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Developmental expression of the N-myc downstream regulated gene (Ndrg) family during *Xenopus tropicalis* embryogenesis

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ABSTRACT The N-myc downstream regulated gene (Ndrg) family consists of four main members *Ndrg1, 2, 3,* and *4*. The *Ndrg* genes are involved in many vital biological events including development. However, comprehensive expression patterns of this gene family during vertebrate embryogenesis remain largely unknown. Here, we analyzed the Ndrg family from the evolutionary perspective and examined the expression patterns of the *Ndrg* genes during *Xenopus tropicalis* embryogenesis. Different Ndrg family members of vertebrates are separated into different homology clusters which can be further classified into two groups and each Ndrg family member is well conserved during evolution. The temporal and spatial expression patterns of *Ndrg1, 2, 3* and *4* are different during early *Xenopus tropicalis* development. *Ndrg1, 2* and *4* are maternally expressed genes while *Ndrg3* is a zygotically expressed gene. The *Ndrg* genes are differentially expressed in the developing central nervous system, the developing sensory organs, and the developing excretory organs. Moreover, they also show other specific expression domains. Our results indicate that the *Ndrg* genes exhibit specific expression patterns and may play different roles during vertebrate embryogenesis.

KEY WORDS: *N-myc downstream regulated gene (Ndrg) family, Xenopus tropicalis, expression pattern, embryogenesis*

The N-myc downstream regulated gene (Ndrg) family comprises four main members *Ndrg1*, *2*, *3*, and *4*. The nomenclature of this family originates from the first member discovered in the family, Ndrg1 (formerly also known as Ndr1/RTP/Drg1), since its expression can be repressed by the proto-oncogenes N-myc/c-myc (Okuda and Kondoh 1999; Shimono *et al.,* 1999). However, the terminology is not comprehensive enough because not all *Ndrg* genes are necessarily regulated by N-myc. At least the expression of mouse *Ndrg2* and *Ndrg3* are not activated in N-*myc* mutants (Okuda and Kondoh 1999).

The Ndrg proteins show high homology to each other, they share one NDR domain and one α/β hydrolase-fold region without hydrolytic catalytic site (Melotte *et al.,* 2010). Nevertheless, the *Ndrg* genes display differential expression. In human tissues, the expression patterns of *Ndrg1*, *2*, and *3* are relatively ubiquitous, while *Ndrg4* is more specifically expressed (Zhou *et al.,* 2001) which indicates that they possibly exert different biological functions. The *Ndrg* genes play important roles in nervous system. *Ndrg1* is essential for Schwann cell signaling in the peripheral nervous system while *Ndrg2* is associated with Alzheimer's disease and the differentiation of neural cells (Melotte *et al.,* 2010). Besides, the Ndrg family members have been indicated in stress response. For example, the expression of *Ndrg1* changes in response to homocysteine and hypoxic conditions (Melotte *et al.,* 2010). In addition, the *Ndrg* genes have different effects on tumor formation. *Ndrg1* is believed to function as a tumor suppressor gene. The expression of *Ndrg1* is significantly downregulated in cancer and metastatic cells (Kovacevic and Richardson 2006). Reduced *Ndrg2* expression has been observed in different cancers (Hu *et al.,* 2004), and the tumor suppressive role of *Ndrg2* and the status of *Ndrg2* as a potential biomarker in cancer have been identified. Similarly, *Ndrg4* is also regarded as a tumor suppressor gene (Melotte *et al.,* 2009). In contrast, *Ndrg3* is considered as a tumor

Abbreviations used in this paper: Ndrg, N-myc downstream regulated gene; NJ, neighbor-joining; CDS, protein-coding sequence; hCG, human chorionic gonadotropin; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; Odc, ornithine decarboxylase; WISH, whole mount *in situ* hybridization.

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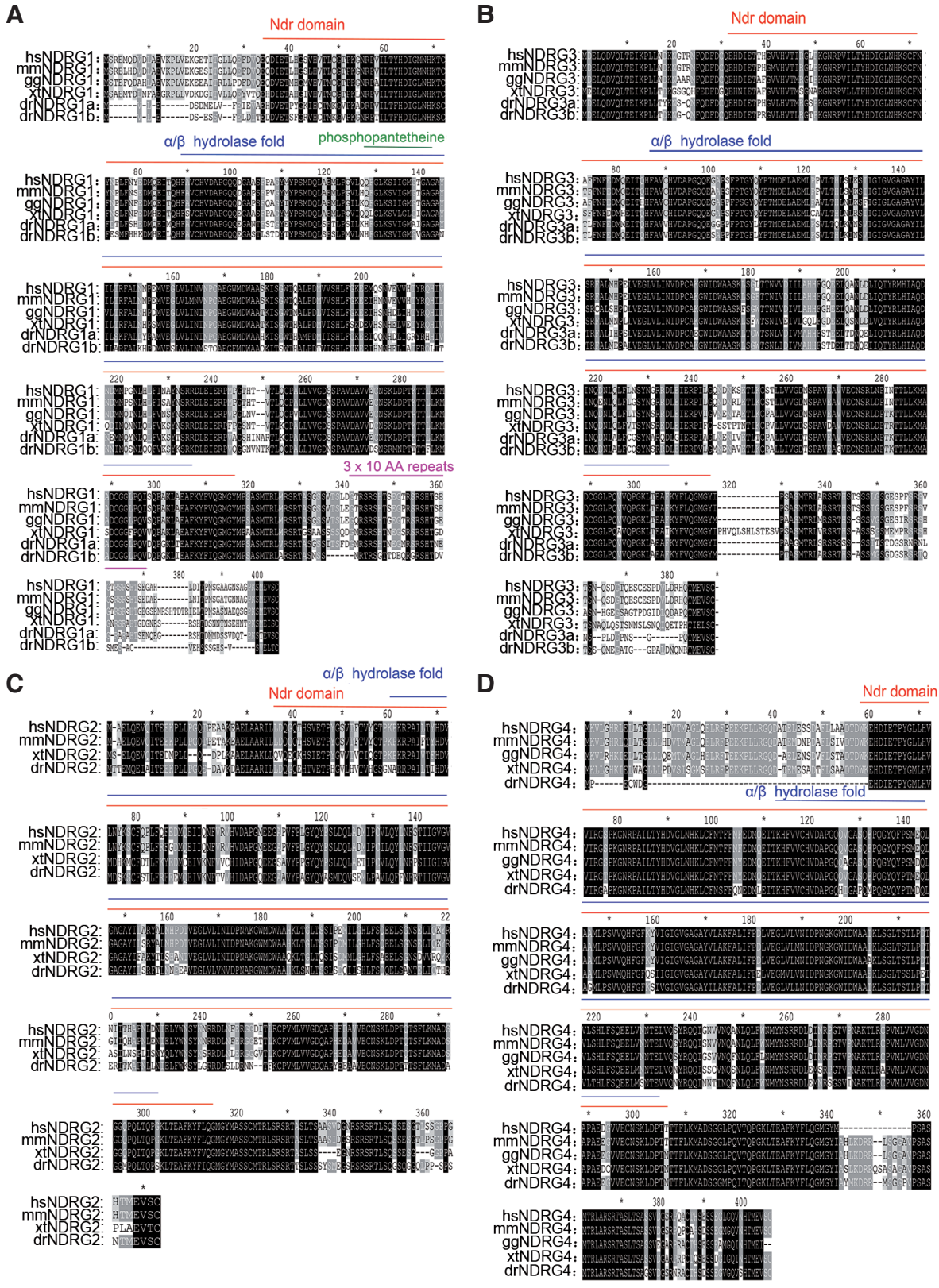

Fig. 1. Amino acid sequences alignment of the *Ndrg* genes. (A-D) *Amino acid sequences of Ndrg1, 2, 3, and 4 from different vertebrates were aligned respectively. Amino acid sequences were collected from human (Homo sapiens), mouse (Mus musculus), chicken (Gallus gallus), frog (*Xenopus *tropicalis), and zebrafish (Danio rerio). Identity residues from zebrafish to mammals are shown in black, and similarity sites are shown in gray. The red, blue, green and pink lines indicate Ndr domain, α/β hydrolase-fold motif, phosphopantetheine site and 3×10 AA (GTRSRSHTSE) tandem repeats, respectively. Abbreviations: hs (Homo sapiens), mm (Mus musculus), gg, (Gallus gallus), xt (*Xenopus *tropicalis), dr (Danio rerio).*

promoter gene because *Ndrg3* enhances *in vitro* and *in vivo* prostate cancer cell growth (Wang *et al.,* 2009). Furthermore, the Ndrg family has been found to play roles in vertebrate histogenesis and organogenesis. *Ndrg1* is involved in the development of *Xenopus laevis* pancreas, oesophagus, stomach, duodenum primordial, urinary and reproductive organs (Zhang *et al.,* 2013). *Ndrg2* is confirmed to be an important regulator of vertebral specification in differentiating somites of mouse (Zhu *et al.,* 2012a). *Ndrg3*, with specific expression in the outer layers of seminiferous epithelium, is considered to be required for human spermatogenesis (Zhao *et al.,* 2001). *Ndrg4* is crucial for cardiac development as it regulates proliferation of cardiomyocytes in zebrafish (Qu *et al.,* 2008).

Although many reports have demonstrated the involvement of the Ndrg family in early life and development, comprehensive expression patterns of the *Ndrg* genes during vertebrate embryonic development have not been well studied. In this study, we analyzed the conservation and phylogeny of the Ndrg family, and described the expression patterns of the *Ndrg* genes during *Xenopus tropicalis* embryogenesis. Our study will be helpful for further functional study of the *Ndrg* genes during development.

Results

Analysis of the conservation and phylogeny of the Ndrg family members

To investigate the conservation of the Ndrg family members during evolution, we performed amino acid sequences alignment of the Ndrg proteins from different species (Fig. 1 A-D). The alignment shows that the shared identities of vertebrate Ndrg1, 2, 3, and 4 are 55.5%, 55.2%, 61.6%, and 64.6% respectively. In addition, we also analyzed the conservation of the Ndrg family proteins between invertebrates and vertebrates. There are two Ndrg family members in invertebrates, and the shared identity between invertebrate and vertebrate Ndrg proteins (24.3%-28.2% for one member and 27.4%-29.7% for the other) is less than that of vertebrate Ndrg proteins.

To study the origin and evolution of the *Ndrg* genes, neighbor joining (NJ) method based phylogenetic tree (Fig. 2) was constructed with the coding sequences (CDSs) of the *Ndrg* genes from representative species. As is shown in the phylogenetic tree, there are four homology clusters in vertebrates while only two in invertebrates. Different Ndrg family members of vertebrates are separated into different homology clusters and the molecular phylogeny of each *Ndrg* gene faithfully presents the evolutionary status of species. The homology clusters can be further classified into two groups. One group consists of *Ndrg1* and *Ndrg3,* and the other consists of *Ndrg2* and *Ndrg4.*

Temporal expression patterns of Ndrg genes during Xenopus tropicalis embryogenesis

The temporal expression patterns of the *Ndrg* genes during *Xenopus tropicalis* embryogenesis were examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Fig. 3). Our results shows that *Ndrg1* is maternally expressed in eggs and the expression level increases significantly from gastrula stage (stage 10) to early tailbud stage (stage 23), then it decreases. *Ndrg2* is also maternally expressed in eggs and the expression level increases from eggs to tailbud stage (stage 28) followed by subsequent decrease. Like *Ndrg1* and *Ndrg2*, *Ndrg4* is a maternally expressed gene as well, and the expression is downregulated from

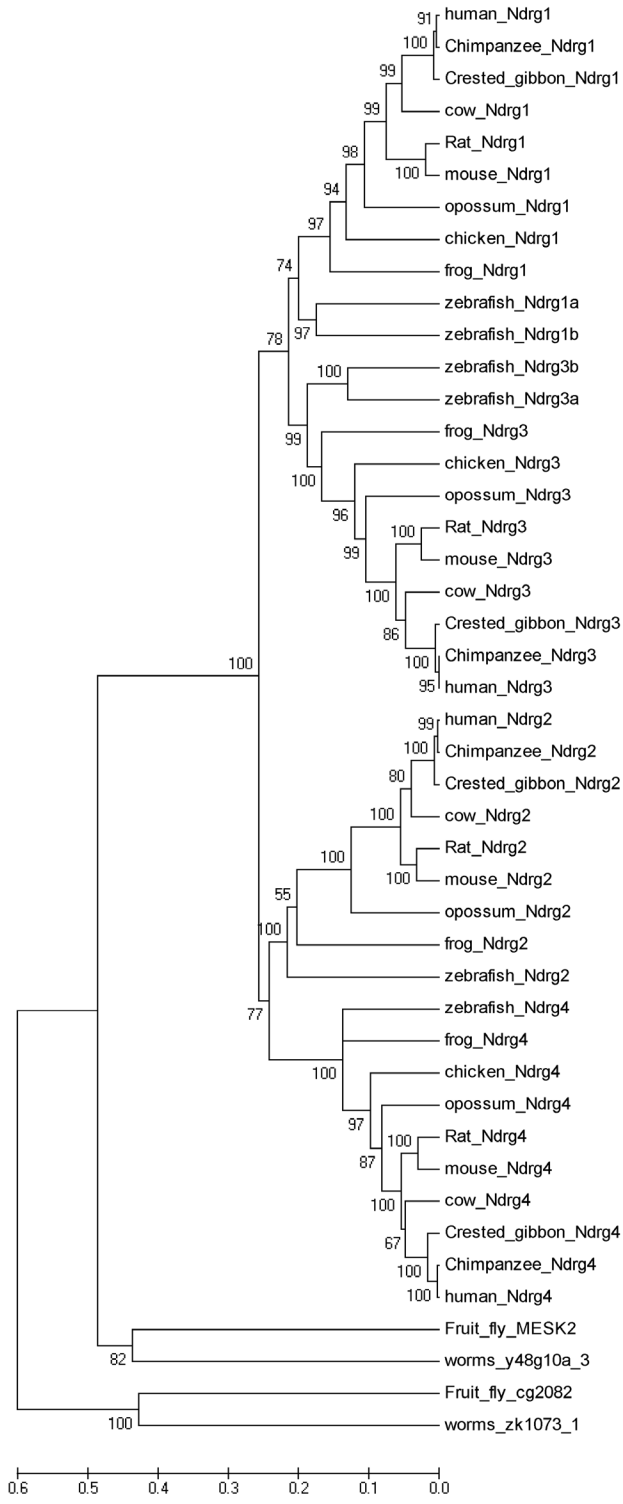

Fig. 2. Phylogenetic tree of the *Ndrg* genes. *The CDSs of the Ndrg genes were obtained from human (Homo sapiens), chimpanzee (Pan troglodytes), gibbion (Nomascus leucogenys), cow (Bos taurus), rat (Rattus norvegicus), mouse (Mus musculus), opossum (Monodelphis domestica), chicken (Gallus gallus), frog (*Xenopus *tropicalis), zebrafish (Danio rerio), fruit fly (*Drosophila melanogaster*), and worm (Caenorhabditis elegans). NJ based phylogenetic tree was constructed by MEGA5 software. Numbers on branch nodes are bootstrap replication values. The scale bar represents the number of amino acids substitutions.*

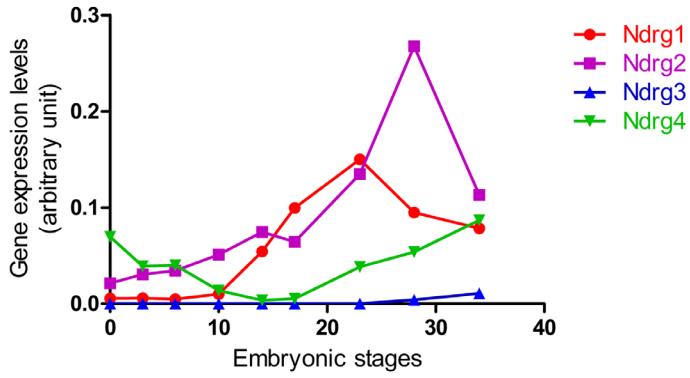

Fig. 3. The temporal expression patterns of *Ndrg1, 2, 3* and *4* during *Xenopus tropicalis* embryogenesis. *qRT-PCR analysis of relative expression levels of Ndrg1, 2, 3 and 4 at eggs (stage 0), cleavage stage (stage 3 and 6), gastrula stage (stage 10), neurula stage (stage 14 and 17), early tailbud stage (stage 23 and 28) and late tailbud stage (stage 34).*

eggs to neurula stage (stage 14). Subsequently, the expression of *Ndrg4* is upregulated. In contrast, the expression of *Ndrg3* is not detected until early tailbud stage (stage 23), and gradually elevated expression is found during tailbud stage (stage 23-34).

The spatial expression patterns of the Ndrg genes during Xenopus tropicalis embryogenesis

The spatial expression patterns of the *Ndrg* genes during *Xenopus tropicalis* embryogenesis were investigated by whole-mount *in situ* hybridization (WISH), and paraffin sections of stage 28 embryos after WISH were used to further examine detailed expression of the *Ndrg* genes.

During *Xenopus tropicalis* embryogenesis, *Ndrg1* is expressed in the animal hemisphere at stage 3 (Fig. 4A), and during gastrulation (stage 10 and 12) it is expressed in the dorsal side (Fig. 4 B,C). Interestingly, the expression of *Ndrg1* is concentrated in the endomesoderm at stage 10 (Fig. 4B). With the development of the embryo, evident signals are detected in the neural crest and the midline at neurula stage (stage 16 and 19) (Fig. 4 D,E). At stage 23, distinct signals are observed in the pronephros and the notochord (Fig. 4F). Later at stage 28, expression is found in the forebrain, the optic vesicle, the branchial arches, the dorsal endoderm, the ventral pancreatic bud and persists in the pronephros and the notochord (Fig. 4 G-J).

TABLE 1

THE MAJOR EXPRESSION DOMAINS OF *NDRG* GENES DURING *XENOPUS TROPICALIS* EMBRYOGENESIS

Gene name	Expression domains
Ndrg1	endomesoderm, neural crest, midline, forebrain, optic vesicle, pronephros, notochord, dorsal endoderm, ventral pancreatic bud, branchial arches
Ndrg2	neural fold, epidermis, brain, spinal cord, optic vesicle, otic vesicle
Ndrg3	brain, spinal cord, otic vesicle, heart, profundal and trigeminal placodes/ganglia
Ndrg4	brain, spinal cord, optic vesicle, otic vesicle, pronephros, branchial arches

Ndrg2 is expressed in the animal side at cleavage stage (stage 5) and gastrula stage (stage 10.5) (Fig. 5 A,B). At the beginning of the neurulation (stage 13), *Ndrg2* is extensively expressed (Fig. 5C). Subsequently, evident signals are found in the neural fold and the epidermis at stage 19 (Fig. 5D). At early tailbud stage (stage 22), signals are observed in the neural tube and remain in the epidermis (Fig. 5E). At the following stage (stage 24 and 28), signals are detected in the optic vesicle, the otic vesicle, the brain, the spinal cord, and the epidermis (Fig. 5 F-K).

At stage 3 and 10.5, the expression of *Ndrg3* is not detected (Fig. 6 A,B). At stage 23, *Ndrg3* is expressed in the spinal cord, the profundal and the trigeminal placodes/ganglia (Fig. 6 C,D). Subsequently (stage 28), signals are observed in the brain, the heart, the otic vesicle, and also in the spinal cord, the profundal and the trigeminal placodes/ganglia (Fig. 6 E-H).

Ndrg4 is highly expressed in the animal side at cleavage stage (stage 6) (Fig. 7A) and extensive expression is detected during gastrulation (stage 10.5) (Fig. 7B). Afterwards, the expression of *Ndrg4* is downregulated at neurula stage (stage 13 and 19) (Fig. 7 C,D). At early tailbud stage (stage 23), strong signals are observed in the forebrain, the optic vesicle, the spinal cord and the pronephros (Fig. 7 E,F). At stage 28, specific expression is found in the forebrain, the midbrain, the hindbrain, the spinal cord, the optic vesicle, the otic vesicle, the pronephros and the branchial arches (Fig. 7 G-J).

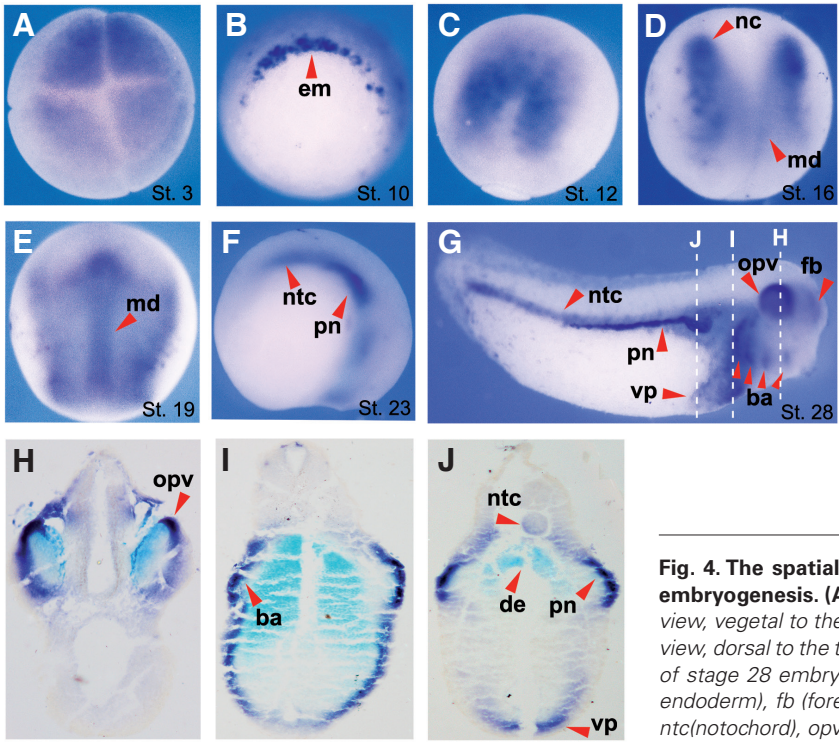

Fig. 4. The spatial expression pattern of *Ndrg1* during *Xenopus tropicalis* embryogenesis. (A) *Animal view.* **(B)** *Vegetal view, dorsal to the top.* **(C)** *Dorsal view, vegetal to the bottom.* **(D, E)** *Dorsal view, anterior to the top.* **(F, G)** *Lateral view, dorsal to the top and anterior to the right.* **(H-J)** *Transverse paraffin sections of stage 28 embryos after WISH. Abbreviations: ba (branchial arch), de (dorsal endoderm), fb (forebrain), em (endomesoderm), nc (neural crest), md (midline), ntc(notochord), opv (optic vesicle), pn (pronephros), vp (ventral pancreatic bud).*

TABLE 2

PRIMER SEQUENCES FOR MOLECULAR CLONING AND qRT-PCR

		Primer sequence (5'-3')	
Gene name	**Purpose**	**Forward**	**Reverse**
Ndrg1	Cloning qRT-PCR	GGCCATCGATAGGCTGACGGCTATGTCTGCGGAGATG GGGATTTCCTCAGGTCGTA	GGCCCTCGAGCATTGGCAGGCAGGTCTTCGTTGTTGAG CTCCTTGAGCCTTCGTTG
Ndrg2	Cloning qRT-PCR	GGCCATCGATGCTCACAGTCTGGAGATGTCTGAACTACAAGA GATGGCGGATTCTGGTGG	GGCCCTCGAGAGACAGAGGTCAGGCAGGGTGTGGGAA ACTCGCTGCTCTGGGACA
Ndrg3	Cloning qRT-PCR	GGCCATCGATATGAAGCTGCTGGGGCATAAGATAGAGC GATGGCTGATTCTGGTGG	GGCCCTCGAGGGATAATGAATGGCGTAAATGGGGGATTA TGCTTTCAGATTGGGTGC
Ndrg4	Cloning qRT-PCR	GGCCGAATTCAGGAAAGAATATGGAGGAGTTGCAAGA CCCAACAAGGACTACACTC	GGCCGTCGACGGATAACCGTAAGCCAATGATAGGAT ATCTGGTTTGACAGGGAG
Odc	qRT-PCR	GCACATGTCAAGCCAGTTCT	TGCGCTCAGTTCTGGTACTT

Taken together, the *Ndrg* genes are differentially expressed during early *Xenopus tropicalis* development (Table 1). For the developing central nervous system, *Ndrg2, 3,* and *4* are expressed in the brain and the spinal cord while *Ndrg1* is expressed in the forebrain. For the developing sensory organs, *Ndrg1, 2,* and *4* are expressed in the optic vesicle while *Ndrg2, 3,* and *4* are expressed in the otic vesicle. For the developing excretory organs, *Ndrg1* and *4* are both expressed in pronephros. In addition, each of them also shows other specific expression domains: *Ndrg1* is expressed in the endomesoderm, the notochord and the ventral pancreatic bud, *Ndrg2* is expressed in the epidermis, and *Ndrg3* is expressed in the heart, the profundal and the trigeminal placodes/ganglia.

Discussion

Our results of phylogenetic analysis are consistent with that of the previous study (Melotte *et al.,* 2010). The phylogenetic tree of the *Ndrg* genes shows different homology clusters for different family members, indicating that the highly homologous *Ndrg* genes emerge through duplication. Moreover, our protein alignment reveals that each member is conserved during evolution. The expression patterns of the *Ndrg* genes described here indicate that they have different functions. The observed expression of *Ndrg1*, *2*, *3* and *4* in the brain may reflect functional roles for the *Ndrg* genes during development of central nervous system. The *Ndrg* genes have previously been shown to be expressed in distinct cell types in the mouse brain (Okuda *et al.,* 2008). These results are complementary, as they allow us to further assess cellular expression patterns of the *Ndrg* genes. Besides, the fact that functional redundancy between the *Ndrg* genes may exist in central nervous system has also been indicated (Okuda *et al.,* 2008). Specific expression of *Ndrg1* detected in the pronephros and the ventral pancreatic bud are well consistent with the suggested roles for *Ndrg1* in regulating pronephros and pancreas development respectively (Kyuno *et al.,* 2003, Zhang *et al.,* 2013). It has been demonstrated that human *Ndrg2* is involved in the regulation of Na^+/K^+-ATPase in epithelial tissues which is important for ion transport and reabsorption (Li *et al.,* 2011), thus the observed expression of *Ndrg2* in the epidermis is likely indicative of a similar role in *Xenopus tropicalis* embryo. Furthermore, the expression of the *Ndrg* genes in the developing sensory organs is observed: *Ndrg1, 2,* and *4* are expressed in the optic vesicle while *Ndrg2, 3,* and *4* are expressed in the otic vesicle, which suggests that the *Ndrg* genes may have other unknown functions during the development of these sensory organs.

It has been shown that *Ndrg1* is a target of the proto-oncogene N-myc while *Ndrg 2* and *3* are not regulated in mouse (Okuda and Kondoh 1999). Interestingly, N-*myc* is highly expressed in the central nervous system, the otic vesicle and is not expressed in the notochord during *Xenopus laevis* embryogenesis (Vize *et al.,* 1990). Compare the expression patterns of *Ndrg1*, *2*, *3* and *4* described here with that of N-*myc*, only the expression of *Ndrg1*

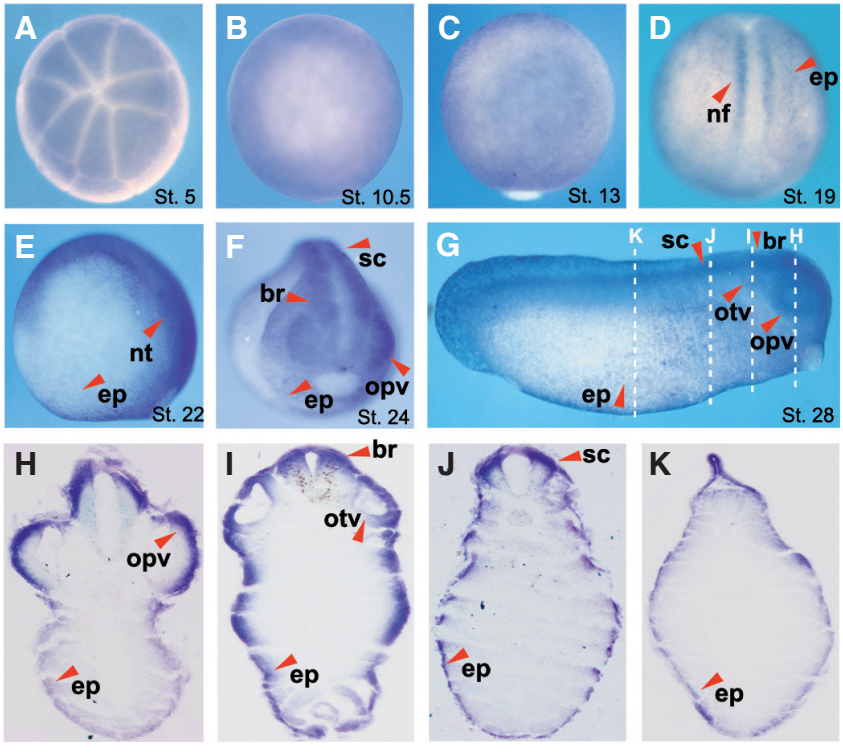

Fig. 5. The spatial expression pattern of *Ndrg2* during *Xenopus tropicalis* embryogenesis. (A,B) *Animal view.* **(C,D)** *Dorsal view, anterior to the top.* **(E,G)** *Lateral view, dorsal to the top and anterior to the right.* **(F)** *Anterior view, dorsal to the top.* **(H-K)** *Transverse paraffin sections of stage 28 embryos after WISH. Abbreviations: br (brain), ep (epidermis), nf (neural fold), nt (neural tube), opv (optic vesicle), otv (otic vesicle), sc (spinal cord).*

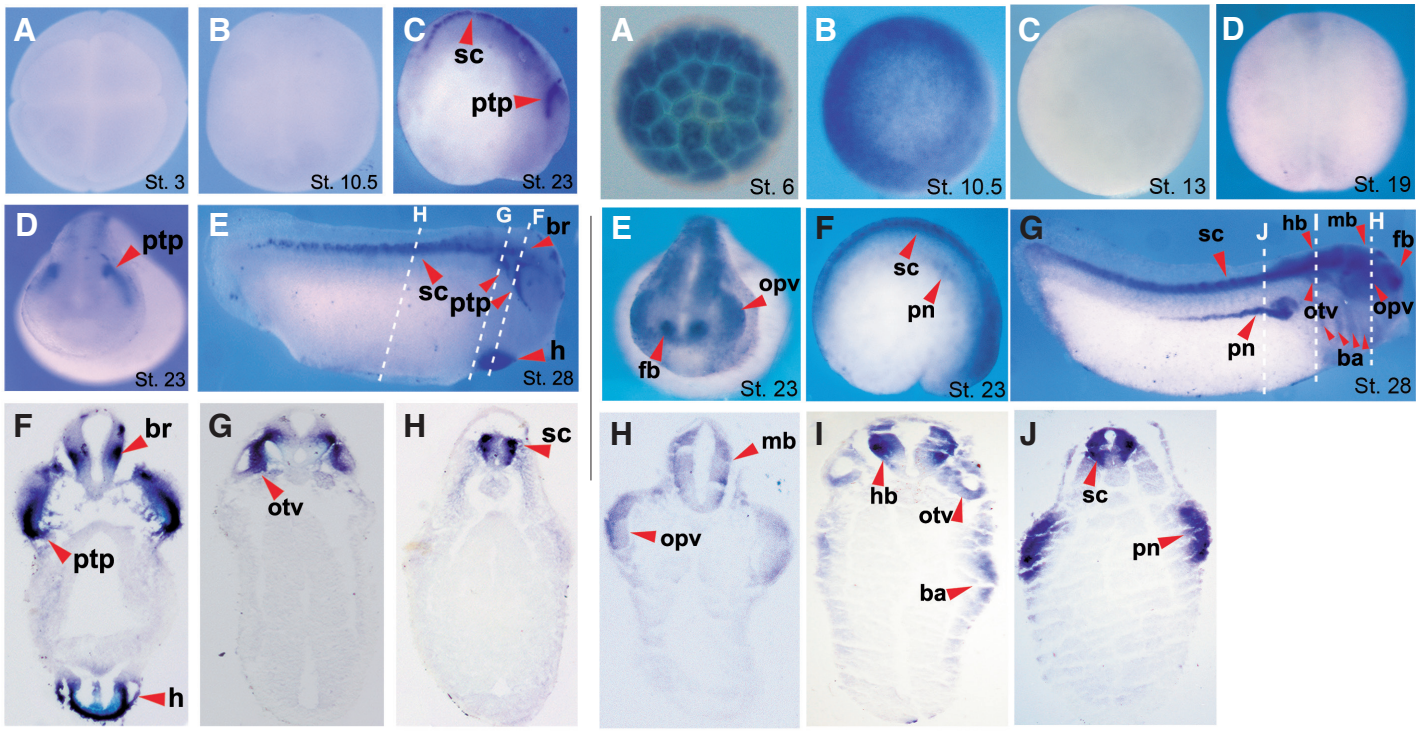

Fig. 6 (Left). The spatial expression pattern of *Ndrg3* during *Xenopus tropicalis* embryogenesis. (A,B) *Animal view.* **(C,E)** *Lateral view, dorsal to the top and anterior to the right.* **(D)** *Anterior view, dorsal to the top.* **(F-H)** *Transverse paraffin sections of stage 28 embryos after WISH. Abbreviations: br (brain), h (heart), ptp (profundal and trigeminal placodes/ganglia), otv (otic vesicle), sc (spinal cord).*

Fig. 7 (Right). The spatial expression pattern of *Ndrg4* during *Xenopus tropicalis* embryogenesis. (A,B) *Animal view.* **(C,D)** *Dorsal view, anterior to the top.* **(E)** *Anterior view, dorsal to the top.* **(F, G)** *Lateral view, dorsal to the top and anterior to the right.* **(H-J)** *Transverse paraffin sections of stage 28 embryos after WISH. Abbreviations: ba (branchial arch), fb (forebrain), mb(midbrain), hb (hindbrain), opv (optic vesicle), otv (otic vesicle), sc (spinal cord), pn (pronephros).*

is likely to be repressed by N-myc in *Xenopus*.

In summary, this study is the first to comprehensively examine the expression patterns of the *Ndrg* genes during *Xenopus tropicalis* embryogenesis. The four Ndrg family members are differentially expressed: *Ndrg1*, *2* and *4* are maternally expressed genes while *Ndrg3* is a zygotically expressed gene. The *Ndrg* genes are differentially expressed in the developing central nervous system, the developing sensory organs, the developing excretory organs and other expression domains specific for each *Ndrg* gene. These results will benefit for further investigation of the roles of the *Ndrg* genes during vertebrate embryogenesis.

Materials and Methods

Amino acid sequences alignment and phylogenetic analysis

The amino acid sequences of the Ndrg family members were retrieved from NCBI or Ensembl database. Multiple sequences alignment and NJ method based phylogenetic analysis were performed as previously described (Zhu *et al*., 2012b). The CDSs of the *Ndrg* genes used were as follows: human (*Homo sapiens Ndrg1* NM_001135242.1, *Ndrg2* NM_201535.1, *Ndrg3* NM_032013.3, *Ndrg4* NM_001130487.1), chimpanzee (*Pan troglodytes Ndrg1* XM_001140617.3, *Ndrg2* NM_001195153.2, *Ndrg3* XM_003316924.2, *Ndrg4* XM_003315122.2), gibbon (*Nomascus leucogenys Ndrg1* XM_003256224.2, *Ndrg2* XM_003260582.2, *Ndrg3* XM_003253534.2, *Ndrg4* XM_003263123.2), cow (*Bos taurus Ndrg1* NM_001035009.2, *Ndrg2* NM_001035304.1, *Ndrg3* NM_001101996.1, *Ndrg4* NM_001075695.2), rat (*Rattus norvegicus Ndrg1* NM_001011991.1, *Ndrg2* NM_001270862.1, *Ndrg3* NM_001013923.1, *Ndrg4* NM_001271091.1), mouse (*Mus musculus Ndrg1* NM_008681.2, *Ndrg2* NM_013864.2, *Ndrg3* NM_180956.1, *Ndrg4* NM_001195006.1), opossum (*Monodelphis domestica Ndrg1* ENSMODG00000001109, *Ndrg2* ENSMODG00000006811, *Ndrg3* ENSMODG00000001353, *Ndrg4* ENSMODG00000014164), chicken (*Gallus gallus Ndrg1* XM_418430, *Ndrg3* ENSGALT00000002265, *Ndrg4* XM_001231664), frog (*Xenopus tropicalis Ndrg1* NM_001008145.1, *Ndrg2* NM_001007897.1, *Ndrg3* NM_001006703.1, *Ndrg4* NM_001006793.1), zebrafish (*Danio rerio Ndrg1a* NM_213348.3, *Ndrg1b* NM_200692.2, *Ndrg2* NM_001008593.1, *Ndrg3a* NM_199517.1, *Ndrg3b* NM_199797.1, *Ndrg4* NM_001045173.2), fruit fly (*Drosophila melanogaster MESK2* NM_166454.2, *CG2082* NM_169100.3), worm (*Caenorhabditis elegans Y48G10A.3* NM_060968.4, *ZK1073.1* NM_078233.5).

Animal care and manipulation

All frogs (*Xenopus tropicalis*) were raised in the Model Animal Care Center of Zhejiang University in accordance with standard guidelines. Adult male and female frogs for mating were injected with 100 U and 150U human chorionic gonadotropin (hCG) through the dorsal lymph sac, respectively. The injected frogs were put together in clean water at room temperature. Embryos were collected every 30 min. Developmental stages of embryos were identified as previously described (Nieuwkoop and Faber 1994).

RNA extraction, molecular cloning, and qRT-PCR

Total RNA isolation from embryos of different developmental stages, DNase I treatment, and synthesis of single-strand cDNA were performed

as previously described (Zhu *et al.,* 2012b). For *Xenopus tropicalis Ndrg1*, *2*, *3*, and *4* CDSs molecular cloning, primers shown in Table 2 were used for PCR amplification with the following conditions: 94°C (30s), 60°C (30s), and 72°C (90s) for 35 cycles. The amplified products of *Ndrg1*, *2*, *3*, and *4* were purified and cloned into pCS107 vectors respectively. All constructs were sequenced to confirm their identities. For analysis of the temporal expression patterns of *Ndrg1*, *2*, *3*, and *4*, qRT-PCR was performed with SYBR Green supermix (Bio-Rad) on the CFX Connect Real-Time PCR System (Bio-Rad) using primers shown in Table 2. PCR thermal cycling conditions were 95°C (10s), 58°C (10s), and 72°C (30s) for 40 cycles. Each PCR was performed in triplicate. The $\Delta\Delta C_T$ method was used to determine relative gene expression using *Odc* as the endogenous control gene. Graphs were made using Graphpad Prism 5 software.

Whole-mount in situ hybridization and sectioning

Antisense digoxigenin-labeled RNA probes were synthesized with CDS constructs of *Xenopus tropicalis Ndrg1*, *2*, *3*, and *4*. Embryos at different developmental stages were processed for WISH as previously described (Zhu *et al.,* 2012b). For detailed information about tissue distribution of *Ndrg1*, *2*, *3*, and *4* transcripts, embryos of stage 28 after WISH were then followed by dehydration, permeabilization, wax infiltration and embedded in paraplast (Leica) for sectioning (20μm thickness).

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