activated Protein Kinase PKR in the Tumor Necrosis Factor-Induced
825 Apoptosis in U937 Cells. *Proc Natl Acad Sci U S A* **1996**, *93* (22), 12451–12455.
- 826 (13) Der, S. D.; Yang, Y.-L.; Weissmann, C.; Williams, B. R. G. A Double-Stranded RNA-
827 Activated Protein Kinase-Dependent Pathway Mediating Stress-Induced Apoptosis. *Proc Natl*
828 *Acad Sci U S A* **1997**, *94* (7), 3279–3283.
- 829 (14) Zuo, W.; Wakimoto, M.; Kozaiwa, N.; Shirasaka, Y.; Oh, S.-W.; Fujiwara, S.; Miyachi, H.;
830 Kogure, A.; Kato, H.; Fujita, T. PKR and TLR3 Trigger Distinct Signals That Coordinate the
831 Induction of Antiviral Apoptosis. *Cell Death Dis* **2022**, *13* (8), 1–15.
832 <https://doi.org/10.1038/s41419-022-05101-3>.
- 833 (15) Guerra, S.; López-Fernández, L. A.; García, M. A.; Zaballos, A.; Esteban, M. Human Gene
834 Profiling in Response to the Active Protein Kinase, Interferon-Induced Serine/Threonine
835 Protein Kinase (PKR), in Infected Cells. *Journal of Biological Chemistry* **2006**, *281* (27),
836 18734–18745. <https://doi.org/10.1074/jbc.M511983200>.

- 837 (16) Hsu, L.-C.; Park, J. M.; Zhang, K.; Luo, J.-L.; Maeda, S.; Kaufman, R. J.; Eckmann, L.;
838 Guiney, D. G.; Karin, M. The Protein Kinase PKR Is Required for Macrophage Apoptosis after
839 Activation of Toll-like Receptor 4. *Nature* **2004**, 428 (6980), 341–345.
840 <https://doi.org/10.1038/nature02405>.
- 841 (17) Lee, E.-S.; Yoon, C.-H.; Kim, Y.-S.; Bae, Y.-S. The Double-Strand RNA-Dependent Protein
842 Kinase PKR Plays a Significant Role in a Sustained ER Stress-Induced Apoptosis. *FEBS*
843 *Letters* **2007**, 581 (22), 4325–4332. <https://doi.org/10.1016/j.febslet.2007.08.001>.
- 844 (18) Liao, Y.; Fung, T. S.; Huang, M.; Fang, S. G.; Zhong, Y.; Liu, D. X. Upregulation of
845 CHOP/GADD153 during Coronavirus Infectious Bronchitis Virus Infection Modulates
846 Apoptosis by Restricting Activation of the Extracellular Signal-Regulated Kinase Pathway. *J*
847 *Virol* **2013**, 87 (14), 8124–8134. <https://doi.org/10.1128/JVI.00626-13>.
- 848 (19) Cuddihy, A. R.; Li, S.; Tam, N. W. N.; Wong, A. H.-T.; Taya, Y.; Abraham, N.; Bell, J. C.;
849 Koromilas, A. E. Double-Stranded-RNA-Activated Protein Kinase PKR Enhances
850 Transcriptional Activation by Tumor Suppressor P53. *Mol Cell Biol* **1999**, 19 (4), 2475–2484.
- 851 (20) Kumar, A.; Yang, Y. L.; Flati, V.; Der, S.; Kadereit, S.; Deb, A.; Haque, J.; Reis, L.;
852 Weissmann, C.; Williams, B. R. Deficient Cytokine Signaling in Mouse Embryo Fibroblasts
853 with a Targeted Deletion in the PKR Gene: Role of IRF-1 and NF-kappaB. *EMBO J* **1997**, 16
854 (2), 406–416. <https://doi.org/10.1093/emboj/16.2.406>.
- 855 (21) McAllister, C. S.; Toth, A. M.; Zhang, P.; Devaux, P.; Cattaneo, R.; Samuel, C. E. Mechanisms
856 of Protein Kinase PKR-Mediated Amplification of Beta Interferon Induction by C Protein-
857 Deficient Measles Virus. *J Virol* **2010**, 84 (1), 380–386. <https://doi.org/10.1128/JVI.02630-08>.
- 858 (22) Silva, A. M.; Whitmore, M.; Xu, Z.; Jiang, Z.; Li, X.; Williams, B. R. G. Protein Kinase R
859 (PKR) Interacts with and Activates Mitogen-Activated Protein Kinase Kinase 6 (MKK6) in
860 Response to Double-Stranded RNA Stimulation. *J Biol Chem* **2004**, 279 (36), 37670–37676.
861 <https://doi.org/10.1074/jbc.M406554200>.
- 862 (23) Taghavi, N.; Samuel, C. E. Protein Kinase PKR Catalytic Activity Is Required for the PKR-
863 Dependent Activation of Mitogen-Activated Protein Kinases and Amplification of Interferon
864 Beta Induction Following Virus Infection. *Virology* **2012**, 427 (2), 208–216.
865 <https://doi.org/10.1016/j.virol.2012.01.029>.
- 866 (24) Zhang, P.; Langland, J. O.; Jacobs, B. L.; Samuel, C. E. Protein Kinase PKR-Dependent
867 Activation of Mitogen-Activated Protein Kinases Occurs through Mitochondrial Adapter IPS-
868 1 and Is Antagonized by Vaccinia Virus E3L. *J Virol* **2009**, 83 (11), 5718–5725.
869 <https://doi.org/10.1128/JVI.00224-09>.
- 870 (25) Zamanian-Daryoush, M.; Mogensen, T. H.; DiDonato, J. A.; Williams, B. R. G. NF-κB
871 Activation by Double-Stranded-RNA-Activated Protein Kinase (PKR) Is Mediated through
872 NF-κB-Inducing Kinase and IκB Kinase. *Mol Cell Biol* **2000**, 20 (4), 1278–1290.
- 873 (26) Arnaud, N.; Dabo, S.; Maillard, P.; Budkowska, A.; Kalliampakou, K. I.; Mavromara, P.;
874 Garcin, D.; Hugon, J.; Gatignol, A.; Akazawa, D.; Wakita, T.; Meurs, E. F. Hepatitis C Virus
875 Controls Interferon Production through PKR Activation. *PLoS One* **2010**, 5 (5), e10575.
876 <https://doi.org/10.1371/journal.pone.0010575>.
- 877 (27) McAllister, C. S.; Samuel, C. E. The RNA-Activated Protein Kinase Enhances the Induction
878 of Interferon-β and Apoptosis Mediated by Cytoplasmic RNA Sensors. *J Biol Chem* **2009**,
879 284 (3), 1644–1651. <https://doi.org/10.1074/jbc.M807888200>.
- 880 (28) Pham, A. M.; Santa Maria, F. G.; Lahiri, T.; Friedman, E.; Marié, I. J.; Levy, D. E. PKR
881 Transduces MDA5-Dependent Signals for Type I IFN Induction. *PLoS Pathog* **2016**, 12 (3),
882 e1005489. <https://doi.org/10.1371/journal.ppat.1005489>.
- 883 (29) Schulz, O.; Pichlmair, A.; Rehwinkel, J.; Rogers, N. C.; Scheuner, D.; Kato, H.; Takeuchi, O.;
884 Akira, S.; Kaufman, R. J.; Reis e Sousa, C. Protein Kinase R Contributes to Immunity against
885 Specific Viruses by Regulating Interferon mRNA Integrity. *Cell Host Microbe* **2010**, 7 (5),
886 354–361. <https://doi.org/10.1016/j.chom.2010.04.007>.

- 887 (30) Cesaro, T.; Michiels, T. Inhibition of PKR by Viruses. *Front Microbiol* **2021**, *12*, 757238.
888 <https://doi.org/10.3389/fmicb.2021.757238>.
- 889 (31) Chaumont, L.; Collet, B.; Boudinot, P. Double-Stranded RNA-Dependent Protein Kinase
890 (PKR) in Antiviral Defence in Fish and Mammals. *Developmental & Comparative*
891 *Immunology* **2023**, *145*, 104732. <https://doi.org/10.1016/j.dci.2023.104732>.
- 892 (32) Rothenburg, S.; Deigendesch, N.; Dey, M.; Dever, T. E.; Tazi, L. Double-Stranded RNA-
893 Activated Protein Kinase PKR of Fishes and Amphibians: Varying the Number of Double-
894 Stranded RNA Binding Domains and Lineage-Specific Duplications. *BMC Biol* **2008**, *6* (1),
895 12. <https://doi.org/10.1186/1741-7007-6-12>.
- 896 (33) Hu, C.-Y.; Zhang, Y.-B.; Huang, G.-P.; Zhang, Q.-Y.; Gui, J.-F. Molecular Cloning and
897 Characterisation of a Fish PKR-like Gene from Cultured CAB Cells Induced by UV-
898 Inactivated Virus. *Fish & Shellfish Immunology* **2004**, *17* (4), 353–366.
899 <https://doi.org/10.1016/j.fsi.2004.04.009>.
- 900 (34) Rothenburg, S.; Deigendesch, N.; Dittmar, K.; Koch-Nolte, F.; Haag, F.; Lowenhaupt, K.;
901 Rich, A. A PKR-like Eukaryotic Initiation Factor 2 Kinase from Zebrafish Contains Z-DNA
902 Binding Domains Instead of dsRNA Binding Domains. *Proceedings of the National Academy*
903 *of Sciences* **2005**, *102* (5), 1602–1607. <https://doi.org/10.1073/pnas.0408714102>.
- 904 (35) Bergan, V.; Jagus, R.; Lauksund, S.; Kileng, Ø.; Robertsen, B. The Atlantic Salmon Z-DNA
905 Binding Protein Kinase Phosphorylates Translation Initiation Factor 2 Alpha and Constitutes
906 a Unique Orthologue to the Mammalian dsRNA-Activated Protein Kinase R. *The FEBS*
907 *Journal* **2008**, *275* (1), 184–197. <https://doi.org/10.1111/j.1742-4658.2007.06188.x>.
- 908 (36) del Castillo, C. S.; Hikima, J.; Ohtani, M.; Jung, T.-S.; Aoki, T. Characterization and
909 Functional Analysis of Two PKR Genes in Fugu (Takifugu Rubripes). *Fish & Shellfish*
910 *Immunology* **2012**, *32* (1), 79–88. <https://doi.org/10.1016/j.fsi.2011.10.022>.
- 911 (37) Gan, Z.; Cheng, J.; Hou, J.; Chen, S.; Xia, H.; Xia, L.; Kwok, K. W. H.; Lu, Y.; Nie, P. Tilapia
912 dsRNA-Activated Protein Kinase R (PKR): An Interferon-Induced Antiviral Effector with
913 Translation Inhibition Activity. *Fish Shellfish Immunol* **2021**, *112*, 74–80.
914 <https://doi.org/10.1016/j.fsi.2021.02.016>.
- 915 (38) Hu, Y.-S.; Li, W.; Li, D.-M.; Liu, Y.; Fan, L.-H.; Rao, Z.-C.; Lin, G.; Hu, C.-Y. Cloning,
916 Expression and Functional Analysis of PKR from Grass Carp (Ctenopharyngodon Idellus).
917 *Fish & Shellfish Immunology* **2013**, *35* (6), 1874–1881.
918 <https://doi.org/10.1016/j.fsi.2013.09.024>.
- 919 (39) Xu, C.; Gamil, A. A. A.; Munang'andu, H. M.; Evensen, Ø. Apoptosis Induction by dsRNA-
920 Dependent Protein Kinase R (PKR) in EPC Cells via Caspase 8 and 9 Pathways. *Viruses* **2018**,
921 *10* (10), 526. <https://doi.org/10.3390/v10100526>.
- 922 (40) Zhu, R.; Zhang, Y.-B.; Zhang, Q.-Y.; Gui, J.-F. Functional Domains and the Antiviral Effect
923 of the Double-Stranded RNA-Dependent Protein Kinase PKR from *Paralichthys Olivaceus*. *J*
924 *Virol* **2008**, *82* (14), 6889–6901. <https://doi.org/10.1128/JVI.02385-07>.
- 925 (41) Hu, Y.; Fan, L.; Wu, C.; Wang, B.; Sun, Z.; Hu, C. Identification and Function Analysis of the
926 Three dsRBMs in the N Terminal dsRBD of Grass Carp (Ctenopharyngodon Idella) PKR. *Fish*
927 *Shellfish Immunol* **2016**, *50*, 91–100. <https://doi.org/10.1016/j.fsi.2016.01.011>.
- 928 (42) Wu, C.; Hu, Y.; Fan, L.; Wang, H.; Sun, Z.; Deng, S.; Liu, Y.; Hu, C. Ctenopharyngodon Idella
929 PKZ Facilitates Cell Apoptosis through Phosphorylating eIF2 α . *Molecular Immunology* **2016**,
930 *69*, 13–23. <https://doi.org/10.1016/j.molimm.2015.11.006>.
- 931 (43) Wang, H.; Xu, Q.; Xu, X.; Hu, Y.; Hou, Q.; Zhu, Y.; Hu, C. Ctenopharyngodon Idella IKK β
932 Interacts with PKR and I κ B α . *Acta Biochim Biophys Sin (Shanghai)* **2017**, *49* (8), 729–736.
933 <https://doi.org/10.1093/abbs/gmx065>.
- 934 (44) Ramnani, B.; Powell, S.; Shetty, A. G.; Manivannan, P.; Hibbard, B. R.; Leaman, D. W.;
935 Malathi, K. Viral Hemorrhagic Septicemia Virus Activates Integrated Stress Response

- 936 Pathway and Induces Stress Granules to Regulate Virus Replication. *Viruses* **2023**, *15* (2), 466.
 937 <https://doi.org/10.3390/v15020466>.
- 938 (45) Wei, J.; Zang, S.; Li, C.; Zhang, X.; Gao, P.; Qin, Q. Grouper PKR Activation Inhibits Red-
 939 Spotted Grouper Nervous Necrosis Virus (RGNNV) Replication in Infected Cells.
 940 *Developmental & Comparative Immunology* **2020**, *111*, 103744.
 941 <https://doi.org/10.1016/j.dci.2020.103744>.
- 942 (46) Liu, T.-K.; Zhang, Y.-B.; Liu, Y.; Sun, F.; Gui, J.-F. Cooperative Roles of Fish Protein Kinase
 943 Containing Z-DNA Binding Domains and Double-Stranded RNA-Dependent Protein Kinase
 944 in Interferon-Mediated Antiviral Response ν . *J Virol* **2011**, *85* (23), 12769–12780.
 945 <https://doi.org/10.1128/JVI.05849-11>.
- 946 (47) Gamil, A. A. A.; Xu, C.; Mutoloki, S.; Evensen, Ø. PKR Activation Favors Infectious
 947 Pancreatic Necrosis Virus Replication in Infected Cells. *Viruses* **2016**, *8* (6).
 948 <https://doi.org/10.3390/v8060173>.
- 949 (48) Dehler, C. E.; Boudinot, P.; Martin, S. A. M.; Collet, B. Development of an Efficient Genome
 950 Editing Method by CRISPR/Cas9 in a Fish Cell Line. *Mar Biotechnol (NY)* **2016**, *18* (4), 449–
 951 452. <https://doi.org/10.1007/s10126-016-9708-6>.
- 952 (49) Biacchesi, S.; Lamoureux, A.; Mérour, E.; Bernard, J.; Brémont, M. Limited Interference at
 953 the Early Stage of Infection between Two Recombinant Novirhabdoviruses: Viral
 954 Hemorrhagic Septicemia Virus and Infectious Hematopoietic Necrosis Virus. *J Virol* **2010**, *84*
 955 (19), 10038–10050. <https://doi.org/10.1128/JVI.00343-10>.
- 956 (50) Concordet, J.-P.; Haeussler, M. CRISPOR: Intuitive Guide Selection for CRISPR/Cas9
 957 Genome Editing Experiments and Screens. *Nucleic Acids Research* **2018**, *46* (W1), W242–
 958 W245. <https://doi.org/10.1093/nar/gky354>.
- 959 (51) Conant, D.; Hsiao, T.; Rossi, N.; Oki, J.; Maures, T.; Waite, K.; Yang, J.; Joshi, S.; Kelso, R.;
 960 Holden, K.; Enzmann, B. L.; Stoner, R. Inference of CRISPR Edits from Sanger Trace Data.
 961 *CRISPR J* **2022**, *5* (1), 123–130. <https://doi.org/10.1089/crispr.2021.0113>.
- 962 (52) Diot, C.; Richard, C.-A.; Risso-Ballester, J.; Martin, D.; Fix, J.; Eléouët, J.-F.; Sizun, C.;
 963 Rameix-Welti, M.-A.; Galloux, M. Hardening of Respiratory Syncytial Virus Inclusion Bodies
 964 by Cyclophamide Proceeds through Perturbation of the Interactions of the M2-1 Protein with
 965 RNA and the P Protein. *Int J Mol Sci* **2023**, *24* (18), 13862.
 966 <https://doi.org/10.3390/ijms241813862>.
- 967 (53) Collet, B.; Collins, C.; Cheyne, V.; Lester, K. Plasmid-Driven RNA Interference in Fish Cell
 968 Lines. *In Vitro Cell Dev Biol Anim* **2022**, *58* (3), 189–193. <https://doi.org/10.1007/s11626-022-00645-2>.
- 970 (54) Collins, C.; Ganne, G.; Collet, B. Isolation and Activity of the Promoters for STAT1 and 2 in
 971 Atlantic Salmon *Salmo Salar*. *Fish Shellfish Immunol* **2014**, *40* (2), 644–647.
 972 <https://doi.org/10.1016/j.fsi.2014.07.025>.
- 973 (55) Collet, B.; Boudinot, P.; Benmansour, A.; Secombes, C. J. An Mx1 Promoter-Reporter System
 974 to Study Interferon Pathways in Rainbow Trout. *Dev Comp Immunol* **2004**, *28* (7–8), 793–801.
 975 <https://doi.org/10.1016/j.dci.2003.12.005>.
- 976 (56) Trobridge, G. D.; Chiou, P. P.; Leong, J. A. Cloning of the Rainbow Trout (*Oncorhynchus*
 977 *Mykiss*) Mx2 and Mx3 cDNAs and Characterization of Trout Mx Protein Expression in
 978 Salmon Cells. *J Virol* **1997**, *71* (7), 5304–5311.
- 979 (57) Cai, R.; Williams, B. R. G. Mutations in the Double-Stranded RNA-Activated Protein Kinase
 980 Insert Region That Uncouple Catalysis from eIF2 α Binding *. *Journal of Biological Chemistry*
 981 **1998**, *273* (18), 11274–11280. <https://doi.org/10.1074/jbc.273.18.11274>.
- 982 (58) Park, S.-H.; Choi, J.; Kang, J.-I.; Choi, S.-Y.; Hwang, S.-B.; Kim, J. P.; Ahn, B.-Y. Attenuated
 983 Expression of Interferon-Induced Protein Kinase PKR in a Simian Cell Devoid of Type I
 984 Interferons. *Mol Cells* **2006**, *21* (1), 21–28.

- 985 (59) Schneider-Poetsch, T.; Ju, J.; Eyler, D. E.; Dang, Y.; Bhat, S.; Merrick, W. C.; Green, R.; Shen,
986 B.; Liu, J. O. Inhibition of Eukaryotic Translation Elongation by Cycloheximide and
987 Lactimidomycin. *Nat Chem Biol* **2010**, *6* (3), 209–217. <https://doi.org/10.1038/nchembio.304>.
- 988 (60) Wong, W. L.; Brostrom, M. A.; Kuznetsov, G.; Gmitter-Yellen, D.; Brostrom, C. O. Inhibition
989 of Protein Synthesis and Early Protein Processing by Thapsigargin in Cultured Cells. *Biochem*
990 *J* **1993**, *289* (Pt 1), 71–79.
- 991 (61) Jensen, I.; Larsen, R.; Robertsen, B. An Antiviral State Induced in Chinook Salmon Embryo
992 Cells (CHSE-214) by Transfection with the Double-Stranded RNA Poly I:C. *Fish & Shellfish*
993 *Immunology* **2002**, *13* (5), 367–378. <https://doi.org/10.1006/fsim.2002.0412>.
- 994 (62) Berthelot, C.; Brunet, F.; Chalopin, D.; Juanchich, A.; Bernard, M.; Noël, B.; Bento, P.; Da
995 Silva, C.; Labadie, K.; Alberti, A.; Aury, J.-M.; Louis, A.; Dehais, P.; Bardou, P.; Montfort, J.;
996 Klopp, C.; Cabau, C.; Gaspin, C.; Thorgaard, G. H.; Boussaha, M.; Quillet, E.; Guyomard, R.;
997 Galiana, D.; Bobe, J.; Volff, J.-N.; Genêt, C.; Wincker, P.; Jaillon, O.; Crollius, H. R.; Guiguen,
998 Y. The Rainbow Trout Genome Provides Novel Insights into Evolution after Whole-Genome
999 Duplication in Vertebrates. *Nat Commun* **2014**, *5* (1), 3657.
1000 <https://doi.org/10.1038/ncomms4657>.
- 1001 (63) Lien, S.; Koop, B. F.; Sandve, S. R.; Miller, J. R.; Kent, M. P.; Nome, T.; Hvidsten, T. R.;
1002 Leong, J. S.; Minkley, D. R.; Zimin, A.; Grammes, F.; Grove, H.; Gjuvslund, A.; Walenz, B.;
1003 Hermansen, R. A.; von Schalburg, K.; Rondeau, E. B.; Di Genova, A.; Samy, J. K. A.; Olav
1004 Vik, J.; Vigeland, M. D.; Caler, L.; Grimholt, U.; Jentoft, S.; Inge Våge, D.; de Jong, P.; Moen,
1005 T.; Baranski, M.; Palti, Y.; Smith, D. R.; Yorke, J. A.; Nederbragt, A. J.; Tooming-Klunderud,
1006 A.; Jakobsen, K. S.; Jiang, X.; Fan, D.; Hu, Y.; Liberles, D. A.; Vidal, R.; Iturra, P.; Jones, S.
1007 J. M.; Jonassen, I.; Maass, A.; Omholt, S. W.; Davidson, W. S. The Atlantic Salmon Genome
1008 Provides Insights into Rediploidization. *Nature* **2016**, *533* (7602), 200–205.
1009 <https://doi.org/10.1038/nature17164>.
- 1010 (64) Macqueen, D. J.; Johnston, I. A. A Well-Constrained Estimate for the Timing of the Salmonid
1011 Whole Genome Duplication Reveals Major Decoupling from Species Diversification.
1012 *Proceedings of the Royal Society B: Biological Sciences* **2014**, *281* (1778), 20132881.
1013 <https://doi.org/10.1098/rspb.2013.2881>.
- 1014 (65) Pasquier, J.; Cabau, C.; Nguyen, T.; Jouanno, E.; Severac, D.; Braasch, I.; Journot, L.;
1015 Pontarotti, P.; Klopp, C.; Postlethwait, J. H.; Guiguen, Y.; Bobe, J. Gene Evolution and Gene
1016 Expression after Whole Genome Duplication in Fish: The PhyloFish Database. *BMC Genomics*
1017 **2016**, *17* (1), 368. <https://doi.org/10.1186/s12864-016-2709-z>.
- 1018 (66) Letunic, I.; Khedkar, S.; Bork, P. SMART: Recent Updates, New Developments and Status in
1019 2020. *Nucleic Acids Research* **2021**, *49* (D1), D458–D460.
1020 <https://doi.org/10.1093/nar/gkaa937>.
- 1021 (67) Letunic, I.; Bork, P. 20 Years of the SMART Protein Domain Annotation Resource. *Nucleic*
1022 *Acids Res* **2018**, *46* (Database issue), D493–D496. <https://doi.org/10.1093/nar/gkx922>.
- 1023 (68) Masliah, G.; Barraud, P.; Allain, F. H.-T. RNA Recognition by Double-Stranded RNA Binding
1024 Domains: A Matter of Shape and Sequence. *Cell Mol Life Sci* **2013**, *70* (11), 1875–1895.
1025 <https://doi.org/10.1007/s00018-012-1119-x>.
- 1026 (69) Nanduri, S.; Carpick, B. W.; Yang, Y.; Williams, B. R.; Qin, J. Structure of the Double-
1027 Stranded RNA-Binding Domain of the Protein Kinase PKR Reveals the Molecular Basis of Its
1028 dsRNA-Mediated Activation. *EMBO J* **1998**, *17* (18), 5458–5465.
1029 <https://doi.org/10.1093/emboj/17.18.5458>.
- 1030 (70) Hanks, S. K.; Quinn, A. M.; Hunter, T. The Protein Kinase Family: Conserved Features and
1031 Deduced Phylogeny of the Catalytic Domains. *Science* **1988**, *241* (4861), 42–52.
1032 <https://doi.org/10.1126/science.3291115>.
- 1033 (71) Romano, P. R.; Garcia-Barrio, M. T.; Zhang, X.; Wang, Q.; Taylor, D. R.; Zhang, F.; Herring,
1034 C.; Mathews, M. B.; Qin, J.; Hinnebusch, A. G. Autophosphorylation in the Activation Loop

- 1035 Is Required for Full Kinase Activity in Vivo of Human and Yeast Eukaryotic Initiation Factor
1036 2alpha Kinases PKR and GCN2. *Mol Cell Biol* **1998**, *18* (4), 2282–2297.
1037 <https://doi.org/10.1128/MCB.18.4.2282>.
- 1038 (72) Patel, R. C.; Stanton, P.; Sen, G. C. Specific Mutations near the Amino Terminus of Double-
1039 Stranded RNA-Dependent Protein Kinase (PKR) Differentially Affect Its Double-Stranded
1040 RNA Binding and Dimerization Properties. *J Biol Chem* **1996**, *271* (41), 25657–25663.
1041 <https://doi.org/10.1074/jbc.271.41.25657>.
- 1042 (73) Pavlovic, J.; Haller, O.; Staeheli, P. Human and Mouse Mx Proteins Inhibit Different Steps of
1043 the Influenza Virus Multiplication Cycle. *J Virol* **1992**, *66* (4), 2564–2569.
1044 <https://doi.org/10.1128/JVI.66.4.2564-2569.1992>.
- 1045 (74) Trobridge, G. D.; Leong, J. A. Characterization of a Rainbow Trout Mx Gene. *J Interferon*
1046 *Cytokine Res* **1995**, *15* (8), 691–702. <https://doi.org/10.1089/jir.1995.15.691>.
- 1047 (75) Elmore, S. Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol* **2007**, *35* (4), 495–
1048 516. <https://doi.org/10.1080/01926230701320337>.
- 1049 (76) Belmokhtar, C. A.; Hillion, J.; Ségal-Bendirdjian, E. Staurosporine Induces Apoptosis through
1050 Both Caspase-Dependent and Caspase-Independent Mechanisms. *Oncogene* **2001**, *20* (26),
1051 3354–3362. <https://doi.org/10.1038/sj.onc.1204436>.
- 1052 (77) Bertrand, R.; Solary, E.; O'Connor, P.; Kohn, K. W.; Pommier, Y. Induction of a Common
1053 Pathway of Apoptosis by Staurosporine. *Experimental Cell Research* **1994**, *211* (2), 314–321.
1054 <https://doi.org/10.1006/excr.1994.1093>.
- 1055 (78) Inoue, S.; Browne, G.; Melino, G.; Cohen, G. M. Ordering of Caspases in Cells Undergoing
1056 Apoptosis by the Intrinsic Pathway. *Cell Death Differ* **2009**, *16* (7), 1053–1061.
1057 <https://doi.org/10.1038/cdd.2009.29>.
- 1058 (79) Manns, J.; Daubrawa, M.; Driessen, S.; Paasch, F.; Hoffmann, N.; Löffler, A.; Lauber, K.;
1059 Dieterle, A.; Alers, S.; Iftner, T.; Schulze-Osthoff, K.; Stork, B.; Wesselborg, S. Triggering of
1060 a Novel Intrinsic Apoptosis Pathway by the Kinase Inhibitor Staurosporine: Activation of
1061 Caspase-9 in the Absence of Apaf-1. *FASEB J* **2011**, *25* (9), 3250–3261.
1062 <https://doi.org/10.1096/fj.10-177527>.
- 1063 (80) Enam, S. U.; Zinshteyn, B.; Goldman, D. H.; Cassani, M.; Livingston, N. M.; Seydoux, G.;
1064 Green, R. Puromycin Reactivity Does Not Accurately Localize Translation at the Subcellular
1065 Level. *eLife* **2020**, *9*, e60303. <https://doi.org/10.7554/eLife.60303>.
- 1066 (81) Nathans, D. Puromycin Inhibition of Protein Synthesis: Incorporation of Puromycin into
1067 Peptide Chains. *Proc Natl Acad Sci U S A* **1964**, *51* (4), 585–592.
- 1068 (82) Biacchesi, S.; Mérour, E.; Chevret, D.; Lamoureux, A.; Bernard, J.; Brémont, M. NV Proteins
1069 of Fish Novirhabdovirus Recruit Cellular PPM1Bb Protein Phosphatase and Antagonize RIG-
1070 I-Mediated IFN Induction. *Sci Rep* **2017**, *7*, 44025. <https://doi.org/10.1038/srep44025>.
- 1071 (83) Chinchilla, B.; Encinas, P.; Estepa, A.; Coll, J. M.; Gomez-Casado, E. Transcriptome Analysis
1072 of Rainbow Trout in Response to Non-Virion (NV) Protein of Viral Haemorrhagic Septicaemia
1073 Virus (VHSV). *Appl Microbiol Biotechnol* **2015**, *99* (4), 1827–1843.
1074 <https://doi.org/10.1007/s00253-014-6366-3>.
- 1075 (84) Chinchilla, B.; Encinas, P.; Coll, J. M.; Gomez-Casado, E. Differential Immune Transcriptome
1076 and Modulated Signalling Pathways in Rainbow Trout Infected with Viral Haemorrhagic
1077 Septicaemia Virus (VHSV) and Its Derivative Non-Virion (NV) Gene Deleted. *Vaccines*
1078 *(Basel)* **2020**, *8* (1), 58. <https://doi.org/10.3390/vaccines8010058>.
- 1079 (85) Kim, M. S.; Kim, K. H. Effects of NV Gene Knock-out Recombinant Viral Hemorrhagic
1080 Septicemia Virus (VHSV) on Mx Gene Expression in Epithelioma Papulosum Cyprini (EPC)
1081 Cells and Olive Flounder (*Paralichthys Olivaceus*). *Fish Shellfish Immunol* **2012**, *32* (3), 459–
1082 463. <https://doi.org/10.1016/j.fsi.2011.12.014>.

- 1083 (86) Ammayappan, A.; Vakharia, V. N. Nonvirion Protein of Novirhabdovirus Suppresses
1084 Apoptosis at the Early Stage of Virus Infection. *J Virol* **2011**, *85* (16), 8393–8402.
1085 <https://doi.org/10.1128/JVI.00597-11>.
- 1086 (87) Hay, S.; Kannourakis, G. A Time to Kill: Viral Manipulation of the Cell Death Program. *J Gen*
1087 *Virol* **2002**, *83* (Pt 7), 1547–1564. <https://doi.org/10.1099/0022-1317-83-7-1547>.
- 1088 (88) Mi, J.; Li, Z. Y.; Ni, S.; Steinwaerder, D.; Lieber, A. Induced Apoptosis Supports Spread of
1089 Adenovirus Vectors in Tumors. *Hum Gene Ther* **2001**, *12* (10), 1343–1352.
1090 <https://doi.org/10.1089/104303401750270995>.
- 1091 (89) Chiou, P. P.; Kim, C. H.; Ormonde, P.; Leong, J.-A. C. Infectious Hematopoietic Necrosis
1092 Virus Matrix Protein Inhibits Host-Directed Gene Expression and Induces Morphological
1093 Changes of Apoptosis in Cell Cultures. *J Virol* **2000**, *74* (16), 7619–7627.
- 1094 (90) Donnelly, N.; Gorman, A. M.; Gupta, S.; Samali, A. The eIF2 α Kinases: Their Structures and
1095 Functions. *Cell. Mol. Life Sci.* **2013**, *70* (19), 3493–3511. <https://doi.org/10.1007/s00018-012-1252-6>.
- 1097 (91) Berlanga, J. J.; Ventoso, I.; Harding, H. P.; Deng, J.; Ron, D.; Sonenberg, N.; Carrasco, L.; de
1098 Haro, C. Antiviral Effect of the Mammalian Translation Initiation Factor 2 α Kinase GCN2
1099 against RNA Viruses. *EMBO J* **2006**, *25* (8), 1730–1740.
1100 <https://doi.org/10.1038/sj.emboj.7601073>.
- 1101 (92) Cheng, G.; Feng, Z.; He, B. Herpes Simplex Virus 1 Infection Activates the Endoplasmic
1102 Reticulum Resident Kinase PERK and Mediates eIF-2 α Dephosphorylation by the Γ 134.5
1103 Protein. *J Virol* **2005**, *79* (3), 1379–1388. <https://doi.org/10.1128/JVI.79.3.1379-1388.2005>.
- 1104 (93) Kesterson, S. P.; Ringiesn, J.; Vakharia, V. N.; Shepherd, B. S.; Leaman, D. W.; Malathi, K.
1105 Effect of the Viral Hemorrhagic Septicemia Virus Nonvirion Protein on Translation via PERK-
1106 eIF2 α Pathway. *Viruses* **2020**, *12* (5), 499. <https://doi.org/10.3390/v12050499>.
- 1107 (94) Baltzis, D.; Li, S.; Koromilas, A. E. Functional Characterization of Pkr Gene Products
1108 Expressed in Cells from Mice with a Targeted Deletion of the N Terminus or C Terminus
1109 Domain of PKR *. *Journal of Biological Chemistry* **2002**, *277* (41), 38364–38372.
1110 <https://doi.org/10.1074/jbc.M203564200>.
- 1111 (95) Li, S.; Koromilas, A. E. Dominant Negative Function by an Alternatively Spliced Form of the
1112 Interferon-Inducible Protein Kinase PKR*. *Journal of Biological Chemistry* **2001**, *276* (17),
1113 13881–13890. <https://doi.org/10.1074/jbc.M008140200>.
- 1114 (96) Abraham, N.; Jaramillo, M. L.; Duncan, P. I.; Méthot, N.; Icely, P. L.; Stojdl, D. F.; Barber, G.
1115 N.; Bell, J. C. The Murine PKR Tumor Suppressor Gene Is Rearranged in a Lymphocytic
1116 Leukemia. *Exp Cell Res* **1998**, *244* (2), 394–404. <https://doi.org/10.1006/excr.1998.4201>.
- 1117 (97) Liu, Z.-Y.; Jia, K.-T.; Li, C.; Weng, S.-P.; Guo, C.-J.; He, J.-G. A Truncated Danio Rerio PKZ
1118 Isoform Functionally Interacts with eIF2 α and Inhibits Protein Synthesis. *Gene* **2013**, *527* (1),
1119 292–300. <https://doi.org/10.1016/j.gene.2013.05.043>.
- 1120 (98) Teige, L. H.; Aksnes, I.; Røsæg, M. V.; Jensen, I.; Jørgensen, J.; Sindre, H.; Collins, C.; Collet,
1121 B.; Rimstad, E.; Dahle, M. K.; Boysen, P. Detection of Specific Atlantic Salmon Antibodies
1122 against Salmonid Alphavirus Using a Bead-Based Immunoassay. *Fish & Shellfish Immunology*
1123 **2020**, *106*, 374–383. <https://doi.org/10.1016/j.fsi.2020.07.055>.
- 1124 (99) Dehler, C. E.; Lester, K.; Pelle, G. D.; Houel, A.; Collins, C.; Dovgan, T.; Machat, R.; Zou, J.;
1125 Boudinot, P.; Collet, B. Viral Resistance and IFN Signaling in STAT2 Knockout Fish Cells.
1126 **2019**, *12*. <https://doi.org/doi:10.4049/jimmunol.1801376>.
- 1127 (100) Macqueen, D. J.; Kristjánsson, B. K.; Johnston, I. A. Salmonid Genomes Have a Remarkably
1128 Expanded Akirin Family, Coexpressed with Genes from Conserved Pathways Governing
1129 Skeletal Muscle Growth and Catabolism. *Physiol Genomics* **2010**, *42* (1), 134–148.
1130 <https://doi.org/10.1152/physiolgenomics.00045.2010>.
- 1131 (101) Jami, R.; Mérour, E.; Bernard, J.; Lamoureux, A.; Millet, J. K.; Biacchesi, S. The C-Terminal
1132 Domain of Salmonid Alphavirus Nonstructural Protein 2 (nsP2) Is Essential and Sufficient To

1133 Block RIG-I Pathway Induction and Interferon-Mediated Antiviral Response. *Journal of*
1134 *Virology* **2021**, 95 (23). <https://doi.org/10.1128/JVI.01155-21>.

1135

1136 **Table legends**

1137 Table I: Primers used in this study

1138 Table II: Plasmids used in this study

1139 Table III: qPCR primers used in this study

1140 Table IV: List of primary antibodies used for Western blots in this study

1141 Table V: Molecular characteristics of Chinook salmon PKR isoforms isolated in this study

1142

1143 **Figure legends**

1144 **Figure 1: Isolation of three Chinook salmon PKR isoforms expressed during rVHSV-Tomato**
1145 **infection in EC cells.** (A) Electrophoresis on 1.5% agar gel (100 V, 60 min) of PKR isoforms (FL =
1146 full length, ML = medium length, SL = short length) amplified from cDNA of EC cells infected with
1147 rVHSV-Tomato (MOI 1). (B) Chromosomal localisation of Chinook salmon PKR (LOC112253229)
1148 and schematic representation of the 3 isolated PKR isoforms at the transcript level. Boxes represent
1149 exons and straight lines represent introns. The transcripts of PKR-FL, PKR-ML and PKR-SL are
1150 represented in black and untranscribed regions are in grey. (C) Schematic representation of the 3
1151 isolated PKR isoforms at the protein level. dsRBM = dsRNA binding motif, KD = kinase domain.
1152 The location of each predicted domain or motif was obtained using SMART (Simple Modular
1153 Architecture Research Tool). (D) Sequence alignment of dsRNA binding motifs from Chinook
1154 salmon PKR isoforms and human PKR. The sequence consensus identified in most dsRNA-binding
1155 motifs from other proteins and the corresponding residues conserved for the fold and/or dsRNA
1156 binding are taken from Masliah et al. and drawn below the alignment. The canonical secondary
1157 structured elements are shown above the alignment ^{68,69}. The circles above the sequences show
1158 residues that have identified as important for dsRNA binding in human PKR ^{71,72}. (E) Schematic
1159 representation of human PKR (hPKR) and Chinook salmon PKR-FL and PKR-ML kinase domains.
1160 The different subdomains (I-XI) conserved in kinase proteins were taken from Hanks et al. ⁷⁰ and
1161 Meurs et al. ². Motifs involved in ATP binding are represented by a black line, the invariant lysine
1162 residue involved in the phosphoryl transfer reaction is represented by an inverted white triangle,
1163 autophosphorylation sites described for hPKR ⁷¹ are represented by inverted black triangles and are
1164 located in the activation loop (black dotted line). The acidic kinase insert is indicated by a black
1165 double arrow and the eIF2 α kinase conserved motif is represented by a grey line; both features are
1166 conserved in eIF2 α kinases.

1167 **Figure 2: Expression profile of the three PKR isoforms in EC cells during IFNA2 stimulation**
1168 **or virus infection determined by western blot (A;B) and by RT-qPCR (C;D).** (A) EC cells were
1169 stimulated with *S. salar* IFNA2 supernatant for (24-72h), infected with rVHSV-Tomato (16-40 hpi),
1170 GSV (16-24hpi) or left untreated (Ctrl). Negative and positive controls are EC cells transfected with
1171 pcDNA3.1-Zeo-BFP or plasmids encoding PKR isoforms (PKR-SL, -ML, -FL), respectively. Cell
1172 lysates were separated by SDS-PAGE and immunoblotted with antibodies against *S. salar* PKR and
1173 α -tubulin (α -tub). PKR-SL was not detected by the anti-PKR antibody and was therefore not
1174 represented. Endogenous PKR-ML was not detected following IFNA2 stimulation and viral

1175 infections; the double bands present on the blot are believed to be non-specific bands. **(B)**
1176 Densitometric quantification of (A). PKR-FL signal intensity normalized to α -tubulin signal intensity
1177 and graphed as fold change relative to non-stimulated or non-infected cells. Bars show means \pm SD
1178 from 2 pooled independent experiments (n=3 for each experiment); ns, non-significant ($p > 0.05$), *,
1179 $p < 0.05$, ****, $p < 0.0001$, Kruskal-Wallis test with Dunn's post-hoc multiple comparison tests.
1180 **(C;D)** EC cells were stimulated with *S. salar* IFNA2 supernatant for 72h, infected with rVHSV-
1181 Tomato (8-24 hpi) or left untreated (Ctrl). **(C)** Relative expression levels of *pkr* isoforms and *mx123*
1182 genes. **(D)** Expression fold change relative to non-stimulated and non-infected controls. Bars show
1183 means \pm SD from 2 pooled independent experiments (n=3 or n=4 for each experiment); *, $p < 0.05$,
1184 ****, $p < 0.0001$, ordinary one-way ANOVA with Tukey's post-hoc multiple comparison tests.

1185 **Figure 3: Development and validation of a *pkr*^{-/-} cell line.** **(A)** Genotype of EC cells (WT), EC-
1186 PKR-C28 (6-nt deletion, 30_35delGTGCGA, resulting in C11_E12del, WT-like) and EC-PKR-C19,
1187 EC-PKR-C3 and EC-PKR-C5 (1-nt insertion, 29_30insT resulting in V11fsX22, *pkr*^{-/-}) obtained from
1188 sequencing of purified PCR products amplified from genomic DNA from each cell line. The location
1189 of the sgRNA is underlined, the protospacer adjacent motif is in dark grey, the start codon and the
1190 premature stop codon are highlighted in black, the 1-nt insertion in EC-PKR-C19, -C3 and -C5 is in
1191 light grey. The corresponding chromatograms are available in Figure S1. **(B)** EC and EC-PKR clones
1192 were stimulated with *S. salar* IFNA2 supernatant for 72h; positive and negative controls are EC-PKR-
1193 C19 cells transfected with pcDNA3.1-Zeo-BFP or pcDNA3.1-Zeo-BFP-P2A-PKR-FL, respectively.
1194 Cell lysates were separated by SDS-PAGE and immunoblotted with antibodies against PKR and α -
1195 tubulin (α -tub). **(C)** Densitometric quantification of (B). PKR signal intensity normalized to α -tubulin
1196 signal intensity and graphed as fold change relative to non-stimulated cells. Bars show means \pm SD
1197 from 3 pooled independent experiments (n=3 or n=4 for each experiment); *, $p < 0.05$, ****, $p <$
1198 0.0001, ordinary two-way ANOVA with Bonferroni's post-hoc multiple comparison tests.

1199 **Figure 4: Overexpression of PKR-FL induces apoptosis.** *pkr*^{-/-} EC-PKR-C19 cells were transfected
1200 with plasmids encoding BFP or different PKR isoforms (PKR-SL, -ML, -FL); at 72hpt, gDNA was
1201 extracted for gDNA laddering assay or caspase activity was measured. In parallel, EC-PKR-C19 cells
1202 were either stimulated with staurosporine (STS, 1 μ M) for 24h, infected with GSV (MOI 1) for 24-
1203 48h or left untreated, as positive and negative controls, respectively. **(A)** Electrophoresis on 1.5%
1204 agar gel (50V, 60 min) of gDNA from transfected cells (left, 3000 ng/well) or GSV-infected and STS-
1205 stimulated cells (right, 600 ng/well). Gels are representative of 2 independent experiments. **(B)**
1206 Caspase 3/7 activity **(C)** Caspase 8 activity and **(D)** Caspase 9 activity signal graphed as fold change

1207 relative to cells transfected with BFP-encoding plasmid (left) or left untreated (right). Dotplots show
1208 means \pm SD from two pooled independent experiments (n=8 for transfection experiments; n=4 for
1209 stimulation experiments); ns, non-significant ($p > 0.05$), ****, $p < 0.0001$, ordinary one-way ANOVA
1210 with Tukey's post-hoc multiple comparison tests.

1211 **Figure 5: Endogenous Chinook salmon PKR triggers apoptosis during rVHSV-Tomato**
1212 **infection.** EC (WT), EC-PKR-C28 (WT-like) and EC-PKR-C19, -C3 and -C5 ($pkrr^{-/-}$) were infected
1213 with rVHSV-Tomato at (A) MOI 1 or (B) MOI 0.1 and caspase 3/7 activity was measured at 24, 48
1214 and 72 h post-infection. Bars show means \pm SD from two pooled independent experiments (n=4 for
1215 each experiment); ns, non-significant ($p > 0.05$), *, $p < 0.05$, **, $p < 0.01$, ****, $p < 0.0001$, ordinary
1216 two-way ANOVA with Tukey's post-hoc multiple comparison tests.

1217 **Figure 6: Overexpression of PKR-FL inhibits host translation.** (A) $pkrr^{-/-}$ EC-PKR-C19 cells were
1218 cotransfected with pcDNA3.1-Hyg-mEGFP and plasmids encoding BFP or different PKR isoforms
1219 (PKR-SL, -ML, -FL). At 8 hpt, mock transfected cells were treated with cycloheximide (CHX,
1220 50 μ g/mL, 24h) or thapsigargin (Tg, 2 μ M, 45 min). At 30 hpt, cells were pulsed with puromycin (5
1221 μ g/mL) for 15 min and cell lysates were separated by SDS-PAGE and immunoblotted with antibodies
1222 against PKR, GFP, puromycin (puro) or actin. (A,B) Densitometric quantification of (A). (B) GFP
1223 signal intensity normalized to actin signal intensity and graphed as fold change relative to BFP
1224 transfected cells. Bars show means \pm SD from two pooled independent experiments (n=3 or n=4 for
1225 each experiment), *, $p < 0.05$; **, $p < 0.01$, ****, $p < 0.0001$, ordinary one-way ANOVA with
1226 Tukey's post-hoc multiple comparison tests. (C) Puromycin signal intensity normalized to actin
1227 signal intensity and graphed as fold change relative to BFP transfected cells. (D) $pkrr^{-/-}$ EC-PKR-C19
1228 cells were cotransfected with pcDNA3-G418-Luc and plasmids encoding BFP or different PKR
1229 isoforms (PKR-SL, -ML, -FL). At 24 hpt, luciferase activity was measured. Luminescence signal
1230 intensity graphed as fold change relative to BFP transfected cells. Data shown are means \pm SD (n=16)
1231 and are representative of 2 independent experiments; ****, $p < 0.0001$, ordinary one-way ANOVA
1232 with Tukey's post-hoc multiple comparison tests.

1233 **Figure 7: Endogenous Chinook salmon PKR inhibits host translation upon transfection with**
1234 **poly(I:C).** (A) Non-stimulated or ssIFNA2-pretreated EC (WT), EC-PKR-C28 (WT-like) and EC-
1235 PKR-C19 ($pkrr^{-/-}$) cells were transfected with rhodamine-labeled poly(I:C)-HMW (pIC) or mock
1236 transfected (Ctl). At 24 hpt, cells were pulsed with puromycin (5 μ g/mL) for 15 min and cell lysates
1237 were separated by SDS-PAGE and immunoblotted with antibodies against PKR, puromycin and α -

1238 tubulin. **(B)** Densitometric quantification of **(A)**. Puromycin signal intensity normalised to α -tubulin
1239 signal intensity and graphed as fold change relative to mock transfected cells. Bars show means \pm SD
1240 from two pooled independent experiments (n=3 for each experiment), ns, non-significant ($p > 0.05$);
1241 *, $p < 0.05$; **, $p < 0.01$, two-way ANOVA with Bonferroni's post-hoc multiple comparison tests.

1242 **Figure 8: Endogenous Chinook salmon PKR is not the main driver of protein synthesis**
1243 **inhibition during rVHSV-Tomato infection.** **(A)** EC (WT), EC-PKR-C28 (WT-like) and EC-PKR-
1244 C19 (*pk^r-/-*) cells were either infected with rVHSV-Tomato at MOI 1, left uninfected (Ctrl) or
1245 stimulated with cycloheximide (CHX, 50 μ g/mL) for 24h. At 16, 24 or 40 hpi, cells were pulsed with
1246 puromycin (5 μ g/mL) for 15 min and cell lysates were separated by SDS-PAGE and immunoblotted
1247 with antibodies against VHSV N, eIF2 α -P, eIF2 α , puromycin (puro), actin, PKR and α -tubulin (α -
1248 tub). **(B,C,D)** Densitometric quantification of **(A)**. **(B)** VHSV N signal intensity normalized to actin
1249 signal intensity. **(C)** eIF2 α -P signal intensity normalised to eIF2 α signal intensity and graphed as fold
1250 change relative to control cells. **(D)** Puromycin signal intensity normalized to actin signal intensity
1251 and graphed as fold change relative to control cells. Bars show means \pm SD from two pooled
1252 independent experiments (n=3 for each experiment); ns, non-significant ($p > 0.05$), ordinary two-way
1253 ANOVA with Tukey's post-hoc multiple comparison tests.

1254 **Figure 9: Overexpression of PKR-FL inhibits rVHSV-Tomato replication.** *pk^r-/-* EC-PKR-C19
1255 cells were transfected with pcDNA3.1-Zeo-BFP or plasmids encoding PKR isoforms. At 24hpt, cells
1256 were infected with rVHSV-Tomato (MOI 1) and fluorescence was measured at different time points
1257 post-infection. **(A)** Fluorescence signal intensity measured over time; data shown are means \pm SD
1258 from 6 independent experiments (n=4 for each experiment); two-way ANOVA with Tukey's post-
1259 hoc multiple comparison tests were performed on log-transformed data: *, $p < 0.05$; **, $p < 0.01$,
1260 comparison to BFP. **(B)** Fluorescence signal intensity graphed as fold change relative to BFP
1261 transfected cells; *, $p < 0.05$, two-way RM ANOVA with Tukey's post-hoc multiple comparison
1262 tests. **(C)** Supernatant was collected at 96hpi, pooled (4 wells per condition) and titrated by plaque
1263 assay. Data shown are means \pm SD from 6 independent experiments (n=1 for each experiment, 4
1264 pooled wells); *, $p < 0.05$; **, $p < 0.01$, ordinary one-way ANOVA with Tukey's post-hoc multiple
1265 comparison tests.

1266 **Figure 10: Endogenous Chinook salmon PKR favors the release of rVHSV-Tomato particles**
1267 **into the supernatant.** **(A;B;C)** EC (WT), EC-PKR-C28 (WT-like) and EC-PKR-C19 (*pk^r-/-*) cells
1268 were infected with rVHSV-Tomato at **(A)** MOI 10, **(B)** MOI 1 or **(C)** MOI 0.1 and fluorescence was

1269 measured at different time points post-infection. Graphs show means \pm SD from 4 independent
1270 experiments (n=8 for each experiment). two-way RM ANOVA with Tukey's post-hoc multiple
1271 comparison tests were performed on each log-transformed data set: (A) *, $p < 0.05$ starting from
1272 48 hpi ; (B) *, $p < 0.05$ starting from 72 hpi ; (C) *, $p < 0.05$ at 96 hpi. **(D;E;F)** EC (WT), EC-PKR-
1273 C28 (WT-like) and EC-PKR-C19 ($pkrr^{-/-}$) cells were infected with rVHSV-Tomato at MOI 1;
1274 supernatants were collected at 96 hpi and the remaining cells were scraped and sonicated; both
1275 supernatants and sonicates were titrated by plaque assay. Log-transformed plaque forming unit (PFU)
1276 counts from **(D)** supernatants, **(E)** sonicated cells and **(F)** total sum of both values of EC, EC-PKR-
1277 C28, EC-PKR-C19 cells (4×10^6 cells) infected with rVHSV-Tomato for 96 hpi. Bars show means \pm
1278 SD from two pooled independent experiments (n=4 for each experiment), *, $p < 0.05$; **, $p < 0.01$,
1279 Kruskal-Wallis test with Dunn's post-hoc multiple comparison tests on log-transformed data.

1280

