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Developmental and tissue-specific expression of NITRs

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Abstract Novel immune-type receptors (NITRs) are encoded by large multi-gene families and share structural and signaling similarities to mammalian natural killer receptors (NKRs). NITRs have been identified in multiple bony fish species, including zebrafish, and may be

restricted to this large taxonomic group. Thirty-nine NITR genes that can be classified into 14 families are encoded on zebrafish chromosomes 7 and 14. Herein, we demonstrate the expression of multiple NITR genes in the zebrafish ovary and during embryogenesis. All 14 families of zebrafish NITRs are expressed in hematopoietic kidney, spleen and intestine as are immunoglobulin and T cell antigen receptors. Furthermore, all 14 families of NITRs are shown to be expressed in the lymphocyte lineage, but not in the myeloid lineage, consistent with the hypothesis that NITRs function as NKRs. Sequence analyses of NITR amplicons identify known alleles and reveal additional alleles within the *nitr1*, *nitr2*, *nitr3*, and *nitr5* families, reflecting the recent evolution of this gene family.

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Novel immune-type receptor (NITR) genes have been identified in zebrafish and 14 other bony fish species (Yoder 2009; Ferrareso et al. 2009). Several lines of investigation suggest that their occurrence may be restricted to teleost species. The majority of NITR transcripts encode type I transmembrane proteins. All NITRs possess a single extracellular immunoglobulin (Ig) domain of the variable (V)-type and most possess a second Ig domain of the intermediate (I)-type. NITRs can be classified as: inhibitory, activating and functionally ambiguous based on peptide signaling motifs (Yoder 2009). Bony fish possess cytotoxic NK-like cells ($\text{TCR}\alpha^{-}\beta^{-}\gamma^{-}\text{IgM}^{-}$) as well as cytotoxic T cells ($\text{TCR}\alpha^{+}\beta^{+}\text{IgM}^{-}$) (Shen et al. 2002) and inhibitory and activating NITRs may play significant roles in the functional regulation of both of these lymphocyte populations (reviewed in Yoder 2009). A phylogenetic comparison of

NITR sequences from different fish species demonstrates that these genes are both recently and rapidly evolving (Yoder 2009). Although the fundamental role of NITRs is not known, their specificity for allogeneic determinants, a characteristic of many NK receptors, has been demonstrated (Cannon et al. 2008).

The expression levels of subsets of NITR genes have been described in various tissues in multiple bony fish species and NITR transcripts are detected predominantly in hematopoietic tissues (Strong et al. 1999; Hawke et al. 2001; Yoder et al. 2002; Piyaviriyakul et al. 2007; Evenhuis et al. 2007; Ferraresso et al. 2009). The majority of NITR transcripts are between 1.4 to 2.0 kB as detected by RNA blot analyses. NITR expression in spleen, kidney and intestine is more abundant than in muscle and liver (Strong et al. 1999; Hawke et al. 2001; Yoder et al. 2002; Evenhuis et al. 2007; Ferraresso et al. 2009). NITRs also are expressed in circulating leukocytes of trout and sea bass (Yoder et al. 2002; Ferraresso et al. 2009) as well as in gills of Japanese flounder and sea bass (Piyaviriyakul et al.

2007; Ferraresso et al. 2009) and on TCR α^+ and IgM $^+$ lymphocytes in Japanese flounder (Piyaviriyakul et al. 2007). RT-PCR analyses reveal that several different classes of NITRs are expressed at different levels in different tissues (Strong et al. 1999; Hawke et al. 2001; Yoder et al. 2002; Piyaviriyakul et al. 2007; Ferraresso et al. 2009). Furthermore, expression of NITR transcripts has been detected by RNA blot and RT-PCR analyses in various clonal NK-like, T, B, and macrophage cell lines of channel catfish (Hawke et al. 2001; Evenhuis et al. 2007). Certain NITRs in catfish appear to be expressed nearly ubiquitously in the various hematopoietic cell lines whereas expression of other catfish NITRs is more restricted (Evenhuis et al. 2007).

Thirty-six zebrafish NITR genes representing 12 families of NITRs have been identified from the zebrafish genome in a single gene cluster on chromosome 7 (Yoder et al. 2004). Three additional NITR genes, defining two additional families, are encoded in a gene cluster on zebrafish chromosome 14 (Yoder et al. 2008). Neither the tissue

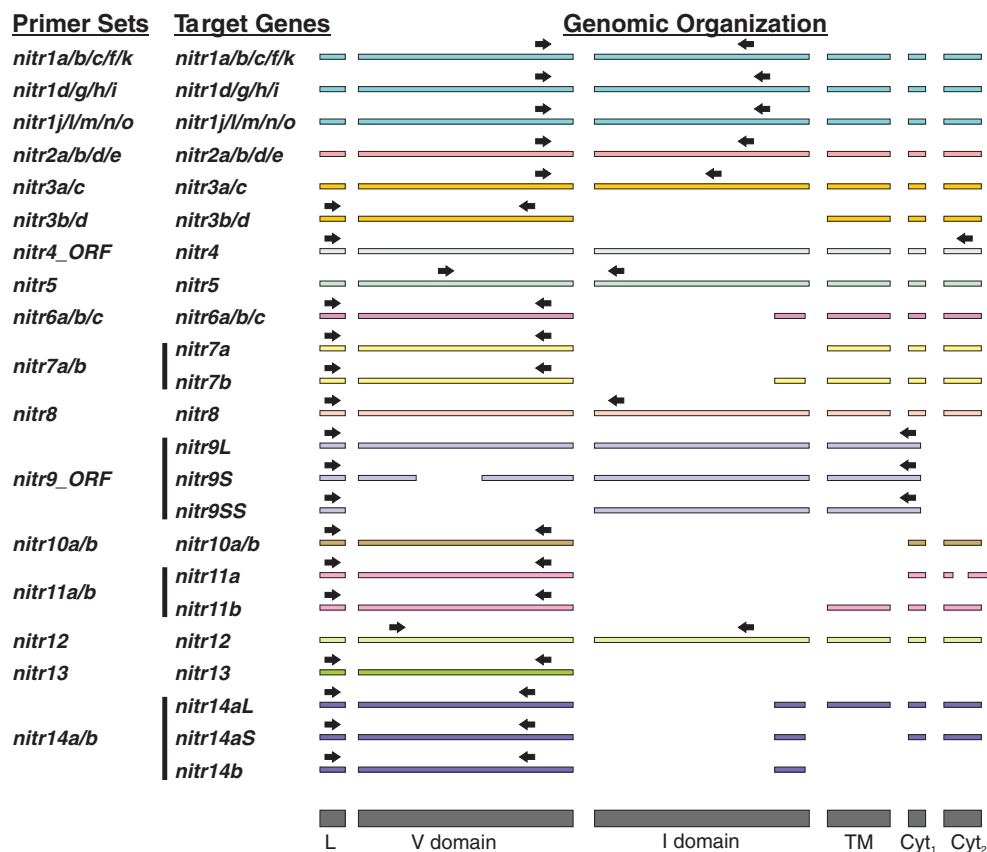


Fig. 1 Overview of oligonucleotide primer design for reverse-transcriptase polymerase chain reaction (RT-PCR). Primer sets are listed on the left. Genes targeted by each primer set and the overall genomic organization of these genes are listed to the right of each primer set. Families of NITRs are defined by number (e.g., *nitr1*). Individual genes within a family are identified with the addition of a lowercase letter (e.g., *nitr1a*). Alternatively spliced isoforms are

identified with *uppercase letters* *L*, *S*, and *SS* for long, short, and super-short, respectively (e.g., *nitr9L*). Colored rectangles represent exons and black arrows approximate the relative location of each primer. The peptide domains associated with each exon are shown at the bottom as gray rectangles (*L* peptide leader sequence, *V* variable domain, *I* intermediate domain, *TM* transmembrane domain, *Cyt*₁ and *Cyt*₂ cytoplasmic regions). Primer sequences are listed in Table 1

distribution nor expression of the different NITR families has been examined in a comprehensive manner. We describe herein the expression patterns of 14 different NITR families in zebrafish embryos, tissues and leukocytes.

In order to detect all possible NITR transcripts, including described polymorphisms and alleles, a panel of primers was developed for detecting NITR transcripts by RT-PCR. For example, three degenerate primer pairs were used to

Table 1 Primer sequences and cycling parameters for PCR

Primer sets	Forward primer Reverse primer	Annealing Temp (°C)	Extension time (s)	No. of cycles (Fig. 2)	No. of cycles (Fig. 3)	Amplicon length (bp)
<i>nitr1a/b/c/f/k</i>	AAGACAAAGYCTTYAGACTCTGC GTTGTTCTTGTGGAGACTGTAGA	65	30	40	40	345
<i>nitr1d/g/h/i</i>	TCAGACTCTGCAACATATTACTGTG ACAGCACAGTAGTAAATGCCAG	65	30	40	40	367/370
<i>nitr1j/l/m/n/o</i>	GGTTATTTAATCTRACCATTTTAAA TAAATGCCAGWATCAGAGCSACTGA	60	30	40	40	389/392/395
<i>nitr2a/b/d/e</i>	GATGAWGKYAGTTTTAATCTGAGCATC GCAGGCTGTAGATGCATTGTGTGCAG	65	30	40	40	346
<i>nitr3a/c</i>	GARGAWTTTGCAAMYTAYTATTG CGCTCCGGTTATCATGAGTGTA	TD65–55 ^a	30	40	50	274
<i>nitr3b/d</i>	ATGAGCTTRCAARRTTGTTTTACC TTCTCCAAACATCATGATGTTCCAG	55	30	40	40	378
<i>nitr4_ORF^b</i>	<u>ATGAATCACATCATCTACGTGATCATC</u> <u>TCATCTTGCAGCATTGGTCTGTGTTGG</u>	65	60	40	40	957/1,014
<i>nitr5</i>	GAAGAGCCACGTCTCATTGCTTCC ATGACTGAACCAGTAGAGACGATG	70	30	35	35	354
<i>nitr6a/b/c</i>	TTGGKTYATTTTGTATGTGTTKGGC TKATGTTGAGGTGAAAAGAGTCTT	58	30	40	40	284
<i>nitr7a/b</i>	TCTGGACTACAYCCGCTGATGCTC CATCCACGCTGATAATCCTCAGTG	70	30	35	35	262/301/304
<i>nitr8</i>	CAACATGAGCACAGYTTTATTCTC TTCTTATCTTCCCTCYGAACTCTAC	65	30	35	40	486
<i>nitr9_ORF^b</i>	<u>ATGATCAACTTTTGGATTTTGGACTTTTC</u> <u>TTACTGCTGGTTAGAAACCGAGTTAATCAT</u>	65	60	40	40	606/858/951
<i>nitr10a/b</i>	GCAGCTTTATTGCTCTTTG CCACAAAAGTAGAAGCCAG	65	30	40	40	323
<i>nitr11a/b</i>	GYGKTAKTAATYTCAGCGCTGTTGATG ATTTTCCAAGAGYMCCAACCACAGAAG	65	30	35	40	348/351
<i>nitr12</i>	GCAAACCCCTGGAGAAGCTCCAAC GGCAGATTGTAGATACAGGACTGC	65	30	40	40	502
<i>nitr13</i>	GGATTTCTCTCATGCTTCTATGCAGG CAGCCACAGTAATATGTTGCYTCATC	70	30	40	50	328
<i>nitr14a/b</i>	ATGATTCTCTGGGCATTTGTTACTG GATGCACAATAGTACACTGCTTCATC	60	30	40	40	326
<i>LC1</i>	GAAGATGCTGGAGATTACTGT ACTCTGAACACTGCTGTCTCC	65	30	40	N.A.	~433
<i>LC3</i>	GAAGATGCTGGAGATTACTGT ACACACAGATCAGCACTTG	65	30	40	N.A.	~405
<i>TCRα</i>	GCNSTNTAYTAYTYGC ATGCCAGTGACAAGAAG	TD65–55 ^a	30	45	45	~339
<i>mpx</i>	CCAGAACCAGTGAGCCTGAGACACG CAGTCTAACCATGGGCAGCGTGCCAC	70	30	N.A.	25	639
<i>β-actin</i>	GGTATGGAATCTTGCGGTATCCAC ATGGGCCAGACTCATCGTACTCCT	65	30	25	25	301

^a Touch-down PCR during which the annealing temperature is lowered from 65°C to 55°C by 0.5°C per cycle for 20 cycles and then an additional 20, 25, or 30 cycles are completed with a 55°C annealing temperature

^b Sequences corresponding to translational start and stop codons are underlined

detect the 14 member genes of the *nitr1* family (Fig. 1 and Table 1). As observed previously in other bony fish species, all zebrafish NITRs are expressed in the kidney, spleen and intestine; a few NITR transcripts are expressed at lower

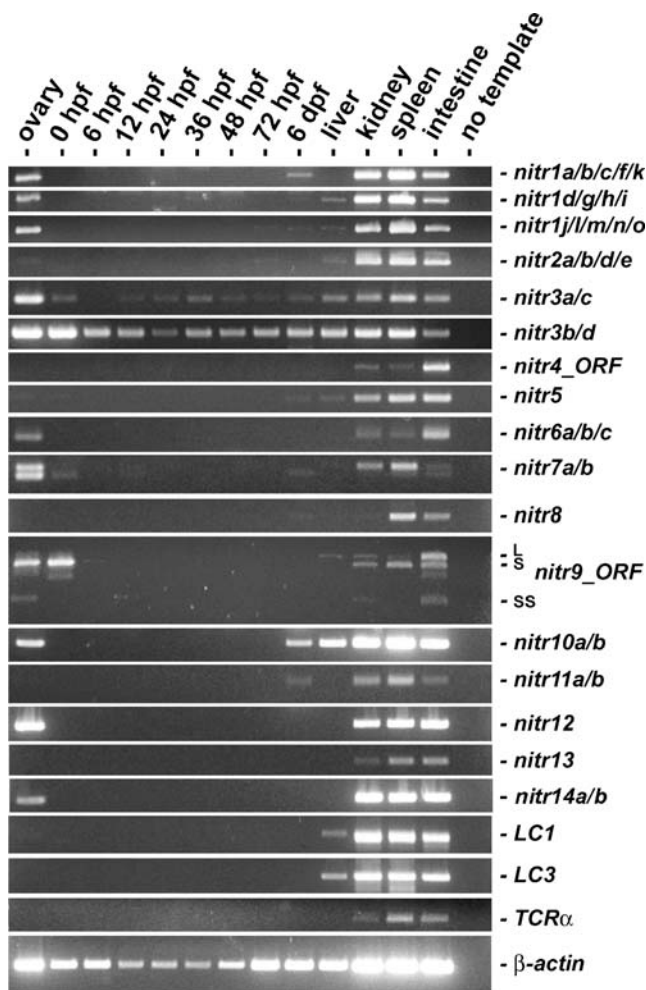
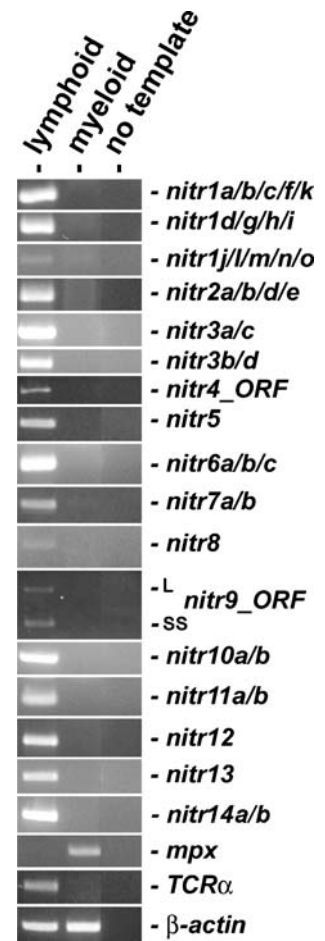


Fig. 2 RT-PCR analyses of NITR gene expression from zebrafish ovaries, embryos, and adult tissues. Zebrafish (AB strain) embryos were collected by natural mating and maintained at 28°C as described (Westerfield 2000). Total RNA (2 µg) from pooled embryos at various developmental stages (hours post-fertilization (hpf) or days post fertilization (dpf)) and dissected tissues (pooled from multiple animals) was reverse transcribed (SuperScript™ II Reverse Transcriptase: Invitrogen; Carlsbad, CA) and subjected to thermal cycling with gene-specific primers (Table 1 and Fig. 1) and TITANIUM™ *Taq* DNA polymerase (Clontech). Primers that amplify the entire open reading frame were utilized for *nitr4* and *nitr9*: note that three mRNA splice variants of *nitr9*, (Wei et al. 2007) are detected in the kidney and intestine. RT-PCR of immunoglobulin light chains (LC1 and LC3), T cell receptor α (TCR-α) and β-actin are included for reference. TCR-α primers were designed to amplify sequences between the variable (V) and constant (C) domains and thus only detect transcripts, which reflect somatic recombination. The degenerate TCR-α forward primers were designed to detect all V domain sequences encoding the conserved motif: A(V/L)YYCA. Limited RNA blot analyses with polyA⁺ mRNA from intestine, kidney and spleen, detected transcripts between 1.4–2.0 Kb for the zebrafish *nitr1–nitr7* families (unpublished observations)

Fig. 3 RT-PCR analyses of NITR gene expression from different leukocyte populations. Lymphoid and myeloid cell populations were purified from the kidney of multiple zebrafish and pooled as described (Yoder et al. 2007). RT-PCR was performed as described in Fig. 2, except 1 µg of total RNA was used in the reverse transcription reaction. RT-PCR of myeloperoxidase (*mpx*) provides a positive control for myeloid cells and TCR-α provides a positive control for T lymphocytes. β-actin is shown as a standard reference. Primer sequences and thermal cycling parameters are listed in Table 1



levels in the liver (Fig. 2). Notably, transcripts of the *nitr1*, *nitr3*, *nitr6*, *nitr7*, *nitr9*, *nitr10*, *nitr12*, and *nitr14* families were detected in the ovary. However, only maternal transcripts of *nitr3*, *nitr7*, and *nitr9* were detected in the 1-cell embryo (0-h post-fertilization) and only *nitr3* was expressed throughout embryogenesis. Efforts to further characterize *nitr3* expression during embryogenesis using whole mount RNA in situ hybridization were unsuccessful (Cannon, Litman and Yoder, data not shown; Thisse and Thisse 2004). These observations are not surprising based on relative levels of expression seen here and noted in previous efforts to recover *nitr3* cDNAs in library screening. The role of the maternal and embryonic NITR transcripts is unknown but of considerable interest.

The expression of NITR genes in zebrafish lymphoid and myeloid cell populations was characterized by RT-PCR (Fig. 3). Expression of the 14 different NITR gene families was detected in the lymphocyte population, but not in the myeloid population. This expression pattern is consistent with the hypothesis that NITRs are expressed and function in cytotoxic NK-like and T cells in bony fish (Yoder 2009); however, expression of a single flounder NITR and a single catfish NITR have been reported in B cells (Piyaviriyakul et al. 2007; Evenhuis et al. 2007). Although the expression of

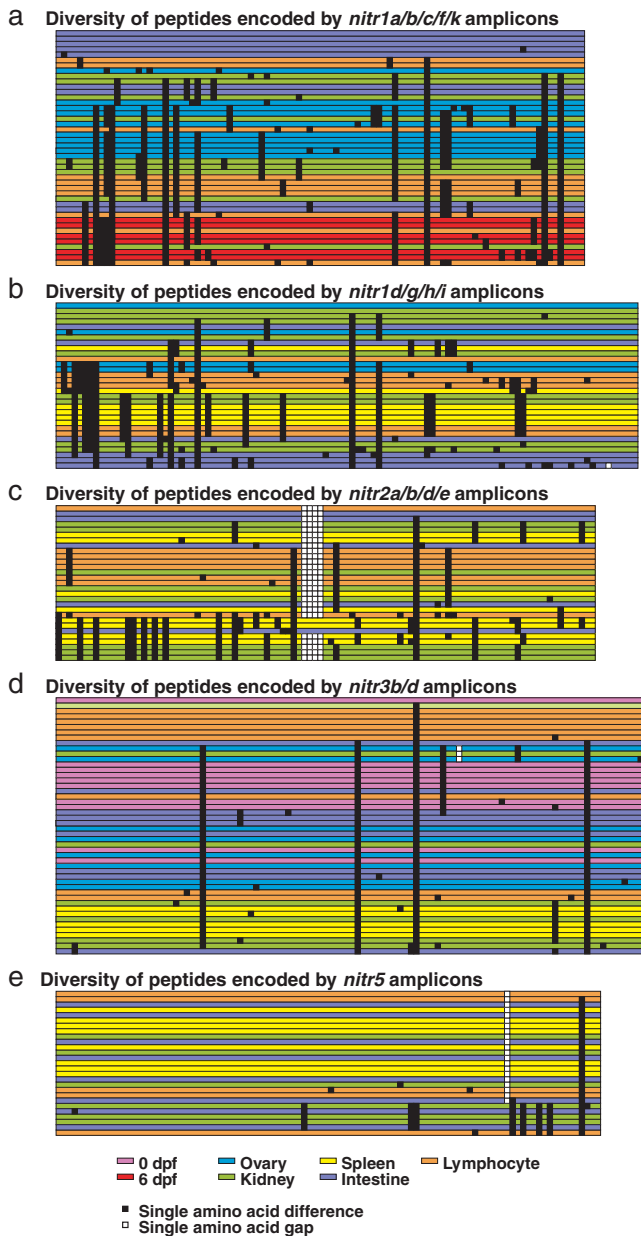


Fig. 4 Summary of variation detected from sequenced **a** *nitr1a/b/c/f/k*, **b** *nitr1d/g/h/i*, **c** *nitr2a/b/d/e*, **d** *nitr3b/d*, and **e** *nitr5* amplicons. Figures are derived from ClustalW alignments of peptides encoded by amplicons (Supplemental Fig. 1). Each row represents a single peptide sequence and is color-coded based on cDNA source, with the top row serving as an arbitrary reference (0 dpf = pink; 6 dpf = red; ovary = blue; kidney = green; spleen = yellow; intestine = violet; lymphocyte = orange). Amino acid positions that differ from the reference sequence are represented as black squares. Gaps in amino acid sequence are represented by white squares

certain catfish NITRs in a macrophage cell line (Evenhuis et al. 2007) may be a derived feature of catfish, it may be that some zebrafish NITRs are expressed in myeloid cells, but at a level too low for detection by our assay.

In order to confirm the identity of the NITR amplicons (Figs. 2 and 3) at least one amplicon was sequenced and

confirmed to be the predicted gene (data not shown). In an effort to evaluate the ability of the degenerate primers to amplify multiple genes and alleles, multiple *nitr1a/b/c/f/k*, *nitr1d/g/h/i*, *nitr2a/b/d/e*, and *nitr3b/d* amplicons were sequenced from various cDNA sources. The peptide sequences encoded by these amplicons is provided in Supplemental Fig. 1 and summarized in Fig. 4. Of the 44 *nitr1a/b/c/f/k* amplicons sequenced from five different cDNA sources, 27 different alleles were identified (different alleles defined as encoding different peptide sequences) including 14 new alleles. Similar results were observed for *nitr1d/g/h/i*, *nitr2a/b/d/e*, and *nitr3b/d* amplicons. In the course of developing rational priming strategies for the *nitr1*, *nitr2*, and *nitr3* multi-gene families, we had the occasion to examine the allelic variation of *nitr5*, which represents a single copy gene. Of the 27 *nitr5* amplicons sequenced from four different cDNA sources, seven new *nitr5* alleles were identified (Supplemental Fig. 1 and Fig. 4). These data further document the polymorphic nature of the NITR gene cluster (Yoder et al. 2004; Yoder 2009) and support a recent and rapid evolution of the NITR gene family.

The work described herein documents that zebrafish NITRs are expressed in the spleen, kidney and intestine; some NITR genes are expressed less abundantly in the liver. The expression of all NITR genes in the lymphoid lineage supports their role as NKRs, as does their high degree of allelic variation (e.g., *nitr1*, *nitr2*, *nitr3*, and *nitr5*). As a large diversified multigene family, zebrafish NITRs exhibit differential, variable expression in the ovary and during embryogenesis. The *nitr3* family is unique in that it is expressed throughout development and suggests that certain NITRs may exhibit alternative functions outside of the leukocyte lineage and their likely primary role as NKRs.

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