Fish and Shellfish Immunology 99 (2020) 119–129

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Intracellular distribution and transcriptional regulation of Atlantic salmon (*Salmo salar*) Rab5c, 7a and 27a homologs by immune stimuli

Arpita Nepal[a], Deanna L. Wolfson[b], Balpreet Singh Ahluwalia[b], Ingvill Jensen[a], Jorunn Jørgensen[a], Dimitar B. Iliev[a,c,*]

[a] Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries & Economics, UiT The Arctic University of Norway, Tromsø, Norway
[b] Department of Physics and Technology, UiT The Arctic University of Norway, Tromsø, Norway
[c] Department of Gene Regulation, Institute of Molecular Biology 'Roumen Tsanev', Bulgarian Academy of Sciences, Sofia, Bulgaria

ARTICLE INFO

Keywords:
Rab5c
Rab7a
Rab27a
Atlantic salmon
TLR ligands
Salmonid alphavirus (SAV)
Endocytosis

ABSTRACT

Rab GTPases control trafficking of intracellular vesicles and are key regulators of endocytic and secretory pathways. Due to their specific distribution, they may serve as markers for different endolysosomal compartments. Since Rab GTPases are involved in uptake and trafficking of endocytosed ligands and cell receptors, as well as secretion of immune mediators, they have been implicated in diverse immunological processes and their functions are often exploited by intracellular pathogens such as viruses. While Rab proteins have been studied extensively in mammals, their functions in vesicle trafficking in teleosts are not well known. In the present work, Atlantic salmon Rab5c, Rab7a and Rab27a homologs were studied in terms of intracellular distribution and gene expression. Structured illumination microscopy demonstrated that transgenic, GFP-tagged salmon Rab5c and Rab7a are, predominantly, located within early endosomes and late endosomes/lysosomes, respectively. In contrast, Rab27a showed a broader distribution, which indicates that it associates with diverse intracellular vesicles and organelles. Infection of salmon with Salmonid alphavirus subtype 3 (SAV3) enhanced the mRNA levels of all of the studied Rab isoforms in heart and head kidney and most of them were upregulated in spleen. This may reflect the capacity of the virus to exploit the functions of these rab proteins. It is also possible that the transcriptional regulation of Rab proteins in SAV3-infected organs may play a role in the antiviral immune response. The latter was further supported by *in vitro* experiments with adherent head kidney leukocytes. The expression of Rab5c and Rab27a was upregulated in these cells following stimulation with TLR ligands including CpG oligonucleotides and polyI:C. The expression of most of the analyzed Rab isoforms in the primary leukocytes was also enhanced by stimulation with type I IFN. Interestingly, IFN-gamma had a negative effect on Rab7a expression which may be linked to the priming activity of this cytokine on monocytes and macrophages. Overall, these data demonstrate that the intracellular distribution of Rab5c, Rab7a and Rab27a is phylogenetically conserved within vertebrates and that these molecules might be implicated in viral infections and the regulation of the antiviral immune response in Atlantic salmon.

1. Introduction

Rab proteins are a large family of phylogenetically conserved small GTPases present in all eukaryotes. These proteins participate in regulation of almost all membrane trafficking processes in eukaryotic cells and perform important functions in various endocytic and secretory pathways [1]. Rab GTPases control membrane identity, vesicle transport, uncoating, fission, motility, target selection and fusion through the recruitment of effector proteins [2].

Rab proteins function as molecular switches through alteration between two conformational states; a GTP-bound form (i.e. active or on form) and a GDP-bound form (i.e. inactive or off form) [3]. Currently, more than 60 Rab genes have been identified in the human genome and these are localized to distinct intracellular membranes [4]. For example, mammalian Rab5 associates with early endosome (EE), and is a major rate-limiting component of the EE pathway [5]. Rab5 regulates the entry of cargo from plasma membrane to the EE [6]. Rab7 associates with lysosomes and late endosomes (LE) and is implicated in the control of the mobility of these organelles [7,8]. Rab27 is expressed in various secretory cells including endocrine and exocrine cells as well as different types of leukocytes [9]. This protein is involved in the transport of LE and lysosome-related organelles [10,11]. In addition, Rab27

* Corresponding author. The Norwegian College of Fishery Science, UiT The Arctic University of Norway, N-9037, Tromsø, Norway.
E-mail addresses: diliev@bio21.bas.bg, dimitar.iliev@uit.no (D.B. Iliev).

https://doi.org/10.1016/j.fsi.2020.01.058
Received 16 August 2019; Received in revised form 23 January 2020; Accepted 27 January 2020
Available online 31 January 2020

plays a significant role in exosome biogenesis and secretion [3].

Since Rab GTPases are critical regulators of endocytic and secretory membrane trafficking events they are implicated in activation and signaling downstream of immune receptors such as the toll-like receptors (TLRs). For instance, Rab7 function has a negative effect on TLR4 signaling by promoting the receptor degradation within lysosomes [12]. On the opposite, Rab5 is necessary for activation of TLR7 and TLR8 [13]. In addition, many intracellular pathogens exploit Rab GTPase functions and some Rab GTPases are involved in replication of viruses infecting humans and fish [1,14].

The Rab GTPases are well conserved evolutionarily within eukaryotes and have been extensively studied in mammalian experimental systems. However, data about these proteins in teleosts and, in particular, Atlantic salmon is scant.

In the present work, Atlantic salmon Rab5c, Rab7a and Rab27a homologs were studied in terms of their intracellular localization and gene expression through 3D Structured Illumination Microscopy (SIM) and Real-time PCR, respectively. SIM is a super-resolution technique that allows for observation of fluorescent samples at a resolution below the diffraction limit [15].

Salmonid alphavirus (SAV) is a positive-sense single-stranded RNA virus which belongs to family *Togaviridae* and causes pancreatic disease (PD) in salmon [16]. There are six subtypes of SAV, assigned SAV1-6 [17] and among them SAV subtype 3 has been reported to cause PD in farmed salmon in Norway [18]. Studies have shown evidence for alphavirus infection through endocytic uptake followed by low pH-triggered membrane fusion [19]. Therefore, one of the objectives of the current study has been to characterize the expression of Rab genes in A. salmon challenged by SAV3. More specifically, we analyzed the influence of SAV3 challenge on the mRNA expression of Rab5c, Rab7a and Rab27a in spleen, head kidney (HK) and heart. In addition, the expression of these Rab homologs was studied in primary HK adherent leukocytes stimulated with TLR ligands and recombinant interferons (IFNs) in order to shed light on the involvement of these Rab proteins in the fish immune response.

2. Materials and methods

2.1. Cloning

The ORFs of *Salmo salar* (Ss) Rab5c (accession number: XM_014192873), SsRab7a (XM_014133381.1) and SsRab27a (XM_014126950.1) were directionally cloned in pENTR/D-TOPO vector (Invitrogen). The ORFs for the genes were generated from cDNA from Atlantic salmon mononuclear phagocytes as template in PCR reaction with Pfu-TURBO polymerase by using the primers listed in Table 1. Amplified fragments were run and purified on an E-gel Agarose Gel Electrophoresis System (Invitrogen). Amplified fragments were then subcloned into pENTR/D-TOPO cloning vector (Invitrogen) and transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen). Plasmid DNA from bacteria was isolated with QIAprep Spin Miniprep Kit (Qiagen). The DNA sequences and orientation of the inserts in pENTR/D-TOPO,were verified using Restriction digestion reaction and further confirmed by sequencing using the Big Dye chemistry and 3130X Genetic Analyser (Applied Biosystems). Subsequently, the gene inserts in pENTR/D/TOPO vector were further transferred into pDEST-EGFP expression vectors by Gateway recombination using LR clonase II enzyme mix following the manufacturer's instructions (Invitrogen).

2.2. Analysis of the intracellular localization of GFP tagged Rab proteins through SIM

To study the subcellular localization of Rab5c/7a/27a, CHSE-214 cells with a density of 60,000 cells/well were seeded on #1.5 coverslips into 24-well culture plates and were grown until they were 70% confluent. The cells were then transfected with TransIT-LT1 (Mirus) transfection reagents following the manufacturer's protocol, with 300 ng of plasmid (pDEST/GFP-Rab5c/Rab7a/Rab27a) and 1.2 μl transfection reagent in 50 μl/well of serum-free opti-MEM medium without antibiotics. The mixture was incubated for 15 min at room temperature before it was added to the cell culture. The cells were incubated for 48 h to achieve optimal expression of the Rab-GFP fusion proteins. To label endolysosomal compartments, the cells were incubated with fluorescent phosphorothioate oligonucleotides (2006 PS-Alexa Fluor 647) and wheat germ agglutinin (WGA-Alexa Fluor 350) both of which are readily taken up by CHSE cells. Lysosomes were loaded with WGA by incubating the cells with WGA-Alexa 350 (20 μg/ml) for 1.5 h and chased for 30 min. To visualize EE, EE and LE, and EE, LE and lysosomes, the cells were incubated with 2006 PS-Alexa647 for 5 min, 15 min and 2 h, respectively. To investigate the association of Rab27a with autophagosomes, cells transfected with Rab27a-GFP plasmid, were treated either with 160 nM bafilomycin A1 (InvivoGen) for 4 h or with IFNγ (300 ng/ml) for 24 h. Control cells were kept in complete L-15 media without simulants. Before fixation, cells were rinsed with 1X PBS and were fixed with 4% formaldehyde (Thermo Scientific) for 10 min followed by washing the cells 3X with PBS. Cells were then permeabilized and blocked with 0.1% of the detergent Triton X-100 in 5% BSA for 30 min. The cells transfected with Rab5c were then immunostained by incubating with rabbit polyclonal anti-EEA1 primary antibody (Abcam, cat.#ab2900) (1:400 dilution) for 1 h at room temperature, washed 3X with PBS followed by 1 h of incubation with secondary anti-rabbit/Alexa-555 antibody (Invitrogen). Similarly, the cells with Rab27a were immunostained with mouse anti-Alix (Abcam, cat#:ab117600) or LC3B (Sigma Aldrich, cat#: L7543) primary antibodies (1:400 dilution) for 1 h followed by washing with PBS for three times and incubated with secondary anti-mouse/Alexa-546/Alexa-555 antibody (Invitrogen) (1:1000 dilution). As a control for the background fluorescence, cells were stained only with secondary antibodies. The coverslips were then mounted with glycerol containing 1% DABCO.

The samples were imaged on the OMX V4 Blaze structured illumination microscope (GE Healthcare, Uppsala, Sweden) equipped with a 60X, 1.42 NA oil immersion objective (Olympus) and three sCMOS cameras. Laser lines 642, 568, 488 and 405 nm provided sequential excitation, with emission filters of 683/12, 608/37, 528/62 and 435/87

Table 1
Primers used in this study.

Primer Name	Sequence 5′-3′	Purpose
Rab 5C (1) Forward	ACAACTCCAAGGTTCCAATGA	qPCR
Rab 5C (1) Reverse	GGTTGTTATGTCCTCGGGTAAG	qPCR
Rab 5C(2) Forward	CACGGCCTAGAGAGTAATGATG	qPCR
Rab 5C(2) Reverse	GTATAGCAGAGGGAGGGATAGA	qPCR
Rab 7a (1) Forward	CTCAAGGCAGAAGAGGCAATA	qPCR
Rab 7a(1) Reverse	CCGTGTCAGGTCTCAATGTT	qPCR
Rab 7a(2) Forward	CGGTGTGAAAGATTGGGACA	qPCR
Rab 7a(2) Reverse	CTTCTGTAGCTGACTGAGGAAGG	qPCR
Rab27a Forward	GTATGACGTGCAAAGCTGAAAG	qPCR
Rab27a Reverse	CACCACACCAAATGGCATAATC	qPCR
Mx Forward	TGCAACCACAGAGGCTTTGAA	qPCR
Mx Reverse	GGCTTGGTCAGGATGCCTAA	qPCR
EF1aB Forward	TGCCCCTCCAGGATGTCTAC	qPCR
EF1aB Reverse	CACGGGCCCACAGGTACTG	qPCR
TNF α Forward	TGCTGGCAATGCAAAAGTAG	qPCR
TNF α Reverse	AGCCTGGCTGTAAACGAAGA	qPCR
IFNα1 Forward	CCTTTCCCTGCTGGACCA	qPCR
IFNα1 Reverse	TGTCTGTAAAGGGATGTTGGGAAAA	qPCR
Rab5c Forward	CACCATGGCAGGTCGAGGAGGA	Cloning
Rab5c Reverse	TTAGTTCCCACCACAGCACTTGCTAC	Cloning
Rab7a Forward	CACCATGACATCTAGGAAGAAAGTACTA	Cloning
Rab7a Reverse	TCAGCAGCTGCAGGCCTCC	Cloning
Rab27a Forward	CACCATGTCTGATGGGGACTATGACTA	Cloning
Rab27a Reverse	CTAACAAGCACATTTGCCCTTCTCC	Cloning

respectively. The manufacturer-provided program SOFTWORX used experimentally-measured point spread functions for image reconstruction.

2.3. In vivo SAV3 experiment

Salmonid alphavirus subtype 3 (SAV3) (PDV-H10-PA3) was obtained from Øystein Evensen (Norwegian University of Life Sciences, Norway). The virus was propagated in CHH-1 cells (derived from heart tissue of a juvenile chum salmon (*Onchorhynchus keta*)) in L-15 medium with 10 U/mL penicillin, 10 μg/mL streptomycin and 5% FBS at 15 °C. Virus titre was determined by the $TCID_{50}$ method [20]. The challenge experiment was performed at the Tromsø Aquaculture Research Station (Tromsø, Norway). Prior to challenge, Atlantic salmon pre-smolt (60–70 g) had been maintained in 10 °C fresh-water with 24 h of light and fed to satiation for 7 weeks. Fish were randomly allocated to two tanks and one group were intra peritoneal (ip) injected with 100 μl suspension containing 1×10^5 infectious SAV3 particles, while the second group were ip injected with 100 μl PBS as control group. The fish were kept at 10 °C with continuous light after injection. Prior to sampling and relocation, fish were starved for minimum 1 day. Organs from control fish and SAV3 infected fish were aseptically collected at 3, 8 and 14 days post infection. The organs were used for subsequent expression experiments by isolating RNA followed by cDNA synthesis and qPCR. The experiment was approved by the Norwegian Animal Care and Welfare Authority (ID: 16409).

2.4. Isolation and culture of primary adherent HK leukocytes and Chinook salmon embryo (CHSE-214)

Adherent leukocytes from HK were isolated as described earlier [21]. In brief, HK from Atlantic salmon of ~500 g were aseptically collected and homogenized through 100 mm pore size cell strainers (Falcon), in L-15 (Leibovitz, L5520, Sigma) medium supplemented with 2% FBS, penicillin (10 U/ml), streptomycin (10 mg/ml) and heparin (20 U/ml). The homogenized cell suspension from organ was placed on 25/54% discontinuous Percoll gradient and was centrifuged at $400 \times g$ at 4 °C for 40 min. The cells at the Percoll interphase were collected and washed with L-15 medium twice. The density of the cells was adjusted to 7×10^6/ml and were added to 24-well plates (1 ml/well) in L-15 media supplemented with 0.1% FBS, penicillin (10 U/ml) and streptomycin (10 mg/ml) at 14 °C. The cells were cultured overnight and non-adherent cells were carefully removed from the adherent cells by washing with L-15 medium thrice. The adherent cells were then grown in the complete medium with 5% FBS at 14 °C for 48 h, prior stimulation.

CHSE cells [22] were cultured as monolayer in L-15 complete growth medium with 8% FBS at 20 °C.

2.5. In vitro stimulation of HK adherent cells

HK adherent cells were stimulated with TLR ligands and IFNs as follows: 2 μM CpG oligodeoxynucleotides (ODNs) (2006 PS: T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T) (Thermo Scientific); 10 μg/ml Polyinosinic-polycytidylic acid (PolyI:C) (Pharmacia Biotech); 100 ng/ml recombinant trout interferon gamma (IFNγ) [23] and 500 U/ml of recombinant salmon interferon a1(IFNa1) [24]. Controls were treated with medium alone or with inverted non-CpG ODNs (2395 PS: T*C*G*T*C*G*T*T*T*TC*G*G*C*G*C*G*C*G*C*C*G) (Thermo Scientific). Cells were harvested 6, 24 and 48 h post-stimulation using lysis buffer, included in the RNeasy Mini kit (Qiagen). Lysates were kept at −80 °C before total RNA isolation, cDNA synthesis and qPCR assays.

2.6. Stimulation of CHSE-214 cells with poly I:C

Approximately 900,000 CHSE-214 cells were seeded in 6-well plates the day before transfection. The cells were transfected with 1 μg poly I:C from GE Healthcare Amersham Biosciences (Uppsala, Sweden) complexed with 3μ Trans-IT-LT1 (Mirus) transfection reagents in 100 μl serum free DMEM. Cells were harvested 6, 24 and 48 h post transfection for RT-PCR assays.

2.7. Quantitative RT-PCR (qPCR)

Organs (HK and spleen) were homogenized using TissueLyser II (Qiagen) before isolating total RNA. Proteinase K was added to isolate total RNA from heart tissues following manufacturer's protocol. Total RNA from tissue homogenates was isolated by following the manufacturer's protocol (Qiagen). Isolated RNA (600 ng per 20 μl reaction) was reverse transcribed using Quantitect® reverse transcriptase kit (Qiagen) following the manufacturer's protocol. A volume of 2 μl cDNA (3 ng of reverse-transcribed RNA) per 20 μl reaction was used with primers for Rab5c1 (recognizing isoforms XM_014192873 and XM_014204108) Rab5c2 (XM_014158741), Rab7a1 (XM_014133381), Rab7a2 (XM_014137854), Rab27a (XM_014126950), Mx, TNFα and salmon Elongation factor 1αβ (listed in Table 1) using SYBR® Green Master Mix (Applied Biosystems).

Total RNA from the stimulated or control salmon HK adherent leukocytes and poly I:C transfected CHSE-214 was isolated using same protocol as for organs, excluding homogenization steps. Isolated RNA (300 ng in a 20 μl reaction) for HK adherent leukocytes and 150 ng in a 20 μl reaction for poly I:C transfected CHSE-214 was reverse transcribed using Quantitect® reverse transcriptase kit (Qiagen) by following the manufacturer's protocol. A volume of 2 μl cDNA (3 ng of reverse-transcribed RNA) per 20 μl reaction was used with same primers as above, as listed in Table 1 using Fast SYBR® Green Master Mix (Applied Biosystems).

The PCR reactions were run on ABI Prism 7500 FAST Cycler (Applied Biosystem). All cDNA samples were run in duplicates and the amplification specificity was confirmed with dissociation curves. The levels of EF1αβ were used to normalize the expression of the genes of interest and fold difference values compared to the non-treated control samples were calculated using Pfaffl's model [25]. For basal expression of Rabs in tissues, the expression of the genes of interest was calculated relative to the Ct values of EF1αβ. In brief, the mRNA levels of the Rabs were expressed as $2^{-\Delta CT}$ (C_T - threshold cycle), where ΔC_T is determined by subtracting the EF1 αβ CT value from the target CT as previously described [26]. Student's *t*-test and one-way ANOVA statistical tests were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA).The value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Cloning and in vitro localization of GFP tagged Rab proteins

The cloned ORFs of Rab5c (XM_014192873), Rab7a (XM_014133381.1) and Rab27a (XM_014126950.1) encode proteins with lengths of 219, 207 and 223 amino acids, respectively. A phylogeny analysis with nucleotide sequences from other vertebrates confirmed the orthology of the cloned molecules (not shown).

The three salmon Rab homologs expressed in CHSE cells as GFP-fusion proteins showed distinct intracellular distribution (Fig. 1). Rab5c-GFP was mostly associated with small vesicles evenly dispersed throughout the cytoplasm, including cell periphery. In contrast, most of Rab7a associated with bigger vesicles clustered around the perinuclear region and there were relatively few Rab7a-positive vesicles in the periphery of the cells. Similar to Rab7a, Rab27a was observed in aggregates of relatively large perinuclear vesicles but, unlike Rab5C and

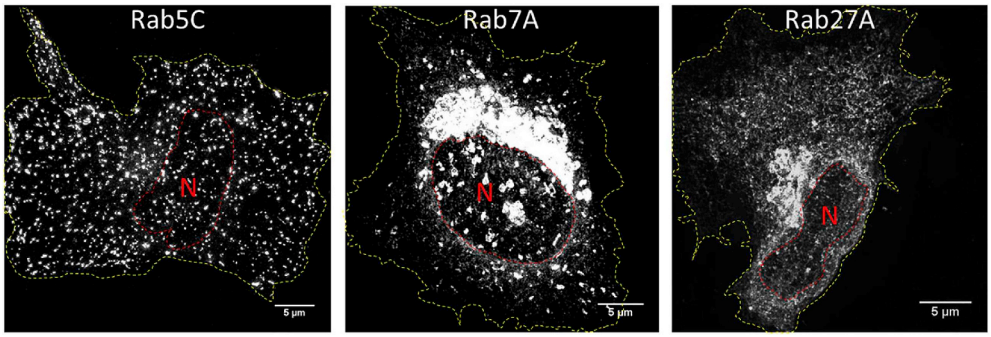

Fig. 1. Intracellular distribution of GFP-tagged Rab5c, Rab7a and Rab27a in CHSE-214 cells. Cells were transfected with constructs expressing the salmon Rab homologs, N-terminally tagged with GFP and imaged with SIM. The images display the maximum intensity z-stack projections of cells transfected with constructs expressing the indicated Rabs. The yellow contours show the cell outline; the red contours show the position of the nuclei (N). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

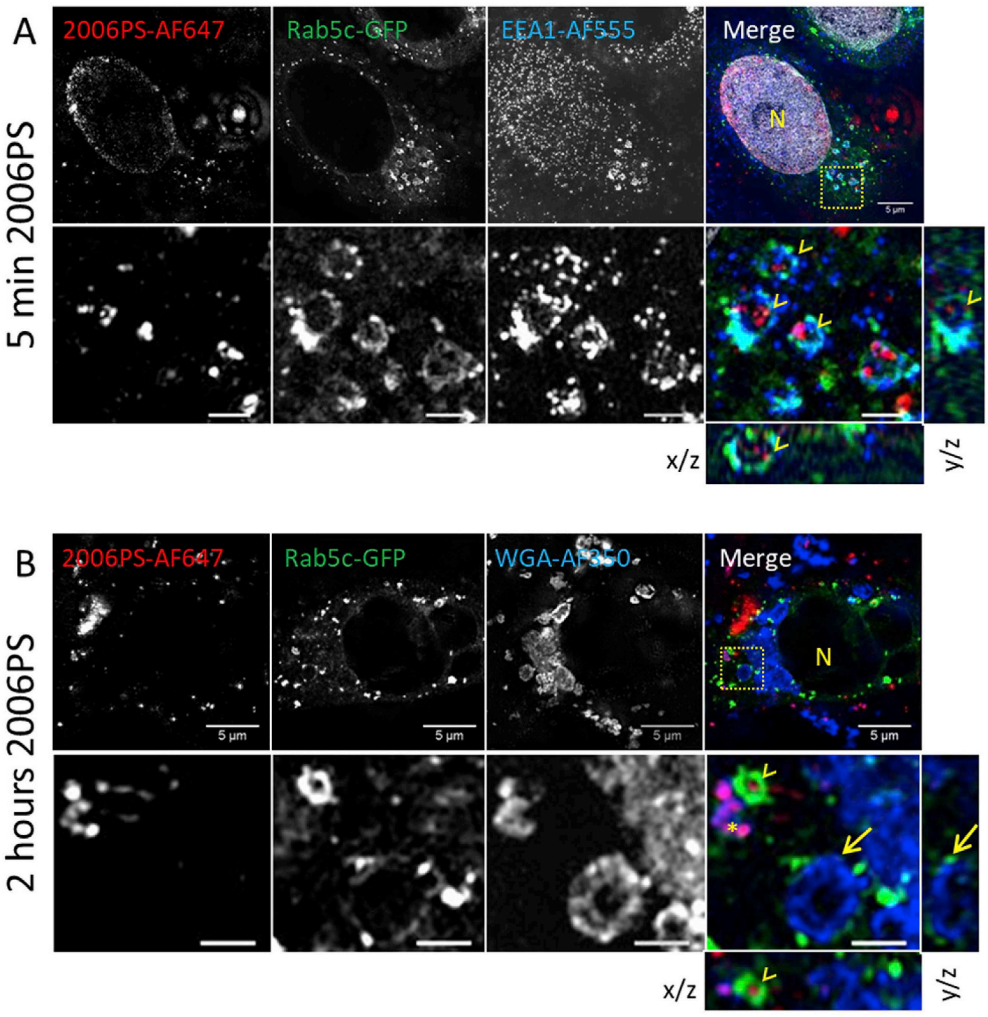

Fig. 2. Salmon Rab5c associates predominantly with early endosomes. CHSE cells expressing Rab5c-GFP (green) were incubated with ODN2006PS-Alexa647 (red) for 5 min (A) and 2 h (B). A, EEA1 was stained with a primary rabbit and a secondary AlexaFluor555 antibodies (blue). Upper row: z-stack projections; nuclei were stained with DAPI (grey in the merged image). Colocalization between Rab5c and EEA1, detected on 2006 PS-containing vesicles, is indicated with arrowheads in the enlarged inset and the x/z and y/z side profile views shown in the lower row. B, Lysosomes were loaded with WGA-AF350 (blue) as described in Materials and methods. The upper row shows z-stack images of cells incubated with 2006 PS-AF 647 for 2 h. Lower row: enlarged inset; arrowhead indicates a Rab5c-positive endosome, asterisk: an adjacent 2006 PS- and WGA-positive lysosome; arrow: a rare example of a large, WGA-positive vesicle, decorated with Rab5c-GFP. Scale bars in enlarged regions, 1 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

7a, which were present mostly on vesicles, a significant proportion of Rab27a was associated with polymorphic structures dispersed throughout the cytoplasm of the CHSE cells.

Cells treated with fluorescent 2006PS ODNs accumulated the ODNs within Rab5c-positive vesicles within 5 min of incubation. Some of these vesicles were also positive for EEA1 as shown by the SIM analysis (Fig. 2A). Longer incubations with the fluorescent ODNs resulted in their accumulation in other types of vesicles, including lysosomes labelled with WGA. Interestingly, in addition to EE, Rab5c could also be observed in association with WGA-positive lysosomes, albeit at much lower levels as compared to EE (Fig. 2B).

Rab7a was predominately present on the membranes of WGA-positive lysosomes (Fig. 3A). After 15 min of incubation, the fluorescent ODNs were observed in some relatively small Rab7a-positive vesicles (< 1 μm). After 2 h of incubation, the ODNs were observed both in lysosomes and in Rab7a-positive vesicles, which were adjacent to lysosomes and appeared to be in a process of fusion with the latter (Fig. 3B).

Compared to Rab5c and 7a, the intracellular distribution of Rab27a was less specific. Although it was difficult to determine whether Rab27a associates specifically with certain types of endolysosomal compartments, in some instances it was observed on perinuclear lysosomes loaded with WGA (Fig. 4A). In addition, we could observe rab27a-positive lysosomes, which also contained alix as indicated by the staining with the monoclonal anti-alix antibody (Fig. 4B) indicating these might be multivesicular bodies containing exosome precursors.

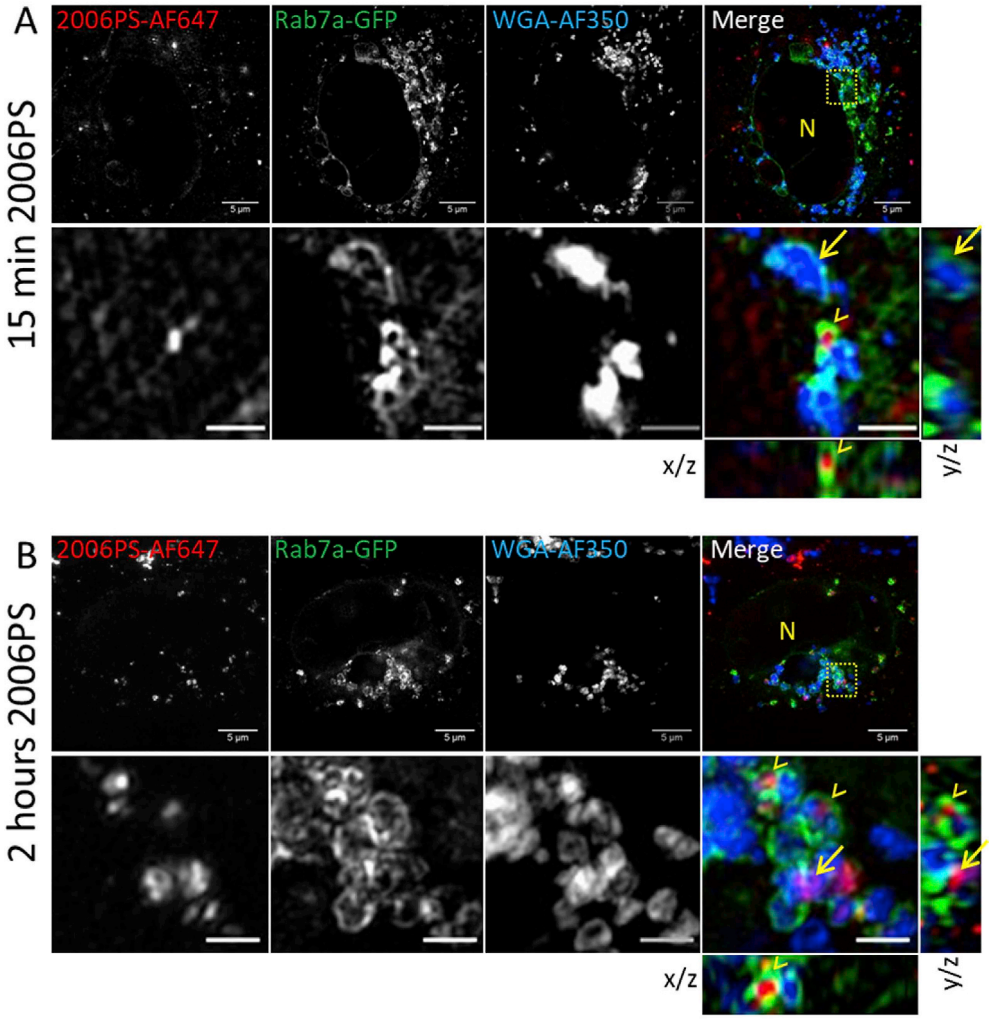

Fig. 3. Salmon Rab7a associates predominantly with LE and lysosomes. The lysosomes of CHSE cells expressing Rab7a-GFP (green) were loaded with WGA-AF350 (blue) and the cells were incubated with 2006 PS-AF647 (red) for 15 min (A) or 2 h (B) prior to fixation and SIM analysis. The lower rows show the enlarged region indicated on the z-stack projections in the upper row. Arrowheads: Rab7a-positive endosomes containing 2006 PS-AF647; arrows: lysosomes containing WGA. N marks the positions of nuclei. Scale bars in enlarged regions, 1 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Staining of Rab27a-GFP-expressing cells with an LC3B antibody showed that the two proteins colocalize in scarcely-observed vesicular structures (Fig. 5A). This colocalization was more pronounced in cells stimulated with recombinant IFN-γ (Fig. 5B). Upon treatment of cells with 160 nM bafilomycin A1 for 4 h, Rab27a-GFP also accumulated in LC3B-positive vesicles (Fig. 5C).

3.2. Expression of Rabs in organs of healthy and SAV3-challenged individuals

In addition to the cloned isoforms, the salmon genome encodes two other Rab5c, seven Rab7a and an additional Rab27a isoforms. One pair of primers for Rab5c (arbitrarily termed Rab5c1) recognize XM_014192873 (the cloned isoform) and is predicted to hybridize with and amplify XM_014204108 (1 nucleotide mismatch) which encode proteins with 99% aa identity (2 aa mismatch). The second pair of primers recognizes specifically XM_014158741 (arbitrarily termed Rab5c2), which encodes a protein with 90% aa identity compared to the other two Rab5c isoforms. The primers for Rab27a recognize the two isoforms XM_014126950 (the cloned one) and XM_014175804 (one nucleotide mismatch) which encode proteins with 99% aa identity (2 aa mismatch). The primers for Rab7a (arbitrarily termed Rab7a1) recognize the cloned isoform (XM_014133381). Another pair of primers (arbitrarily termed Rab7a2) amplifies XM_014137854 which encodes a protein with 90% aa identity compared to the cloned one. The aa alignments are presented in Supplementary Fig. 1.

Basal expression of these transcripts was analyzed in the immune organs HK and spleen and in the heart from healthy salmon using qPCR (Fig. 6). The expression of Rab5c detected with both primer pairs as well as the mRNA levels of Rab27a were highest in spleen. Notably, while Rab5c1 showed high relative expression in the heart, the Rab5c2 mRNA levels in the same organ was low. The two Rab7a isoforms were most highly expressed in heart and their levels in the immune organs were similar.

In order to investigate the potential of a SAV3 infection to modulate the expression of Rab genes *in vivo*, selected samples were obtained from a larger SAV-trial. The establishment of infection was confirmed by detection of virus transcripts (nsP1) in serum and organs by qPCR. While no viral transcripts were detected in HK, spleen and heart at 3 dpi, nsP1 transcripts were present in all the organs at 8 and 14 dpi [27]. The qPCR analysis of the expression of the Rabs in organs of fish challenged with SAV3 demonstrated that the infection affected the expression of all of the tested isoforms (Fig. 7). In the spleen, transcripts detected with the Rab5c1 primers were upregulated at 3 and 14 days dpi whereas the Rab5c2 isoform was significantly induced at 8 and 14 days. While in spleen, the expression of Rab7a1 was not affected by the infection, Rab7a2 showed delayed SAV3-induced upregulation 14 days dpi. In HK, Rab5c1, Rab7a2 and Rab27a showed significant induction at all of the tested time points which gradually increased with time. In this organ, Rab5c2 was transiently upregulated by SAV3 with a peak at 8 days and subsequent downregulation. Rab7a1 showed biphasic upregulation at 3 and 14 days with a downregulation 8 days dpi. In heart, Rab5c1 and 2 as well as Rab7a1 were quickly but transiently upregulated 3 days dpi. Following upregulation at 3 days, Rab7a2 was

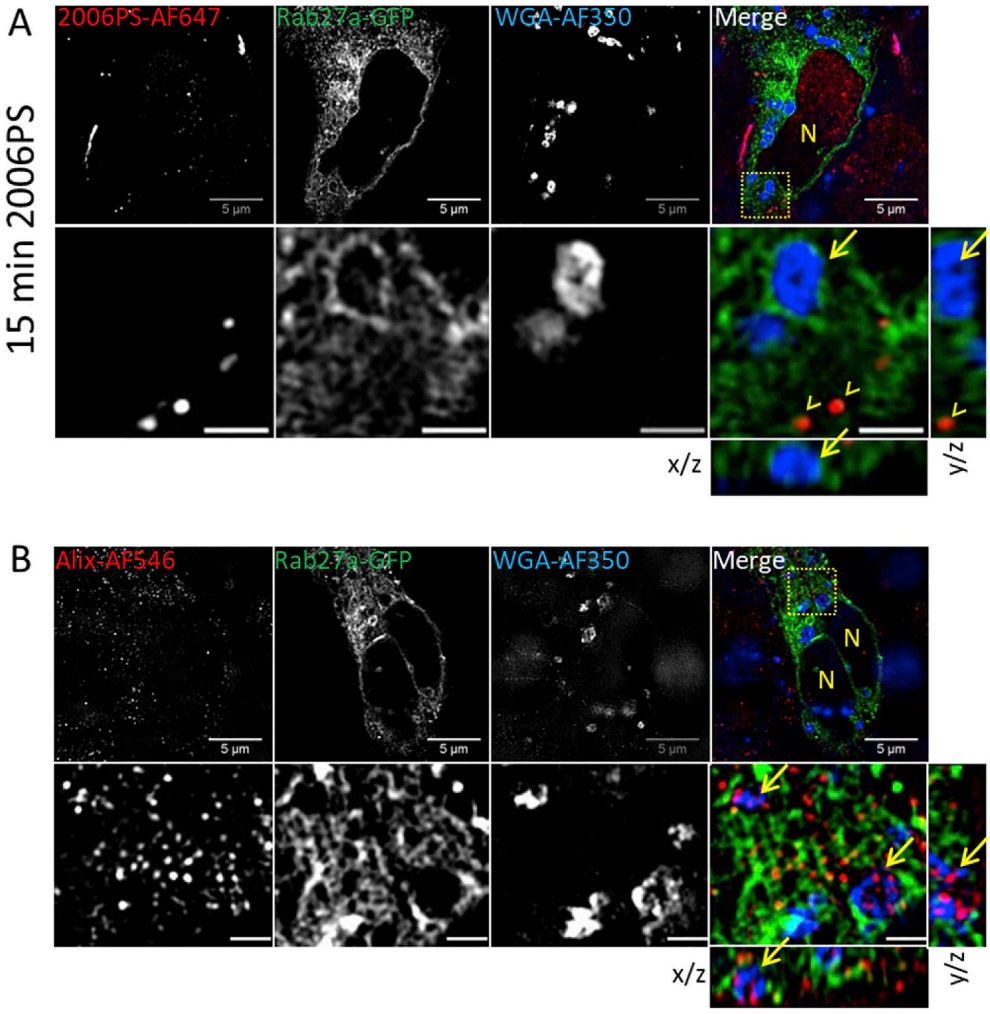

Fig. 4. Salmon Rab27a has a broad intracellular distribution and it is observed on lysosomes containing alix. A, CHSE cells expressing Rab27a-GFP (green) were loaded with WGA-AF350 (blue) and were incubated with 2006 PS-AF647 (red) for 15 min prior to fixation and SIM analysis. The images in the lower row show the enlarged region indicated on the z-stack projection in the upper row. Arrowheads show internalized 2006 PS-AF647 in Rab27a-negative vesicles. Arrow – Rab27a-positive lysosome. B, Rab27a-GFP were stained with primary anti-alix and AlexaFluor-546 secondary antibodies (red). The arrows in the enlarged merge indicate lysosomes containing alix. N marks the positions of nuclei. Scale bars in enlarged regions, 1 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

downregulated at 8 days and it was highly upregulated again 14 days dpi. The upregulation of Rab27a in heart was slow and it was significant only 14 days after the SAV3 challenge.

3.3. Rab expression in adherent HK leukocytes stimulated in vitro with TLR ligands and IFNs

The expression of the salmon Rab isoforms was analyzed in primary cultures of adherent HK leukocytes, which are enriched in mononuclear phagocytes [28]. The cells were stimulated with CpG ODNs 2006 PS (a ligand for TLR9 and TLR21) and poly I:C (a ligand for TLR3 and TLR22) as well as recombinant IFNα and IFNγ. As shown in Fig. 8, the CpG stimulation upregulated Rab27a and Rab5c1 but not Rab5c2. In contrast, poly I:C induced both Rab5c1 and Rab5c2 as well as Rab27a. Although not as prominent, IFNa1 upregulated all of the isoforms, except for Rab7a1. Interestingly, while the IFNγ stimulation upregulated weakly Rab5c, it had a transient, negative effect on the expression of Rab7a1.

3.4. Rab mRNA expression in CHSE cells

Unlike primary salmon leukocytes, direct stimulation of CHSE cells with poly I:C cannot elicit an IFN response [29]; however, transfection of these cells with the poly I:C does activate IFN promoters [30]. In the current study, CHSE cells responded to transfection with poly I:C by strong upregulation of IFNa1 and Mx; however, the expression of the Rab isoforms was not significantly affected by the stimulation (Fig. 9).

4. Discussion

With the current work we demonstrate that, similar to mammalian counterparts, salmon homologs of Rab5c and Rab7a are primarily located within EE and LE/lysosomes, respectively. Rab27a showed broader intracellular distribution; nevertheless, its association with alix-positive lysosomes and LC3B-positive vesicles suggests that, like in mammals, the salmon homolog might be implicated in exocytic pathways such as exophagy and secretion of exosomes.

Phosporothioate CpG ODNs are potent immunostimulants [31], however, to activate immune cells, these ODNs need to enter the endocytic pathway and accumulate within acidified compartments containing endosomal TLRs such as TLR9 [32]. The CHSE cells used in the current study do not express endogenous TLR9 and they do not respond to stimulation with CpGs; however, we have observed that these cells readily endocytose 2006 PS ODN. More specifically, within 15 min, 2006 PS ODNs were detected in small vesicles with neutral pH, most likely representing endocytic vesicles and EE while after prolonged incubations (> 1 h) CHSE cells accumulated the ODNs within LE and lysosomes [33]. In the current study, within 5 min of incubation with fluorescent 2006 PS, the ODNs could be observed in Rab5c-coated vesicles where Rab5c colocalized with EEA1. Rab5 is essential for the homotypic fusion of EE with each other *in vitro* [34]. In addition to EE, Rab5 is also present on plasma membrane [35], which highlights its role in regulation of membrane traffic in the EE pathway [36]. EEA1 is an early endosome marker and an effector protein of Rab5 that facilitates the fusion of endosomes [37]. EEA1 provides the mechanical

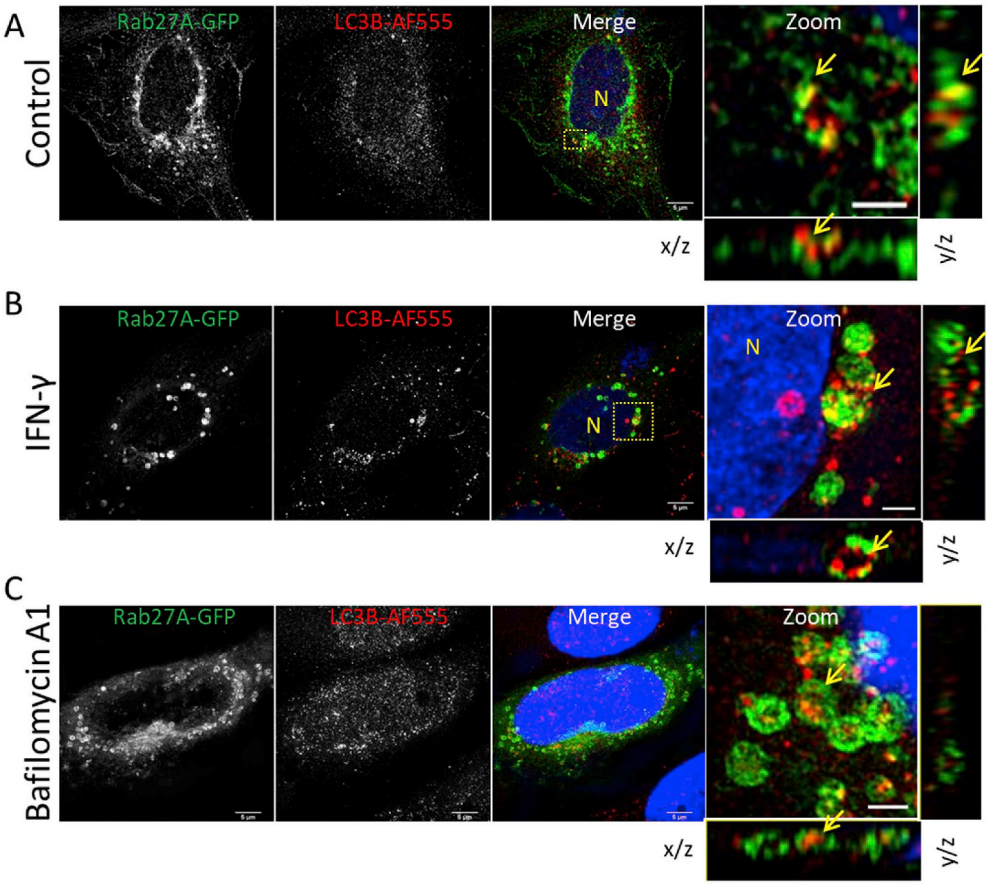

Fig. 5. Salmon Rab27a associates with the autophagosomal marker LC3B in IFN-γ-induced vesicles. A, CHSE cells expressing Rab27a-GFP (green) were stained with primary anti-LC3B and a secondary AlexaFluor555 antibodies (red). The arrow in the magnified region indicates a rarely observed association of Rab27a with LC3-positive structures. B, Stimulation of cells with 300 ng of recombinant IFNγ for 24 h results in formation of LC3B and Rab27A-positive vesicles, likely representing autophagosomes (arrows in the magnified region). C, Treatment of CHSE cells with 160 nM bafilomycin A1 for 4 h also leads to accumulation of Rab27a in LC3B-positive autophagosomes (arrows). Nuclei were stained with DAPI (blue). Scale bars in magnified regions, 1 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

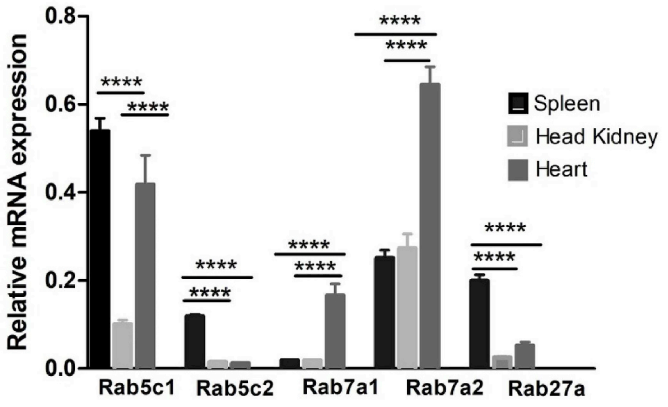

Fig. 6. Basal expression of Rabs in salmon spleen, head kidney and heart. The mRNA levels were quantified with Real-time PCR and the expression of Rabs is shown relative to the expression of the housekeeping gene EF1aB. The columns show mean values ± standard error (n = 12). ****p < 0.0001.

force required to bring incoming vesicles close together when bound by Rab5-GTP [38]. Overall, Rab5 and EEA1 together are required in the progression of internalized extracellular substances into the endosomal pathway [38]. Therefore, the data presented here demonstrate that salmon Rab5c can be regarded as an EE marker and indicate that that the functional relationships between Rab5c and EEA1 are, very likely, conserved between teleosts and mammals.

It should also be acknowledged that, in addition to EE, salmon Rab5c was, in some occasions, observed at very low levels on lysosomal membranes. It is difficult to speculate whether this association might have any functional significance or it is merely an artifact of the transient overexpression of the Rab5c-GFP fusion protein.

In mammals, Rab7 is involved in transport from EE to LE and it is a marker for LE and lysosomes [7,39] It has also been found that recruitment of Rab7 on LE is accompanied by subsequent the loss of Rab5 (31). WGA is a carbohydrate-binding lectin having affinity for N-acetylglucosamine and sialic acid. WGA has been found to interact with the lysosome membrane (30) and, in the current study, it was used as an endocytic substrate to label lysosomes. The results presented here demonstrate that salmon Rab7a associates with lysosomes and it is likely involved in LE-lysosome fusion since after 2 h of incubation with 2006 PS, the ODNs were observed in Rab7a positive vesicles, which appeared to be in process of fusion with WGA-loaded lysosomes.

Rab27a is involved in the transport and the secretion of endosome and lysosome-like organelles such as melanosomes [40] and lytic granules of cytotoxic lymphocytes [41]. In addition, Rab27a is implicated in the secretion of exosomes [42] as well as IFNγ-induced secretory autophagy (exophagy) [43] and it is likely to play a role in virus-induced exophagy and exosome secretion [44]. The salmon Rab27a could be observed on vesicles containing alix (an exosome marker) and in LC3B-positive autophagosomes whose accumulation was induced by treatment with bafilomycin A1. Bafilomycin A1 blocks the fusion of autophagosomes with lysosomes leading to accumulation of autophagosomes [45]. Therefore, our data suggest that the salmon Rab27A may associate both with multivesicular bodies containing exosome precursors as well as autophagosomes. The association of salmon Rab27a with IFNγ-induced vesicles containing the autophagosome marker LC3B suggests that, similar to its mammalian homolog, salmon Rab27a might be involved in regulation of unconventional secretion of proteins and extracellular vesicles such as exosomes.

The transcript levels of all of the studied Rab isoforms showed significant differences across the analyzed organs indicating differential expression by different cell types and, possibly, tissue and organ-specific functions. Of note, the expression pattern of salmon Rab5 in HK

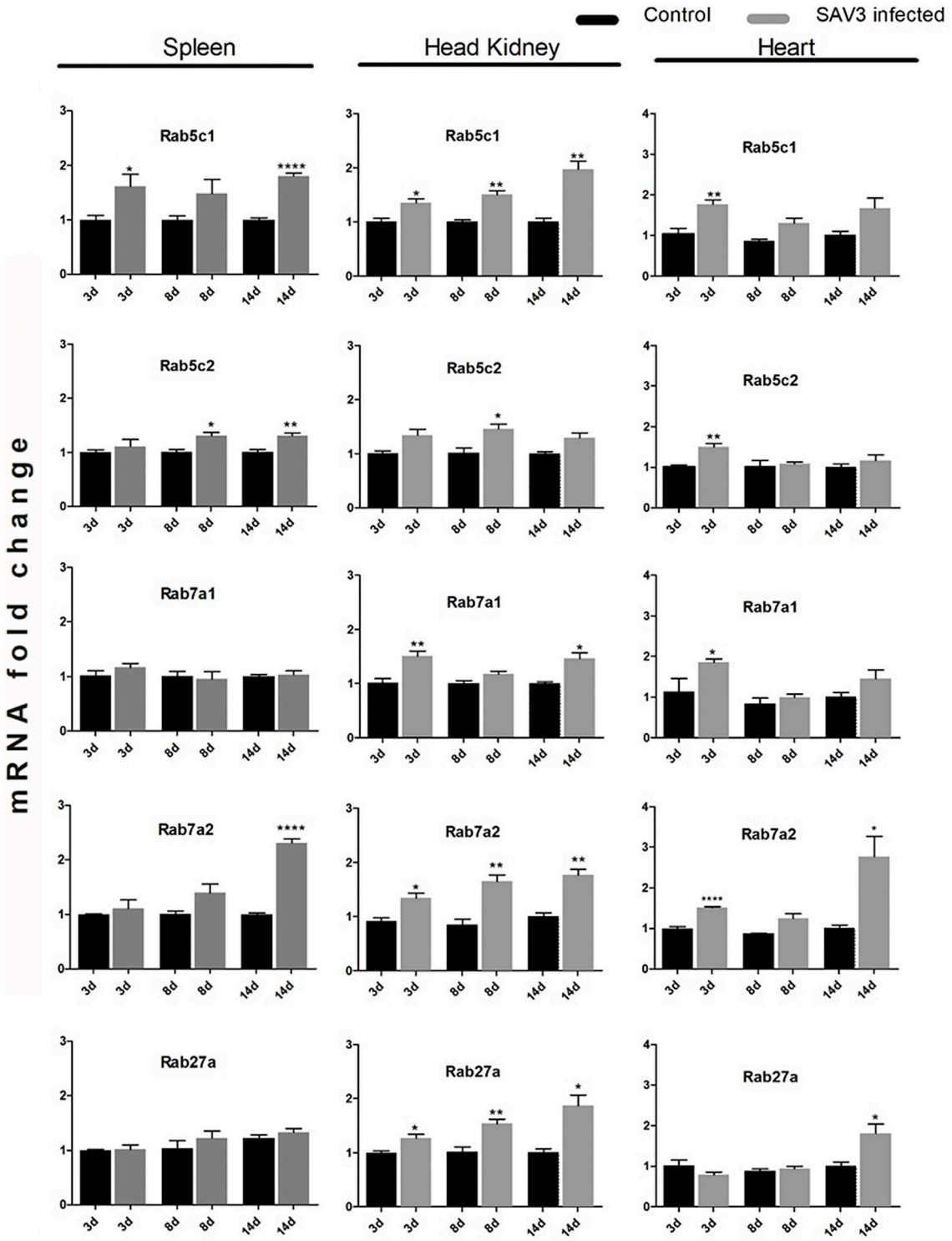

Fig. 7. Real-time PCR analysis of the expression of Rab isoforms in spleen (n = 4), HK and heart (n = 8) of fish infected with SAV3 for 3, 8 and 14 days. The graph bars display mean "fold change" values ± standard error compared to control group (injected with PBS) for each time point (n = 4). Gene expression was normalized against EF1aB. *p < 0.05 **p < 0.01 ***p < 0.001 ****p < 0.0001.

had a similar pattern in channel catfish [46] and yellow croaker [47].

As obligate intracellular parasites, viruses are known to interact and exploit the functions of different Rab proteins, including Rab5, Rab7 and Rab27 [48]. Viral infections are also known to modulate the expression of Rabs – for example in spleen of orange-spotted grouper, a Rab7 homolog was upregulated by Singapore grouper iridovirus infection [14]. Transcriptional regulation of Rab proteins, for example Rab7b, is involved in regulation of TLR signaling [12,49]. Moreover, in orange spotted grouper, overexpression of a Rab7 homolog enhanced the cytopathic effect of a viral infection and the replication of the viral genome [14]. Therefore, modulation of mRNA expression of Rabs by viral infections may reflect the capacity of the virus to exploit the functions of these molecules and might be implicated in the immune response against the pathogen. In the current study, we investigated the effect of SAV3 infection on the expression of the salmon Rabs in two immune organs – HK and spleen which are the major sites of innate and

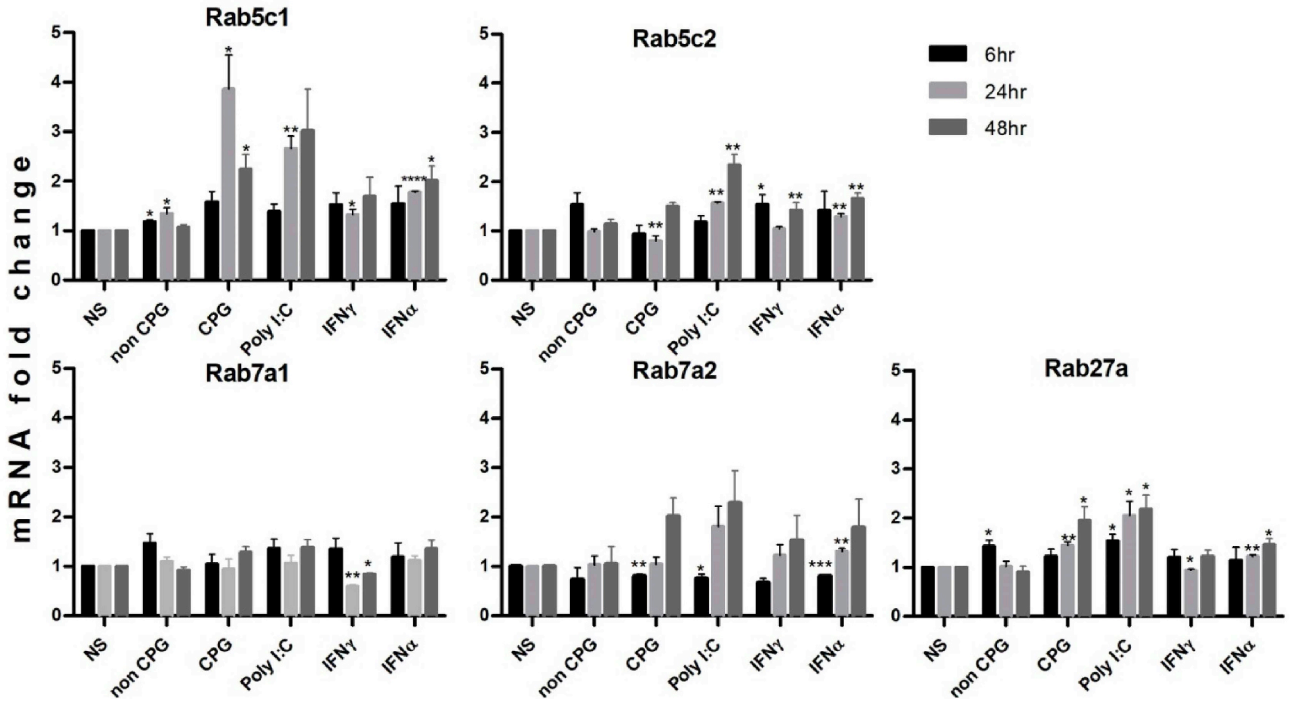

Fig. 8. Transcriptional regulation of salmon Rab isoforms in primary adherent HKL stimulated with TLR ligands (CpG and PolyI:C) and cytokines (IFNγ and IFN α) for 6, 24 and 48 h. Gene expression was analyzed with Real-time PCR and the Rab expression was normalized against EF1aB. The graph bars display mean "fold change" values ± standard error compared to non-stimulated samples (NS) for each time point (n = 3). *p < 0.05 **p < 0.01 ***p < 0.001 ****p < 0.0001.

adaptive immune responses in fish [50] and in heart which is the target organ of SAV3 [51]. Analysis of SAV3 replication in samples from this trial demonstrated that virus transcripts could be detected at 8 and 14 days post-infection but not after 3 days [27]. Since in HK and heart most of the analyzed Rab transcripts showed significant upregulation at 3 days post-infection, it could be speculated that this might be due to a direct immune response to the viral infection. In spleen and heart, more of the rab transcripts were significantly upregulated at 14 as compared to 8 days post-infection. This might be a consequence of the viral replication since the results from the study by Svenning et al. showed that the SAV3 replication peaked on day 14 in these organs [27].

To find out whether Rab5c/7a/27a expression might be directly modulated by innate immune stimuli in salmon leukocytes, the expression profiles of these genes were investigated in primary adherent leukocytes from HK. CpG ODNs which activate TLR9 and TRL21 induced the expression of Rab5c1 and Rab27a but not Rab7a. The activation of endosomal innate immune receptors, such as TLR9, depends on spatiotemporal regulation of the distribution of the CpGs within

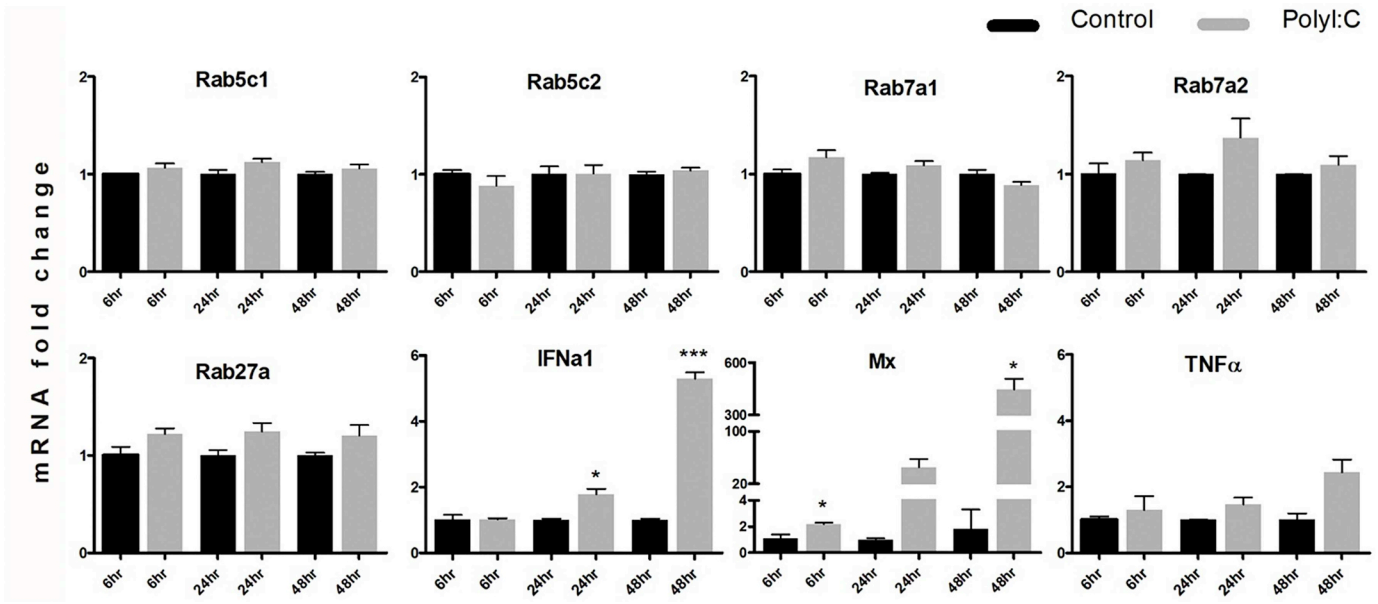

Fig. 9. Real-time PCR analysis of Rab expression in CHSE cells transfected with polyI:C for 6, 24 and 48 h (n = 3). The graph bars display mean "fold change" values ± standard error compared to non-stimulated samples (NS) for each time point (n = 2). Gene expression was normalized against EF1aB. *p < 0.05 ***p < 0.001.

different endolysosomal compartments. More specifically, longer retention of CpGs in endosomes prior to their transfer into lysosomes promotes the CpG-induced IFN response [52]. Therefore, it is possible that the induction of Rab5c and Rab27a by CpGs and polyI:C in salmon leukocytes might be implicated in the regulation of the immunostimulatory activity of the of these TLR ligands.

It has been found that pretreatment of human macrophages with type I IFN sensitizes the cells to treatment with TLR ligands through upregulation of TLRs, including TLR3 [53]. Therefore, it is possible that the modest, yet significant, upregulation of all of the Rabs, except for Rab7a1 by IFNa, as observed in the current study, might also be implicated in the priming activity of type I IFN.

IFNγ is one of the most important cytokines for the intercourse between the adaptive and innate immunity [54]. It plays a central role in the priming of macrophages [55] and we have previously shown that it induces the expression of the TLR9 protein in salmon adherent HK leukocytes [56]. In the current study, IFNγ upregulated modestly Rab5c and Rab27a; however, it had a negative effect on the expression of Rab7a. In mice, Rab7b is involved in the negative regulation of TLR signaling, including TLR4 [12] and TLR9 [49] by promoting their lysosomal degradation. Whether the negative effect of IFNγ on the expression of salmon Rab7a mRNA is involved in the priming activity of this cytokine on salmon macrophages will be an interesting direction for future studies.

Overall, the stimulation of primary adherent HK leukocytes with TLR ligands resulted in moderate but significant modulation of the expression of most of the analyzed Rab isoforms. That indicates that these Rab genes might be involved in the regulation of endosomal TLR signaling through a positive feedback loop. This is further corroborated by the fact that transfection of CHSE cells with polyI:C did not result in significant changes in the expression of the salmon Rab homologs. Unlike primary salmon leukocytes, CHSE cells do not express TLR9 [33] and they are unresponsive to direct stimulation with CpGs and polyI:C [29]. The robust IFN response in CHSE cells induced by transfection with polyI:C is most likely mediated by cytoplasmic helicases such as RIG-I and MDA-5 [57]. Therefore, these data suggest that the modulation of the expression of the salmon Rab homologs by TLR ligands in primary leukocytes is most likely dependent on the function of endosomal immune receptors such as TLR9 and TLR3.

In summary, with this work we have demonstrated that salmon homologs of Rab5c and Rab7a may serve as reliable markers for EE and LE/lysosomes, respectively. As it concerns salmon Rab27a, our observations indicate that, like in mammals, it might be implicated in unconventional secretory pathways. The modulation of the expression of the salmon Rab homologs by SAV3, TLR ligands and IFNs indicates that these genes may play important roles in the regulation of the salmon immune response.

Funding

AN and DLW were supported by the UiT 'Tematisksatsing' (Strategic Funding) to establish a 'Centre on Nanoscopy to decode nanoscale biological systems'. DBI received support from the Research Council of Norway (Project No.: 230735/F20) and the Bulgarian National Science Fund (project No: КП-06-H21/17–19.12.2018).

Declaration of competing interest

The authors have no competing interests to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.01.058.

References

[1] S.B. Sieczkarski, G.R. Whittaker, Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses, Traffic 4 (2003) 333–343.
[2] J.C. Herve, N. Bourmeyster, Rab GTPases, master controllers of eukaryotic trafficking, Small GTPases 9 (2018) 1–4.
[3] L. Blanc, M. Vidal, New insights into the function of Rab GTPases in the context of exosomal secretion, Small GTPases 9 (2018) 95–106.
[4] H. Stenmark, V.M. Olkkonen, The Rab GTPase family, Genome Biol. 2 (2001) REVIEWS3007.
[5] M.A. Barbieri, R.L. Roberts, A. Gumusboga, H. Highfield, C. Alvarez-Dominguez, A. Wells, et al., Epidermal growth factor and membrane trafficking. EGF receptor activation of endocytosis requires Rab5a, J. Cell Biol. 151 (2000) 539–550.
[6] J.T. Murray, C. Panaretou, H. Stenmark, M. Miaczynska, J.M. Backer, Role of Rab5 in the recruitment of hVps34/p150 to the early endosome, Traffic 3 (2002) 416–427.
[7] C. Bucci, P. Thomsen, P. Nicoziani, J. McCarthy, B. van Deurs, Rab7: a key to lysosome biogenesis, Mol. Biol. Cell 11 (2000) 467–480.
[8] R. Vitelli, M. Santillo, D. Lattero, M. Chiariello, M. Bifulco, C.B. Bruni, et al., Role of the small GTPase Rab7 in the late endocytic pathway, J. Biol. Chem. 272 (1997) 4391–4397.
[9] J.L. Johnson, J. Monfregola, G. Napolitano, W.B. Kiosses, S.D. Catz, Vesicular trafficking through cortical actin during exocytosis is regulated by the Rab27a effector JFC1/Slp1 and the RhoA-GTPase-activating protein Gem-interacting protein, Mol. Biol. Cell 23 (2012) 1902–1916.
[10] M.S. Marks, H.F.G. Heijnen, G. Raposo, Lysosome-related organelles: unusual compartments become mainstream, Curr. Opin. Cell Biol. 25 (2013) 495–505.
[11] M. Fukuda, Regulation of secretory vesicle traffic by Rab small GTPases, Cell. Mol. Life Sci. 65 (2008) 2801–2813.
[12] Y. Wang, T. Chen, C. Han, D. He, H. Liu, H. An, et al., Lysosome-associated small Rab GTPase Rab7b negatively regulates TLR4 signaling in macrophages by promoting lysosomal degradation of TLR4, Blood 110 (2007) 962–971.
[13] F. Heil, P. Ahmad-Nejad, H. Hemmi, H. Hochrein, F. Ampenberger, T. Gellert, et al., The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily, Eur. J. Immunol. 33 (2003) 2987–2997.
[14] J. Fu, Y. Huang, J. Cai, S. Wei, Z. Ouyang, F. Ye, et al., Identification and characterization of Rab7 from orange-spotted grouper, Epinephelus coioides, Fish Shellfish Immunol. 36 (2014) 19–26.
[15] M.G.L. Gustafsson, Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy, J Microsc-Oxford 198 (2000) 82–87.
[16] R.T. Nelson, M.F. Mcloughlin, H.M. Rowley, M.A. Platten, J.I. Mccormick, Isolation of a toga-like virus from farmed Atlantic salmon Salmo-salar with pancreas disease, Dis. Aquat. Org. 22 (1995) 25–32.
[17] E. Fringuelli, H.M. Rowley, J.C. Wilson, R. Hunter, H. Rodger, D.A. Graham, Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences, J. Fish. Dis. 31 (2008) 811–823.
[18] J.H. Weston, M.D. Welsh, M.F. McLoughlin, D. Todd, Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, Salmo salar L, Virology 256 (1999) 188–195.
[19] M. Kielian, C. Chanel-Vos, M. Liao, Alphavirus entry and membrane fusion, Viruses 2 (2010) 796–825.
[20] G. Strandskog, S. Villoing, D.B. Iliev, H.L. Thim, K.E. Christie, J.B. Jorgensen, Formulations combining CpG containing oligonucleotides and poly I:C enhance the magnitude of immune responses and protection against pancreas disease in Atlantic salmon, Dev. Comp. Immunol. 35 (2011) 1116–1127.
[21] S. Graham, C.J. Secombes, The production of a macrophage-activating factor from rainbow trout Salmo gairdneri leucocytes, Immunology 65 (1988) 293–297.
[22] C.N. Lannan, J.R. Winton, J.L. Fryer, Fish cell lines: establishment and characterization of nine cell lines from salmonids, In Vitro 20 (1984) 671–676, https://doi.org/10.1007/BF02618871.
[23] I. Skjaeveland, D.B. Iliev, G. Strandskog, J.B. Jorgensen, Identification and characterization of TLR8 and MyD88 homologs in Atlantic salmon (Salmo salar), Dev. Comp. Immunol. 33 (2009) 1011–1017.
[24] B. Robertsen, V. Bergan, T. Rokenes, R. Larsen, A. Albuquerque, Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity, J. Interferon Cytokine Res. 23 (2003) 601–612.
[25] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.
[26] J. Montero, J. Garcia, M.C. Ordas, I. Casanova, A. Gonzalez, A. Villena, et al., Specific regulation of the chemokine response to viral hemorrhagic septicemia virus at the entry site, J. Virol. 85 (2011) 4046–4056.
[27] S. Svenning, A.T. Gondek-Wyrozemska, Y.A. van der Wal, B. Robertsen, I. Jensen, J.B. Jorgensen, et al., Microbial danger signals control transcriptional induction of distinct MHC class I L lineage genes in Atlantic salmon, Front. Immunol. 10 (2019) 2425.
[28] D.B. Iliev, L. Lagos, H.L. Thim, S.M. Jorgensen, A. Krasnov, J.B. Jorgensen, CpGs induce differentiation of Atlantic salmon mononuclear phagocytes into cells with dendritic morphology and a proinflammatory transcriptional profile but an exhausted allostimulatory activity, Front. Immunol. (2019) 10.
[29] A.L. Monjo, S.J. Poynter, S.J. DeWitte-Orr, CHSE-214: a model for studying extracellular dsRNA sensing in vitro, Fish Shellfish Immunol. 68 (2017) 266–271.
[30] V. Bergan, S. Steinsvik, H. Xu, O. Kileng, B. Robertsen, Promoters of type I interferon genes from Atlantic salmon contain two main regulatory regions, FEBS J. 273

(2006) 3893–3906.
[31] D.B. Iliev, I. Skjaeveland, J.B. Jorgensen, CpG oligonucleotides bind TLR9 and RRM-containing proteins in Atlantic salmon (Salmo salar), BMC Immunol. 14 (2013) 12.
[32] E. Latz, A. Schoenemeyer, A. Visintin, K.A. Fitzgerald, B.G. Monks, C.F. Knetter, et al., TLR9 signals after translocating from the ER to CpG DNA in the lysosome, Nat. Immunol. 5 (2004) 190–198.
[33] D.B. Iliev, I. Skjaeveland, J.B. Jorgensen, CpG oligonucleotides bind TLR9 and RRM-Containing proteins in Atlantic Salmon (Salmo salar), BMC Immunol. 14 (2013).
[34] J.P. Gorvel, P. Chavrier, M. Zerial, J. Gruenberg, rab5 controls early endosome fusion in vitro, Cell 64 (1991) 915–925.
[35] P. Chavrier, R.G. Parton, H.P. Hauri, K. Simons, M. Zerial, Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments, Cell 62 (1990) 317–329.
[36] C. Bucci, R.G. Parton, I.H. Mather, H. Stunnenberg, K. Simons, B. Hoflack, et al., The small Gtpase Rab5 functions as a regulatory factor in the early endocytic pathway, Cell 70 (1992) 715–728.
[37] F.T. Mu, J.M. Callaghan, O. Steelemortimer, H. Stenmark, R.G. Parton, P.L. Campbell, et al., Eea1, an early endosome-associated protein - Eea1 is a conserved alpha-helical peripheral membrane-protein flanked by cysteine fingers and contains a calmodulin-binding Iq Motif, J. Biol. Chem. 270 (1995) 13503–13511.
[38] D.H. Murray, M. Jahnel, J. Lauer, M.J. Avellaneda, N. Brouilly, A. Cezanne, et al., An endosomal tether undergoes an entropic collapse to bring vesicles together, Nature 537 (2016) 107–111.
[39] Y. Feng, B. Press, A. Wandinger-Ness, Rab 7: an important regulator of late endocytic membrane traffic, J. Cell Biol. 131 (1995) 1435–1452.
[40] P. Bahadoran, E. Aberdam, F. Mantoux, R. Busca, K. Bille, N. Yalman, et al., Rab27a: a key to melanosome transport in human melanocytes, JCB (J. Cell Biol.) 152 (2001) 843–849.
[41] M. Van Gele, P. Dynoodt, J. Lambert, Griscelli syndrome: a model system to study vesicular trafficking, Pigm Cell Melanoma R 22 (2009) 268–282.
[42] A. Bobrie, S. Krumeich, F. Reyal, C. Recchi, L.F. Moita, M.C. Seabra, et al., Rab27a supports exosome-dependent and -Independent mechanisms that modify the tumor microenvironment and can promote tumor progression, Canc. Res. 72 (2012) 4920–4930.
[43] Y.D. Chen, Y.T. Fang, Y.L. Cheng, C.F. Lin, L.J. Hsu, S.Y. Wang, et al., Exophagy of annexin A2 via RAB11, RAB8A and RAB27A in IFN-gamma-stimulated lung epithelial cells, Sci. Rep. 7 (2017) 5676.
[44] S. Shrivastava, P. Devhare, N. Sujijantarat, R. Steele, Y.C. Kwon, R. Ray, et al., Knockdown of autophagy inhibits infectious hepatitis C virus release by the exosomal pathway, J. Virol. 90 (2016) 1387–1396.
[45] A. Yamamoto, Y. Tagawa, T. Yoshimori, Y. Moriyama, R. Masaki, Y. Tashiro, Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells, Cell Struct. Funct. 23 (1998) 33–42.
[46] R. Wang, Y. Zhang, S. Liu, C. Li, L. Sun, L. Bao, et al., Analysis of 52 Rab GTPases from channel catfish and their involvement in immune responses after bacterial infections, Dev. Comp. Immunol. 45 (2014) 21–34.
[47] Q.S. Fang Han, Yu Zhang, Xiaolong Wang, Zhiyong Wang, Molecular characterization and immune responses of Rab5 in large yellow croaker (Larimichthys crocea), Aquacult. Fish. 2 (2017) 165–172.
[48] P. Spearman, Viral interactions with host cell Rab GTPases, Small GTPases 9 (2018) 192–201.
[49] M. Yao, X.G. Liu, D. Li, T.Y. Chen, Z. Cai, X.T. Cao, Late endosome/lysosome-localized Rab7b suppresses TLR9-initiated proinflammatory cytokine and type I IFN production in macrophages, J. Immunol. 183 (2009) 1751–1758.
[50] C.M. Press, O. Evensen, The morphology of the immune system in teleost fishes, Fish Shellfish Immunol. 9 (1999) 309–318.
[51] L. Andersen, A. Bratland, K. Hodneland, A. Nylund, Tissue tropism of salmonid alphaviruses (subtypes SAV1 and SAV3) in experimentally challenged Atlantic salmon (Salmo salar L.), Arch. Virol. 152 (2007) 1871–1883.
[52] K. Honda, Y. Ohba, H. Yanai, H. Negishi, T. Mizutani, A. Takaoka, et al., Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction, Nature 434 (2005) 1035–1040.
[53] J. Siren, J. Pirhonen, I. Julkunen, S. Matikainen, IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29, J. Immunol. 174 (2005) 1932–1937.
[54] H.A. Young, J.H. Bream, IFN-gamma: recent advances in understanding regulation of expression, biological functions, and clinical applications, Curr. Top. Microbiol. Immunol. 316 (2007) 97–117.
[55] P.J. Murray, T.A. Wynn, Protective and pathogenic functions of macrophage subsets, Nat. Rev. Immunol. 11 (2011) 723–737.
[56] I. Skjaeveland, D.B. Iliev, J. Zou, T. Jorgensen, J.B. Jorgensen, A TLR9 homolog that is up-regulated by IFN-gamma in Atlantic salmon (Salmo salar), Dev. Comp. Immunol. 32 (2008) 603–607.
[57] P.M. Barral, D. Sarkar, Z.Z. Su, G.N. Barber, R. DeSalle, V.R. Racaniello, et al., Functions of the cytoplasmic RNA sensors RIG-I and MDA-5: key regulators of innate immunity, Pharmacol. Therapeut. 124 (2009) 219–234.