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Characterisation of the teleostean κB-Ras family: The two members NKIRAS1 and NKIRAS2 from rainbow trout influence the activity of NF-κB in opposite ways

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ABSTRACT

Two structurally similar NF-κB-inhibitor-interacting Ras-like proteins (NKIRAS) regulate the activity of the transcription factor NF-κB and thereby control several early immune mechanisms in mammals. We identified the orthologous sequences of *NKIRAS1* and *NKIRAS2* from the rainbow trout *Oncorhynchus mykiss*. The level of sequence identity was similarly high (≥68%) between the two and in comparison to their mammalian counterparts. Strikingly, *NKIRAS2* was present as four transcript variants. These variants differed only in length and in the nucleotide composition of their 5' termini and were most likely generated by splicing along unconventional splice sites. The shortest *NKIRAS2* variant was most strongly expressed in a lymphocyte-enriched population, while *NKIRAS1* was most strongly expressed in cells of myeloid origin. Fluorescent-labelled NKIRAS1 and NKIRAS2 proteins from rainbow trout were detected in close association with the p65 subunit of NF-κB in the nucleus and cytoplasm of CHSE-214 cells. Subsequent reporter-gene experiments revealed that NKIRAS1 and a longer NKIRAS2 variant in rainbow trout decreased the level of activated NF-κB, while the two shortest NKIRAS2 variants increased the NF-κB activity. In addition, the overexpression of the shortest NKIRAS2 variant in CHSE-214 cells induced a stronger transcription of the genes encoding the pro-inflammatory cytokines TNF, CXCL8, and IL1B compared to non-transfected control cells. This is the first characterisation of NKIRAS orthologues in bony fish and provides additional information to the as yet underexplored inhibition pathways of NF-κB in lower vertebrates.

1. Introduction

The 'NF-κB-inhibitor-interacting Ras-like proteins' NKIRAS1 and NKIRAS2 (also known as κB-Ras 1 and κB-Ras 2 [1]) are members of the hyperfamily of Ras proteins accounting for more than 150 GTP-binding proteins largely involved in cell-signal-transduction networks [2]. NKIRAS1 and NKIRAS2 contribute to the maintenance of homeostasis in mammalian cells by controlling the activity of NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B cells) factors [3].

The NF-κB/Rel factors represent one of the best characterised families of immunorelevant transcription factors [4], which are of particular importance for both innate and adaptive immune responses [5]. Overshooting of immune reactions and pathological responses is prevented by controlling the activity of NF-κB by a range of different

Abbreviations: aa, amino acid; CDS, coding sequence; CXCL8, C-X-C motif chemokine ligand 8; GDP, guanosine diphosphate; GFP, green fluorescent protein; GTP, guanosine-triphosphate; hpi, hours post infection; IL, interleukin; MAb, monoclonal antibody; mPlum, mutant variant of red fluorescent protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NKIRAS, NF-κB-inhibitor-interacting Ras-like proteins; nt, nucleotide(s); ORF, open-reading frame; PEST, proline (P), glutamic acid (E), serine (S), and threonine (T)-rich domain; Ras, Rat sarcoma; SNP, single-nucleotide polymorphism; TGFB, transforming growth factor beta; TNF, tumour necrosis factor; qPCR, quantitative polymerase-chain reaction; WGD, whole-genome duplication.

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signal-responsive mechanisms [6]. Under homeostatic conditions, the 'inhibitors of NF-κB proteins' (IκBs) maintain NF-κB factors in an inactive form in the cytosol [7]. Eight IκB-like family members have been discovered in mammals [8,9], and the alpha member is a prototypic representative in mammals [10] and fish [11]. IκB masks the nuclear localisation signals of NF-κB factors [12], thereby preventing their access to NF-κB-specific response elements in the promoters of relevant genes. NKIRAS proteins interact with the PEST domains of IκB proteins, mainly of the beta type [1,13], to impede their degradation and to restrain the inhibitory complex and its substrate NF-κB within the cytoplasm [1,14]. This inhibition can be removed through activated pattern-recognition receptors and several pro-inflammatory mediators that promote the degradation of IκBα [15].

The NKIRAS proteins are activated through the binding of GTP and Mg^{2+} to the 'switch I' and 'switch II' domains [16]. The subsequent conformational rearrangement of NKIRAS1 and NKIRAS2 allows recruitment of downstream effector proteins [17,18]. NKIRAS2 bound to GDP has been reported to suppress the phosphorylation of the NF-κB p65 subunit, thereby interfering with its interaction with the transcriptional coactivator p300 [19].

Comprehensive phylogenetic comparisons of teleostean Ras factors have been published so far [20,21], but functional studies on the role of Ras proteins in fish immunity are rare [22] and nothing is known about the involvement of NKIRAS during the early and presumably NF-κB-driven immune processes in fish. Here, we characterised the two NKIRAS factors in the rainbow trout *Oncorhynchus mykiss* and investigated their structural and functional properties in terms of interference with the early immune-activating mechanisms of salmonid fish against *Aeromonas salmonicida* infections.

2. Material and methods

2.1. Fish

Rainbow trout (*O. mykiss*) were obtained from a local commercial fish farm (Forellenzucht Uthoff GmbH, Neubrandenburg, Germany) and kept at 15 °C in 1000 L tanks in partially recirculating water systems and a light period of 12 h per day. For the stimulation trial, fish (30–50 g) were intraperitoneally injected with 5×10^5 colony-forming units (CFU) of the highly virulent JF5505 strain of *A. salmonicida* ssp. *salmonicida* (diluted in 200 μL phosphate buffered saline [PBS]) using a 20G needle. Control fish were injected with 200 μL PBS. At 6, 12 and 24 h post stimulation, four fish per treatment group were euthanised using an overdose of benzocaine (100 mg/L). All experimental procedures complied with the relevant European guidelines on animal welfare (Directive 2010/63/EU on the protection of animals used for scientific purposes) and were approved by the institute's ethics board (approval ID: FLI 28/17).

2.2. Cell sorting

Cells were isolated from individual head kidneys, and 4×10^4 cells were used for labelling with 50 ng MAb21 [23] per 2×10^5 cells at 4 °C for 30 min. The cells were then washed and centrifuged (515×*g*, 4 °C, 5 min). The cell pellet was resuspended in 180 μL autoMACS Running Buffer (Miltenyi Biotec) before adding 20 μL of anti-mouse secondary antibody conjugated to magnetic beads (Miltenyi Biotec) for each 1×10^7 cells. After incubation for 20 min at 4 °C, the cells were washed with 700 μL autoMACS Running Buffer and centrifuged for 10 min at 300×*g*. Cells were resuspended in 500 μL autoMACS Running Buffer and sorted using a single magnetic column and the posselS program on the AutoMACS Pro Separator (Miltenyi Biotec). The separated positive and negative fractions were centrifuged for 7 min at 515×*g* and 4 °C and the cell pellets were resuspended in 350 μL RNA RLT lysis buffer (Qiagen) and stored at −70 °C. RNA extraction and cDNA synthesis were conducted as described previously [24]. Subsequent expression analysis was conducted on MAb21-negative cells (a lymphocyte-enriched population containing mainly B cells, T cells and thrombocytes) and MAb21-positive cells (a population enriched with cells of myeloid origin).

2.3. Construction of NKIRAS-expression constructs

We amplified the open-reading frames (ORFs) of *NKIRAS1* and three *NKIRAS2* transcript variants using the oligonucleotide primers listed in Table 1 and the Platinum Taq High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Amplicons were subcloned in pGEM-T Easy (Promega), retrieved by digestion with the restriction enzymes *Hind*III and *Eco*RI and inserted 'in frame' into the mammalian expression vector v280 [25,26] (previously double-digested with the above restriction enzymes). The resulting plasmids (v280_NKIRAS1 and v280_NKIRAS2) were utilised for functional analyses.

We identified the subcellular localisation of the NKIRAS1 (XM_021560247) and NKIRAS2 (XM_021557708) variants in salmonid model cells by inserting a fragment coding for a derivative of the red fluorescent protein (mPlum) and a green fluorescent protein (GFP) at the 3' end of the CDS of our v280_NKIRAS1 and v280_NKIRAS2 plasmids. The mPlum and GFP fragments had previously been amplified from commercial vectors (mPlum: pmPlum, ClonTech/Takara; GFP: pAM 505, NCBI-nucleotide accession code: AF140578) and inserted into the v280 clones using the restriction sites *Cla*I and *Hind*III or *Hind*III and *Eco*RI, respectively.

2.4. Cell culture, transfections and luciferase assay

The CHSE-214 cell line derived from *Oncorhynchus tshawytscha* was cultured as described previously [25]. Endotoxin-free preparations (ZymoPure II Plasmid Maxi Prep Kit, ZymoResearch) of the expression constructs were transfected into CHSE-214 cells using the X-tremeGENE HP DNA Transfection Reagent (Roche). For co-transfection assays in six-well plates, we used 50 ng of the modified NF-κB-responsive promoter of the endothelial-leukocyte adhesion molecule gene (ELAM-1--luc) [27] and increasing concentrations (from 100 ng to 750 ng) of the v280_NKIRAS1 and v280_NKIRAS2 plasmids.

Induction experiments were carried out in 24-well plates. Three wells of each row were left as unstimulated controls, while the other three were stimulated for 3 h or 24 h with 100 ng/mL flagellin isolated from the Gram-negative bacterium *Salmonella typhimurium* (tlrl-stfla; Invivogen), which has been proven to induce inflammatory responses in CHSE-214 cells [25,26,28]. Three hours after incubation, the cells were harvested in ice-cold PBS for RNA isolation and the subsequent profiling of immune-gene expression. Twenty-four hours after stimulation, the luciferase activity of the cell lysates was measured with the Dual-Luciferase Reporter Assay System (Promega) in a Lumat LB9501 luminometer (Berthold). The values were normalised against the protein concentration of the CHSE-214 cell extracts. Each transfection was assayed in triplicate; each transfection experiment was repeated three times.

2.5. Confocal microscopy

The CHSE-214 cells were transfected with vectors expressing either mPlum-tagged NKIRAS1 or GFP-tagged NKIRAS2a from rainbow trout to monitor the transfection efficacy and the intracellular localisation of both factors. Live-cell imaging was performed as described previously [25]. Hoechst 33342 dye (1 mg/mL; Sigma Aldrich/Merck) was used to visualize the nuclei. For co-staining with the NF-κB subunit p65, the CHSE-214 cells were fixed (10 min, 4% paraformaldehyde, Merck), permeabilised (5 min, 0.1% Triton X-100, Sigma-Aldrich/Merck) and incubated in the dark with monoclonal anti-p65 (D14E12) antibody from rabbit (1:200, 30 min, Cell Signalling) and donkey anti-rabbit Alexa 488 secondary antibody (1:100, 30 min, Sigma-Aldrich/Merck).

Table 1
Primers used in this study.

Gene symbol	NCBI accession code	Primer sequence 5'→3' (sense, antisense)	Fragment length [bp]
Construction of NKIRAS-expression constructs:			
NKIRAS1	XM_021560247	ATGCAAGCTTATGGGGAAAGGTTGCAAAGT, TTAACGACATCCACATCCAAGG	576
NKIRAS2a	XM_021557708	ATGCAAGCTTATGGGCAAGAGTTGTAAAGTGGTGG, ATGCGAATTCGCTATCGACAGAGCCACTCCCC	576
NKIRAS2b	XM_021557707	ATGCAAGCTTATGACGTCACTCAGCGAGGACG, ATGCGAATTCGCTATCGACAGAGCCACTCCCC	669
NKIRAS2c	XM_021557706	ATGCAAGCTTATGTTCTGGAATGACGTCACTCAG, ATGCGAATTCGCTATCGACAGAGCCACTCCCC	675
Quantitative PCR analysis:			
NKIRAS1	XM_021560247	GATCGTGGTGTAAAGGAACAGTT, TCCACACTGTAGACCAGCACAA	119
NKKIRAS2a	XM_021557708	CATCATCATGGGCAAGAGTTGTA, CTGAGCCCGCAATATGATTGG	108
NKIRAS2b	XM_021557707	TTTTCTGGCTCGTGCAGTTACC, AATATGATTGGCATACAGCAGTTG	130
NKIRAS2c	XM_021557706	CGAGGACGAGTGGGCAGAGT, GCCCGCAATATGATTGGCATAC	171
NKIRAS2d	XM_021557705	TGCATGTCTGCCTGTCTCTTTTT, TGAGCCCGCAATATGATTGGCA	201
TNF-1	NM_001124357	AACGATGCAGGATGAAATTGAGC, GGCCGTCATCCTTTCTCCACT	161
IL10	NM_001245099	TGCCCAGTGCAGACGTGTACC, CGGGGCTCTTCAAGTGGTGTA	179

2.6. Quantitative PCR (qPCR) analysis

We profiled the quantity of the various *NKIRAS* transcripts in rainbow trout leucocytes by establishing a panel of discriminating oligonucleotide primers that allowed a selective quantification of the *NKIRAS1* and the four *NKIRAS2* transcript variants (Table 1). The qPCR primers were designed (using Pyrosequencing Assay Design software v.1.0.6; Biotage) to amplify fragments between 108 nt and 201 nt. We further profiled the expression of cytokine-encoding genes in CHSE-214 cells. The *Oncorhynchus*-specific primer pairs for *IL1B*, *CXCL8*, *TGFB-2*, and *IL4/13* were published previously [25]; the primer pairs for *TNF-1* and *IL10* were optimised for *O. tshawytscha* (Table 1). *EEF1A1* [29] and *RPS5* [24] were chosen as reference genes for normalisation of the qPCR data. Quantitative PCR analysis was performed on the LightCycler-96 system (Roche), essentially as previously described [25]. The cDNA input into the individual RT-qPCR assays was equivalent to 2.5 ng total RNA isolated from cells.

2.7. Data analysis

A parametric *t*-test or nonparametric Mann-Whitney *U* test provided by GraphPad Prim 8 software was used to evaluate the statistical significance of the qPCR data. In all these tests, a two-tailed p value of <0.05 was considered statistically significant. The ClustalW and T-Coffee expresso alignment tools were used to compare multiple NKIRAS nucleotide and amino acid (aa) sequences.

The Molecular Evolutionary Genetics Analysis package MEGA7 [30] was used to construct the phylogenetic tree using the Neighbour-Joining method with a bootstrap test based on 1000 replications. Three-dimensional structures were obtained using PHYRE2 (Protein Homology/analogY Recognition Engine V 2.0) [31]. Signal peptides and disordered protein regions were predicted using the online tools SignaIP-5.0. and PrDOS [32].

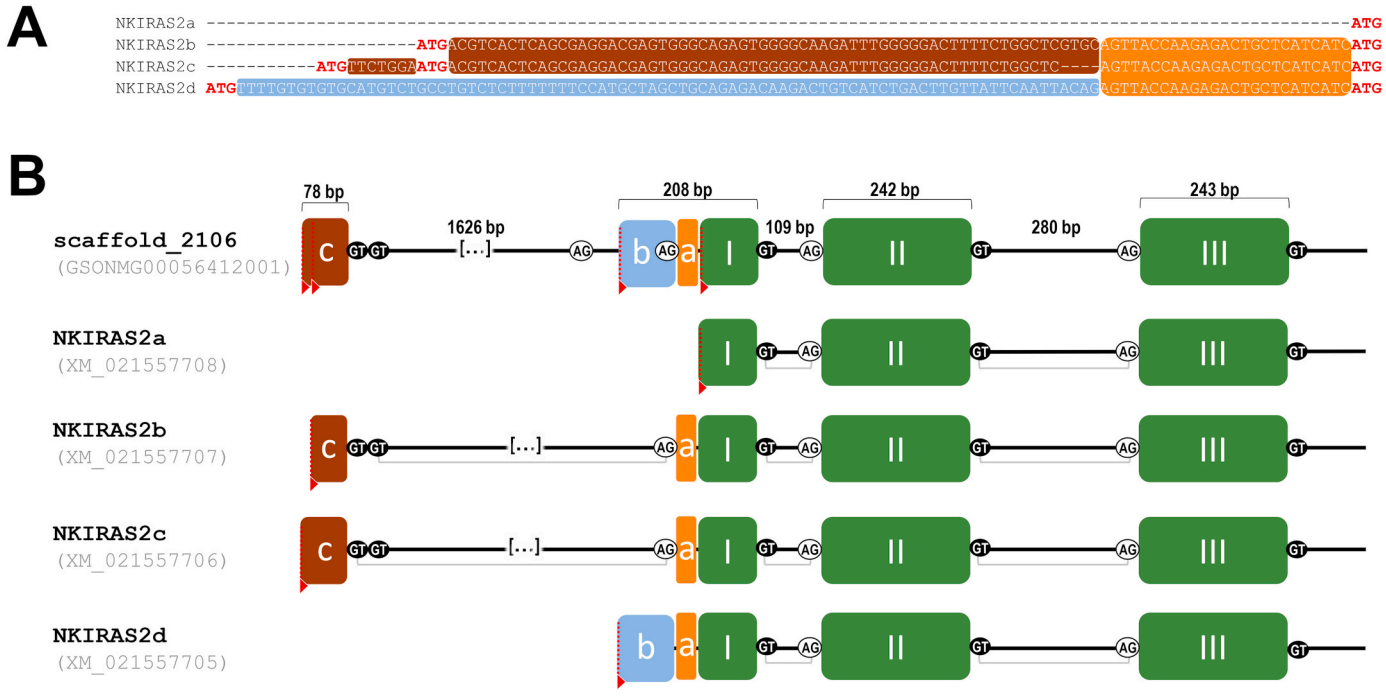

Fig. 1. Structural comparison of the four NKIRAS2 transcript variants from rainbow trout. (**A**) Only the coding sections were aligned, in which the four variants a to d differ from each other. Identical sections are colour coded. Potential start codons are marked in red. (**B**) Schematic representation of the NKIRAS2 scaffold 2106 (Genoscope *O. mykiss* genome; ID: GSONMG00056412001) in rainbow trout and the four resulting transcript variants (along with NCBI nucleotide IDs). The coding exons conserved in extant vertebrates are indicated by green boxes and Roman numerals. Other sequence segments are coloured according to (A). The lengths of the exons/coding sequence fragments and introns are given above the illustrations. The (possible) transcription start is indicated by a red arrowed line. Splice donor (GT) and acceptor sites (AG) are indicated by filled and open circles, respectively. For each transcript, the used splice sites are linked by grey brackets. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Differential splicing generates four NKIRAS2 variants alongside one NKIRAS1 transcript in rainbow trout

Our search of the NCBI-gene database for NKIRAS-like sequences from rainbow trout revealed one coding sequence (CDS) (accession code: XM_021560247) orthologous to the human *NKIRAS1* gene and four transcript variants X1 to X4 (XM_021557705 to XM_021557708) orthologous to the human *NKIRAS2* gene (Suppl. Fig. 1). The CDS of *NKIRAS1* from rainbow trout comprises 579 nt and shares the same length with the shortest *NKIRAS2* variant (X4), which is henceforth referred to as *NKIRAS2a*. The 579-nt long sequence of *NKIRAS2a* is also included in the variants X1 to X3, which also bear specific 5' termini of different lengths and nucleotide compositions. The longest *NKIRAS2* variant (X1) comprises 693 nt and is henceforth termed *NKIRAS2d*, followed by *NKIRAS2c* (X2, 678 nt) and *NKIRAS2b* (X3, 672 nt). The coding sequences of all five *NKIRAS* transcripts share an identity of >69% with the human *NKIRAS1* and an identity of >77% with the human *NKIRAS2*. The coding sequences of *NKIRAS1* and *NKIRAS2a* from rainbow trout share an identity of 68% due to 192 exchanged nucleotides (Suppl. Fig. 1). We verified the lengths and nucleotide compositions of *NKIRAS1* and *NKIRAS2a* to *NKIRAS2c* by cloning and repeated sequencing.

The individual 5' regions of the *NKIRAS2* variants *b*, *c* and *d* share a 25-nt sequence immediately upstream of the common start codon (Fig. 1A, orange box). Interestingly, a ≥96% identical sequence fragment resides in other coding gene sections from bacteria (e.g. sequence ID: SIT85884), protozoans (e.g. CDW78563) and vertebrates (e.g. XM_028038946), but they are translated into completely different amino acid (aa) sequences. In variants *b* and *c*, a 64-nt long sequence (Fig. 1A, brown box) is located upstream of this 25-nt motif. The 25-nt and 64-nt segments are separated by 4 additional nt in the case of variant *b*, whereas variant *c* contains an additional 10-nt long sequence at its 5' end. In variant *d*, a different 89-nt long sequence is located upstream of the 25-nt motif (Fig. 1A, blue box).

A BLAST search of these three different 5′-UTR segments against the rainbow trout genome [33] identified an approximately 3030-nt long genomic sequence on scaffold 2106 (Fig. 1B). The ORF of *NKIRAS2a* is distributed across three exons (I, 94 nt; II, 242 nt; III, 243 nt) and has the same organisation seen in mammals. The additional 25-nt, 64-nt and 89-nt long segments, which characterise the individual *NKIRAS2* versions *–b*, *–c* and *–d*, respectively, are all encoded in the genomic region upstream of the conventional three *NKIRAS2* exons and allow the generation of four different transcript variants via differential splicing.

We also investigated whether undiscovered transcript variants of *NKIRAS* might exist by screening an RNA-sequence read collection of two rainbow trout strains [34]. We did not identify any further transcript variants of *NKIRAS1* and *NKIRAS2*; however, we observed that both the *NKIRAS1* and *NKIRAS2* transcripts bore a certain degree of sequence variability. The CDS of *NKIRAS1* contained 11 single-nucleotide changes, contributing to a 1.9% variability (Fig. 2A),

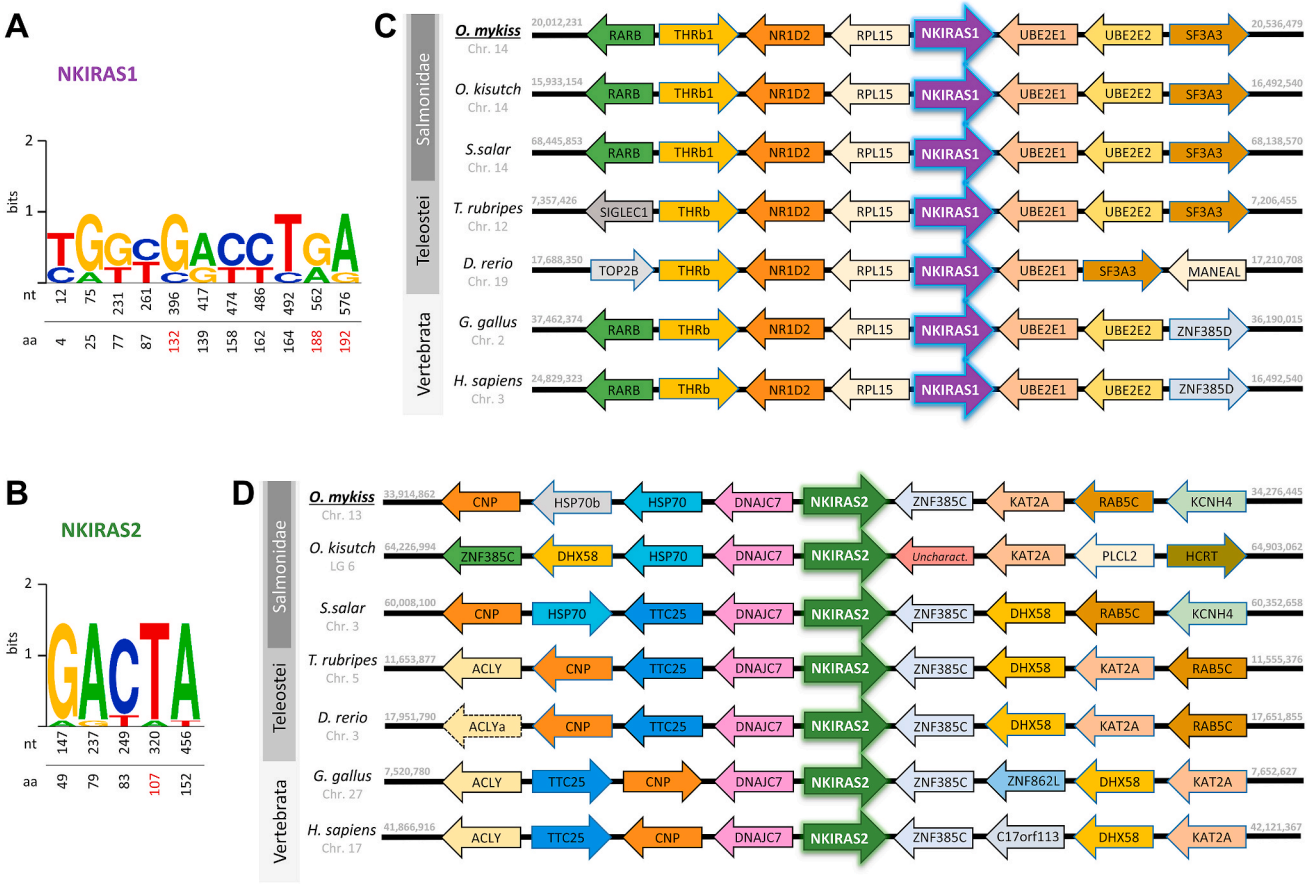

Fig. 2. Sequence conservation of (A) *NKIRAS1* and (B) *NKIRAS2* from rainbow trout. The identified single-nucleotide changes in the coding sequences of both genes are represented as sequence logos. The frequency of a nt variation at its specific sequence position is given below the drawing, alongside the position of the affected aa. Red numbers represent conservative mutations; black numbers represent silent mutations. **Synteny between (C) *NKIRAS1* and (D) *NKIRAS2* loci from different vertebrate species.** The gene order was identified using Genomicus v1.01. Species name and chromosomal location (in nt) are listed on the left. Arrows represent genes found in synteny; the colour indicates orthologous genes and their direction indicates the gene orientation. The figure is not scaled; the position of the depicted genes is given in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

which corresponds approximately to the average variability assessed for the rainbow trout genome [35]. The variability of *NKIRAS2* was comparably lower (five single nucleotide changes, 0.9% variability; Fig. 2B).

As a last test, we checked the number of *NKIRAS* transcripts in other salmonid species. Our search in the NCBI gene database revealed only one entry each for the genomic sequences of both *NKIRAS1* and *NKIRAS2* in salmonid fishes (*O. mykiss*, *Salmo salar* and *Salvelinus alpinus*). The genes flanking *NKIRAS1* (Fig. 2C) and *NKIRAS2* (Fig. 2D) are similarly arranged across different vertebrate genomes, although a few of the neighbouring genes are reversed in their arrangement.

3.2. NKIRAS1 and NKIRAS2 from rainbow trout share well-conserved structural motifs

The five *NKIRAS* sequences from trout encode proteins with lengths ranging from 192 to 230 aa. The aa sequences among the vertebrate NKIRAS orthologues are relatively well conserved (>60%; Table 2). In line with this observation, the encoded NKIRAS proteins from trout contain the same motifs and domains that have been characterised in their mammalian NKIRAS counterparts (Fig. 3A). We also found two and three SxDx motifs, which are recognised and modified by *A. salmonicida* to impede the catalytic activity of Ras proteins from the infected host and control the activity of leukocytes [36].

Despite the numerous structural similarities, 64 aa residues still allowed discrimination between NKIRAS1 and NKIRAS2 (Fig. 3A). Notably, a signal peptide of 17 aa has been predicted only in *NKIRAS2d*. In our phylogenetic tree, both genes separate, despite the high degree of sequence identity, due to these differing aa residues (Fig. 3B1). The three-dimensional reconstructions of NKIRAS1 (Fig. 3B2) and NKIRAS2a (Fig. 3B3) from rainbow trout illustrate the well-conserved quaternary structure of particular domains. The characteristics of the active domains, which are critical for the GTPase activity, are located in a similar configuration as observed in their human counterparts [37].

3.3. NKIRAS copy number is slightly modulated after infection with A. salmonicida

The human and murine *NKIRAS1* and *NKIRAS2* genes are widely expressed in various tissues, including immune cells [3]. Our qPCR measurements (Fig. 4A) revealed that *NKIRAS2a* (~3000 copies/μg RNA) was the most strongly expressed *NKIRAS* gene in the MAb 21-negative cell population from rainbow trout enriched with lymphocytes, whereas *NKIRAS2b* was the least expressed variant (~200 copies/μg RNA). In the MAb21-positive cells of myeloid origin, *NKIRAS1* was most strongly expressed (~670 copies/μg RNA), whereas all other *NKIRAS* transcripts were expressed at a similarly low level (<100 copies/μg RNA).

Infection with a highly virulent strain of *A. salmonicida* significantly upregulated the levels of the *NKIRAS2* variants *a*, *b*, and *c* ($p < 0.05$) in the MAb 21-negative cell population by about twofold at 12 or 24 h post infection (hpi) compared to PBS controls (Fig. 4B). In the MAb21-positive cell population, the *NKIRAS1* level was slightly downregulated within 24 h, while the *NKIRAS2a* level was 2.7-fold and 4.0-fold upregulated after 12 h and 7 d, respectively (Fig. 4C). However, the fourfold increase resulted only in about 230 copies/μg RNA. The concentrations of the other *NKIRAS* transcripts at 7 days post infection returned to values similar to those measured in the cells of naïve trout.

3.4. Overexpressed NKIRAS1 and NKIRAS2 are localised in the cytoplasm and nucleus of salmonid cells

We overexpressed fluorescent-labelled *NKIRAS1* and *NKIRAS2a* from rainbow trout in CHSE-214 cells to investigate the localisation of the salmonid NKIRAS factors. Confocal microscopy revealed that NKIRAS1 (flagged with mPlum) and NKIRAS2 (flagged with GFP) apparently shared the same subcellular localisation in the nucleus and cytoplasm (Fig. 5A1, A2) as is observed in humans [38]. We also stained native NF-κB p65 factors using a monoclonal anti-RELA antibody from rabbit. In unstimulated cells, NKIRAS1 and NKIRAS2 seem to restrain p65 within the cytoplasm (Fig. 5B1, B2), but we could not detect transfer of p65 to the nucleus after stimulation with flagellin for 1 h (data not shown).

3.5. NKIRAS1 and NKIRAS2a modulate the activity of NF-κB in opposite directions in CHSE-214 cells

Stimulation of CHSE-214 cells with flagellin increased the level of active NF-κB by an average of 2.8-fold (Fig. 5C1-C4, 2nd column). We transfected the CHSE-214 cells with increasing concentrations (250 ng to 750 ng) of the plasmids expressing *NKIRAS1* to drop the flagellin-induced NF-κB activation down to the level of the control cells (Fig. 5C1). Transfection with the same amounts of the NKIRAS2c-encoding plasmid had a similar dampening effect on the level of activated NF-κB, even though the lowest concentration (250 ng) of transfected *NKIRAS2c* had a less pronounced effect compared with the NKIRAS1-encoding plasmid (Fig. 5C2). By contrast, increasing concentrations of NKIRAS2a (Fig. 5C3) and NKIRAS2b (Fig. 5C4) raised the level of activated NF-κB by 21-fold (with a quantity of 750 ng of transfected plasmid).

3.6. Overexpression of NKIRAS1 and NKIRAS2 increases the in vitro expression of specific cytokines

We also investigated the influence of the three NKIRAS factors NKIRAS1, NKIRAS2a and NKIRAS2c, which had modulated the NF-κB level in different directions, on the expression of cytokine-encoding genes in CHSE-214 cells. These genes included those for the pro-

Table 2
Amino-acid sequence identity of NKIRAS1 and NKIRAS2a from rainbow trout with orthologous sequences from selected vertebrates.

Species	Accession Number		Sequence identity [%]	
	NKIRAS1	*NKIRAS2*	*NKIRAS1*	*NKIRAS2a*
O. kisutch	XP_020355835[a]	XP_020342081[a]	99.48	98.96
S. salar	XP_013997908[a]	XP_014048269[a]	99.48	97.40
T. rubripes	XP_003969113[a]	XP_003964849[a]	90.62	89.06
P. reticulata	XP_008419502[a]	XP_008401777[a]	89.58	86.98
G. aculeatus	ENSGACP00000010534[b]	ENSGACP00000011568[b]	92.71	88.02
D. rerio	NP_001093546[a]	NP_001003433[a]	84.90	85.94
A. sinensis	XP_006021262[a]	XP_006032823[a]	61.46	78.65
P. vitticeps	XP_020663250[a]	XP_020667364[a]	70.83	79.69
G. gallus	XP_004939460[a]	NP_001006333[a]	73.44	79.69
H. sapiens	NP_065,078[a]	NP_001001349[a]	73.96	77.60

[a] NCBI accession code.
[b] ENSEMBL accession code.

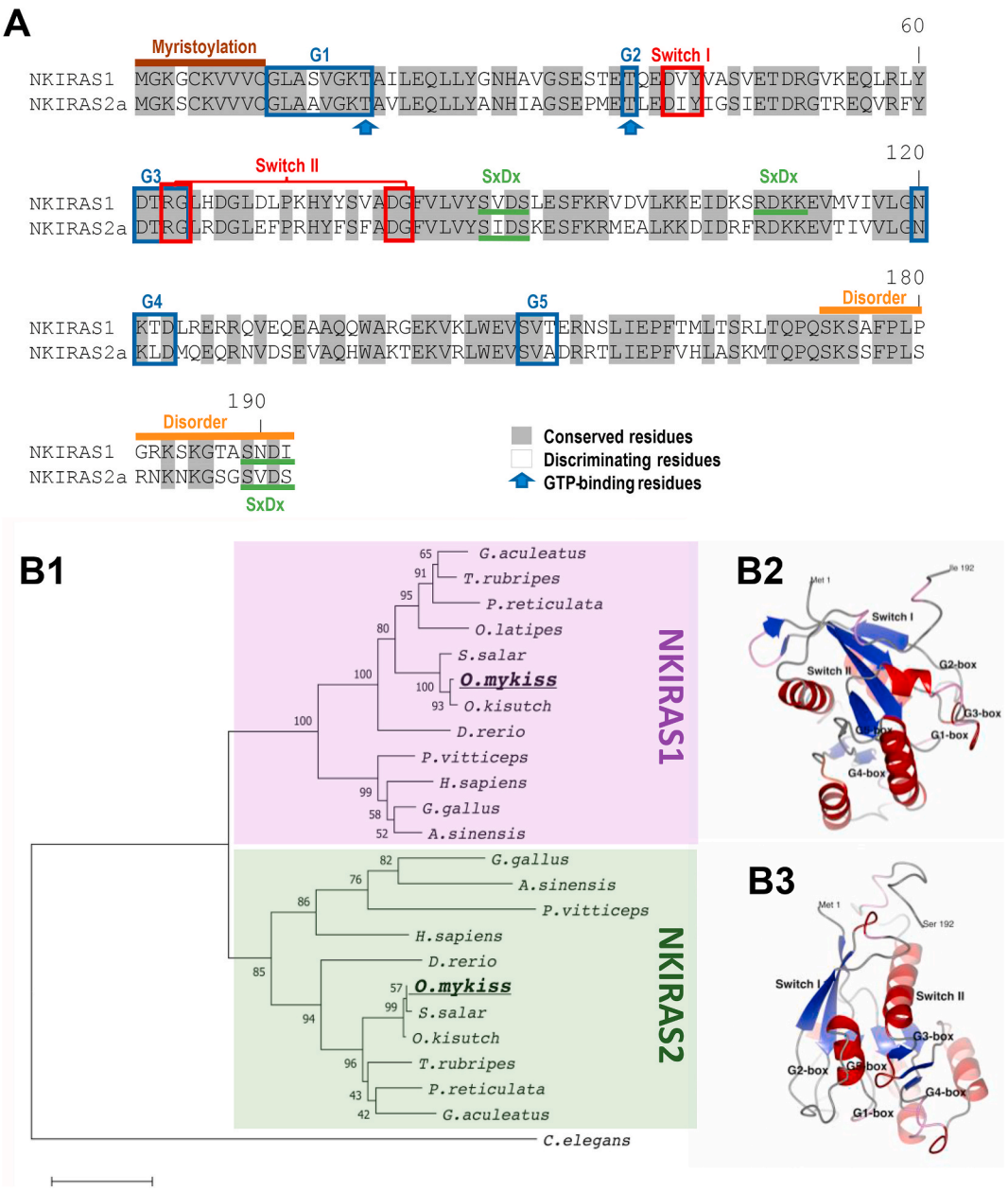

Fig. 3. Comparison of the protein sequences of NKIRAS1 and NKIRAS2a. (**A**) The primary sequences of the proteins are expressed in one-letter codes. Characteristic motifs are framed and labelled: a myristoylation signal MGxxCxxxxC (position 1–10); G1-box GxxxxGKS/T (11–18); G3-box DxxG (61–64); G4-box N/TKXD (121–123); G5-box/SAK motif (150–152); switch I (41–43) and switch II region (63–64 and 80–81); disorder region (173–192); and the *A. salmonicida*-responsive SxDx motifs (87–90, 107–110, 189–192). Conserved aa residues are highlighted in grey. (**B1**) Phylogenetic tree constructed with NKIRAS1 and NKIRAS2 protein sequences of rainbow trout *O. mykiss* (underlined) and its orthologue sequences from other vertebrates. NKIRAS from *Caenorhabditis elegans* was used as an outgroup. The bootstrap values are given at the node of each clade. The scale bar represents a genetic distance of 0.1 aa substitutions per site. The tertiary structures of (**B2**) NKIRAS1 and (**B3**) NKIRAS2 from rainbow trout are drawn to the right side of the respective phylogenetic cluster. β-sheets and α-helices are highlighted in blue and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inflammatory cytokines *IL1B*, *TNF* and *CXCL8* and the anti-inflammatory cytokines *TGFB2*, *IL10* and *IL4/13* (Fig. 6).

Stimulation of the non-transfected control cells with flagellin for 3 h significantly increased the copy numbers of the pro-inflammatory cytokine genes *IL1B* by fourfold, and *CXCL8* and *TNF* by approximately twofold, whereas the copy numbers of the genes *TGFB2*, *IL10* and *IL4/13* remained almost at the same levels under all the examined conditions. We detected almost no changes in cells that were stimulated and transfected with *NKIRAS1* versus stimulated non-transfected cells. The stimulation and transfection with *NKIRAS2c* increased the level of *TNF* and *IL1B* by 125–150%. The stimulation and transfection with *NKIRAS2a* provoked an increase in the transcript abundance of *TNF* (254%; $p = 0.03$), *CXCL8* (162%; $p = 0.09$), and *IL1B* (133%; $p = 0.74$) over the stimulated non-transfected cells.

4. Discussion

NKIRAS1 and NKIRAS2 constitute a small Ras subfamily which has been widely investigated with regard to the regulation of NF-κB in mammals [3]. In all extant vertebrate species, the genes encoding NKIRAS1 and NKIRAS2 are most likely paralogues [39] that descended from a whole-genome duplication (WGD) occurring in the ancestral

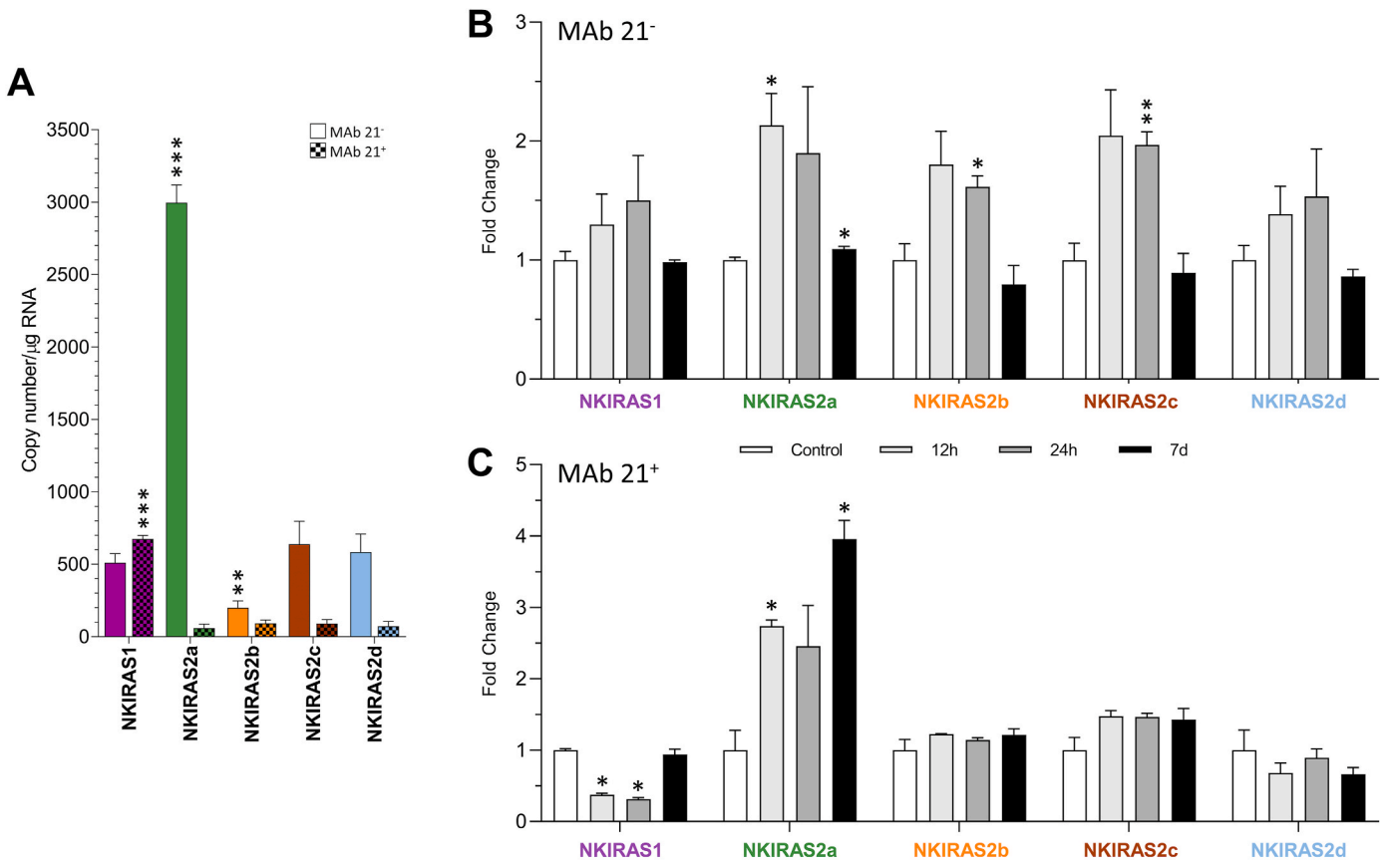

Fig. 4. Expression of NKIRAS genes in lymphoid cells of rainbow trout before and after stimulation. (**A**) Transcript levels of NKIRAS1 (purple bars), NKIRAS2a (green), NKIRAS2b (orange), NKIRAS2c (brown), and NKIRAS2d (light blue) were determined in MAb 21-negative lymphoid cells (non-patterned columns) and MAb21-positive cells of myeloid origin (chequered columns) from healthy rainbow trout. Asterisks represent a significantly different transcript level compared with the NKIRAS1 transcript level (**p < 0.01; ***p < 0.001; *t*-test). After stimulation with *A. salmonicida*, the aformentioned genes were profiled at 0 h (white bars), 12 h (light grey), 24 h (dark grey) and 7 days (black) post challenge in (**B**) MAb21-negative and (**C**) MAb21-positive cells. Bars represent the averaged copy numbers (n = 3) normalised against two reference genes; error bars represent the standard deviation. Asterisks represent a significantly different post-stimulation transcript level compared with the respective 0 h control value (*, p < 0.05; **p < 0.01, one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

vertebrate [40]. Although salmonid fishes underwent two additional WGDs compared with mammals [33,41,42], *NKIRAS1* and *NKIRAS2* each appear to be present as only one gene copy. Nevertheless, differential splicing of the original NKIRAS2 mRNAs from rainbow trout most likely allows the generation of at least four different *NKIRAS2* variants with differentially long 5′-ends. This splicing mechanism does not affect the succession of the exon order or the length of particular exons, as observed previously in rainbow trout [24,25], but it elongates the N-terminus of the *NKIRAS2* variants *b*, *c*, and *d* compared to their paralogue *NKIRAS2a* in rainbow trout and their orthologues in all other vertebrate species. From a structural point of view, the shortest variant *a* therefore seems to be the prototypic *NKIRAS2* version. In the Atlantic salmon *S. salar*, *NKIRAS1* (but not *NKIRAS2*) is present as two different coding sequences (XM_014177875 and XM_014177876), one of which extends beyond the 5′-end, similar to the longer *NKIRAS2* variants from rainbow trout. Accordingly, the mechanism of the N-terminal NKIRAS-protein extension does not seem to be restricted to rainbow trout; however, the functional consequence associated with this structural change remains unknown.

The present gene profiling of the five *NKIRAS* transcripts in the lymphoid and myeloid cells of rainbow trout confirmed that the expression of *NKIRAS* either remained at a generally low transcript level or was only slightly altered after bacterial infection. A revision of ~50 transcriptome studies on salmonid fish after exposure to various immune stimuli [43] confirmed that the copy numbers of *NKIRAS1* and *NKIRAS2* rarely exceed those of the transcript level detected in the control fish. This indicates that the transcription of *NKIRAS* genes is subject to low variability. The permanent presence of NKIRAS seems to be a crucial mechanism for maintaining homeostasis, as mammalian NKIRAS has been proven to control NF-κB [13] and, by extension, numerous immune-gene programs [5,6]. Our structural analysis of the NKIRAS sequences from rainbow trout suggested that all variants may function similarly to their mammalian counterpart because the essential aa residues and motifs of the NKIRAS orthologues were well preserved during vertebrate evolution.

The microscopy examination of *NKIRAS*-overexpressing CHSE cells did not provide evidence of a direct influence of NKIRAS on the translocation of NF-κB. This may reflect the artificial overexpression of NKIRAS or the stress to which the cells were exposed by the previous transfection. Our reporter-gene experiments in CHSE-214 cells revealed that expression of rainbow trout NKIRAS1 and NKIRAS2c reduced the activity of NF-κB, in agreement with previous reports on mammalian NKIRAS proteins [1]. By contrast, expression of NKIRAS2a and NKIRAS2b had an inducing effect on the activity of NF-κB. We found evidence to support an increase in the transcript amount of pro-inflammatory cytokine genes, at least in response to NKIRAS2a expression, in stimulated CHSE-214 cells compared to cells without overexpressed NKIRAS2a.

The reason for these contrary NF-κB regulation mechanisms may be the specificity of the NKIRAS variants for the various combinations of

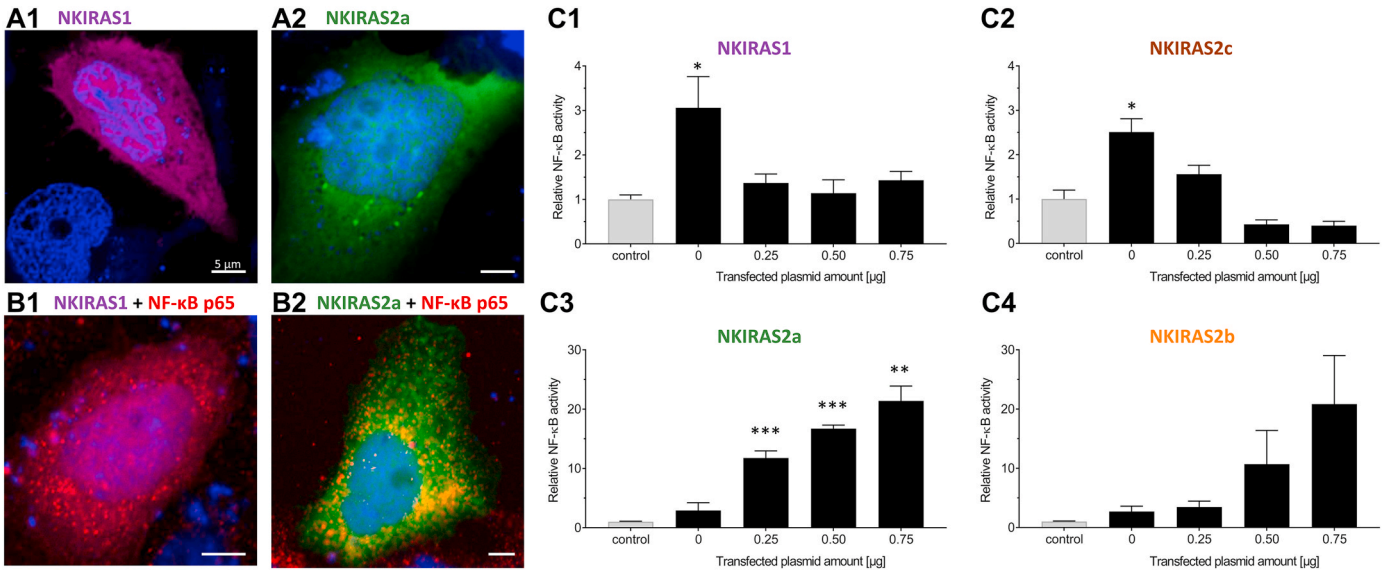

Fig. 5. Impact of overexpressed NKIRAS on the activation of NF-κB in salmonid cells. Confocal analysis of (**A1**) mPlum-tagged NKIRAS1 (purple) and (**A2**) GFP-tagged NKIRAS2a (green) in CHSE-214 cells, 48 h after transfection. Nuclei were stained with Hoechst 33342 dye (blue). The co-localisation of (**B1**) NKIRAS1 and (**B2**) NKIRAS2 with NF-κB was displayed by labelling CHSE-214 cells with anti-RELA antibodies (red) 24 h after transfection with NKIRAS-expressing plasmids. White scale bar represents 5 µm in all images. The luciferase activity of CHSE-214 cells overexpressing (**C1**) NKIRAS1, (**C2**) NKIRAS2c, (**C3**) NKIRAS2a, and (**C4**) NKIRAS2b was determined after a 24 h stimulation with 100 ng/mL flagellin and compared to the non-transfected, unstimulated control cells (set 1.0). The concentrations of the plasmids used for the transfection of the cells are indicated on the abscissa. Statistical significance compared with the control group was assessed using one-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Standard error of the mean (SEM) is indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

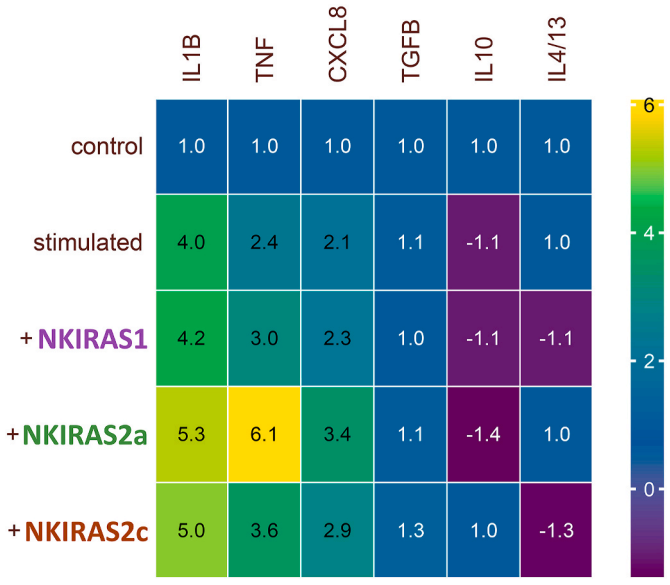

Fig. 6. Expression profiling of stimulated NKIRAS-overexpressing CHSE-214 cells. The HeatMap illustrates the averaged fold-change values (according to the legend on the right) of the mRNA concentrations measured in non-transfected cells (2nd row) and transfected cells (either with *NKIRAS1*-, *NKIRAS2a*- or *NKIRAS2c*-expression plasmids, 3rd to 5th row) after a 3 h stimulation with 100 ng/mL flagellin and compared to unstimulated control cells (set as 1.0, 1st row). The quantified transcripts are listed as gene symbols above the scheme. All expression values were normalised against the geometric mean of two reference genes.

NF-κB/IκB complexes. This would require, as a first step, that the five NKIRAS variants interact specifically with the multiple IκBα proteins in rainbow trout that have been identified apart from the prototype [11]. No IκBβ proteins have yet been isolated from salmonid fish; however, IκBα from rainbow trout contains six ankyrin repeats, as occurs in the human IκBβ, whereas the human IκBα has only five ankyrin domains [44]. Particular IκBα variants may therefore compensate for the loss of IκBβ in rainbow trout. A second point is that these IκB proteins, in turn, interact intimately with the NF-κB/Rel factors. However, few of those factors contain a transcription activation domain, such as p65 (RelA); others such as p50 (NFKB1) or p52 (NFKB2) lack this domain entirely and require interactions with further transcription factors to induce transcription [5,6]. The different NKIRAS-mediated mechanisms controlling the activity of certain NF-κB/Rel factors could explain the difference in the expression of the reporter construct and the inflammatory genes after overexpression of the individual NKIRAS variants.

The finding that transcript variants of NKIRAS2 exert different impacts on the activity of NF-κB could be viewed as unexpected, especially in light of the fact that these variants differ only in their N-termini, which account for only about one sixth to one seventh of the shortest variant 2a. Nevertheless, the N-terminus has been reported to determine several key properties of a protein [45], such as the half-life. In this context, we refer to the predicted 17 aa signal peptide of variant d of NKIRAS2. This signal peptide could guide the insertion of NKIRAS2d into cellular membranes along the secretory pathway, and its cleavage would ensure that the mature NKIRAS2 variant d factor starts with a glutamic acid residue at the N-terminus. The presence of this residue decreases the half-life of a protein to 1 h [46]. NKIRAS2d would therefore be a rather fragile factor compared to its NKIRAS2 isoforms that start with a methionine residue and have an expected half-life of 30 h [46]. Alternatively, the preservation of signal peptides in the mature protein could provide additional features, such as selective coupling to G proteins or regulative functions [47]. Regardless of the presence of signal peptides, the N-terminus is well established to determine the affinity of a protein for involvement in different cell signalling cascades [48]. The different N-termini probably also affect the interaction of NKIRAS with PEST domain-containing factors other than IκB, such as hormone receptors. This hypothesis might be addressed in future analyses.

5. Conclusions

We describe here the structure of five NKIRAS variants in rainbow trout which also modulate the activity of NF-κB and, thus, the pro-inflammatory immune responses in a teleostean model cell. Beyond NF-κB signal transduction, NKIRAS proteins may conceivably control further signalling cascades. Unfortunately, insufficient knowledge is available regarding the NF-κB network in fish; therefore, the obtained data cannot be integrated and adequately evaluated. Our future research will address the interaction of NF-κB and its associated factors in bony fish.

CRediT authorship contribution statement

Fabio Sarais: Cloning, Cell culture, Reporter-gene analyses, qPCR, Data analysis, Visualization, Writing- Original draft preparation.

Henrike Rebl: Confocal microscopy.

Marieke Verleih: SNP analysis.

Sven Ostermann: Cell sorting.

Aleksei Krasnov: Data analysis.

Bernd Köllner: Acquisition of funding.

Tom Goldammer: Supervision, Acquisition of funding.

Alexander Rebl: Conceptualization, Supervision, Data Validation, Visualization, Writing- Reviewing and Editing.

All authors discussed the results and commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.08.052.

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