Doctor Thesis

Effects of NDRG1 family proteins on photoreceptor outer segment morphology in zebrafish
(ゼブラフィッシュにおける NDRG1 ファミリー蛋白質の視細胞外節の形態に対する影響)

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日本語要旨

脊椎動物の網膜には桿体・錐体の 2 種類の視細胞が存在する。近年当研究室では、*N-myc downstream regulated gene 1 b* (*ndrg1b*)遺伝子がコイ錐体特異的に発現していることを見出した。相同蛋白質であるヒト NDRG1 蛋白質は常染色体劣性遺伝の脱ミエリン性神経変性の原因遺伝子として知られている。本研究では、NDRG1b 蛋白質が錐体特異的にどのような機能に関わっているのかを明らかにすることを目的とし、コイと近縁で遺伝学的手法が確立したゼブラフィッシュを実験動物として用いて研究を行った。

コイ *ndrg1b* のゼブラフィッシュ相同遺伝子の単離

成魚での RT-PCR 解析により、ゼブラフィッシュ網膜には、*ndrg1b* 遺伝子に加えて、N 末端配列の異なる 2 種類の splice variants, *ndrg1a-1*, *2* 遺伝子の合計 3 種類の遺伝子が発現していることが示唆された。そのため、これら 3 種類全ての遺伝子について蛋白質コード領域の全長を RT-PCR によりクローニングした。

成魚視細胞での局在解析

成魚網膜視細胞での NDRG1a-1, NDRG1a-2, NDRG1b 蛋白質の局在を検討する目的で、特異的抗体を作製し免疫組織染色を行った。その結果、NDRG1b 蛋白質はコイ同様に錐体特異的に発現し、外節を含む形質膜に一様に局在することが判明した。また NDRG1a-1 蛋白質も錐体に発現し、NDRG1b 蛋白質と同様の局在を示した。加えて NDRG1a-1 は桿体にも発現しており錐体と似た局在を示したが、外節には局在しなかった。NDRG1a-2 蛋白質は錐体のみで発現しており、軸索様突起に発現が見られた。視細胞では、光情報伝達に関わる蛋白質の多くが外節に局在する。従って、これら 3 種類の蛋白質は光情報伝達に直接に関与するのではなく、形態維持や脂質代謝など別の機能に関わる可能性が考えられる。

初期発生過程での発現解析

全身由来の total RNA を用いた RT-PCR 解析により、初期発生過程における mRNA 発現を検討した。その結果、視細胞の形成時期である受精後 48 時間(48 hpf)付近から *ndrg1a-1* と *ndrg1b* の発現が急激に亢進することが判明した。一方 *ndrg1a-2* は、発生段階によらず未受精の段階からほぼ一定量発現していた。そして特異的抗体による免疫組織染色により、mRNA 発現と対応して網膜での NDRG1a-1・NDRG1b 蛋白質の発現が 48 hpf 付近から観察された。しかし、NDRG1a-1 が発現開始直後から視細胞層に限局して発現するのに対し、NDRG1b は最初視細胞層を含む網膜の広範囲で発現し、発生と共に視細胞層に限局していくことが判明した。網膜での NDRG1a-2 蛋白質の発現は明確には確認されなかった。

視細胞の分化・成熟過程での錐体オプシン発現時期との比較

ゼブラフィッシュでは、一般に 43-48 hpf 付近に視細胞への最終分化が開始するため、48 hpf 付近での視細胞層における NDRG1a・NDRG1b 蛋白質の発現と視細胞のマーカーである錐体オプシンの発現の時期とを特異的抗体を使って比較した。先の発現解析より 48 hpf 付近では NDRG1a-v2 蛋白質の発現量は少ないと判断されたため、NDRG1a 蛋白質の検出には、1 と 2 を両方とも認識する C 末端に対する抗血清を用いた。その結果、網膜層において 72 hpf までの全ての発生段階で、NDRG1a・NDRG1b 陽性細胞は錐体オプシン陽性細胞よりも、より広範囲で観察された。この結果は、錐体が未成熟な分化直前ない

しは直後の段階からNDRG1a・NDRG1b蛋白質は発現しており、また、視細胞層でオプシンに先行して発現が広がって行くことを強く示唆している。そのため、NDRG1a・NDRG1b蛋白質は錐体の成熟過程に何らかの寄与をする可能性がある。

NDRG1a-1・NDRG1b蛋白質発現阻害の視細胞形成過程への影響

視細胞におけるNDRG1a-1・NDRG1b蛋白質の機能を推定する目的で、受精直後の胚に *ndrg1a-1* または *ndrg1b* に対するMorpholinoアンチセンスオリゴ(MO)をインジェクションして簡便に発現阻害し、96 hpfにおいて眼球や網膜の免疫組織学的な観察を行った。その結果、ランダムな配列であるcontrol MOを注入した個体に比べて、*ndrg1a-1* に対するMOを注入した個体では、桿体・錐体両視細胞の外節が顕著に縮小し、オプシン濃度が若干低下した。NDRG1bに対するMOを注入した個体では、control MOを注入した個体に比べて、錐体外節が顕著に縮小し、赤/緑オプシンの濃度が若干低下した。以上の結果から、NDRG1a-1, NDRG1b蛋白質は、ともに視細胞の外節形成に必要であることが示唆される。

NDRG1ファミリー蛋白質の異所的もしくは過剰発現による成魚桿体への影響

錐体でのNDRG1bとNDRG1a-2蛋白質の機能を詳細に検討する目的で、錐体特異的に発現しているNDRG1bとNDRG1a-2蛋白質を *rhodopsin* プロモーターの支配下で全桿体細胞に異所的に発現させ、成魚網膜に対して免疫組織染色を行った。その結果、NDRG1b陽性個体網膜ではmCherryを強制発現した野生型(WT) 個体網膜に比べて桿体外節のマーカーであるGt1αのシグナルが減少していた。そこで、NDRG1b発現個体の網膜から桿体を単離して外節の形態を比較したところ、NDRG1b発現桿体ではコントロール(2%)に比べて10倍以上の割合で、通常は円柱状である桿体外節の先端が細くなった先細の外節を持つ桿体が観察された。また、円柱状の外節の長さが 65%程度に短くなっていた。一方NDRG1a-2を異所的に発現した桿体では、WT桿体との明確な形態的相違は認められなかった。また、NDRG1a-1 を桿体に過剰発現させたところ、NDRG1b 発現桿体と同様の結果が得られた。

NDRG1a-1・NDRG1b蛋白質の強制発現桿体の外節膜構造の検討

桿体の外節は、およそ千枚程度積み重なった円板状の膜の周囲を形質膜が覆った構造をしており、一方錐体の外節は、形質膜が貫入することで何層も折りたたまれたラメラ状の構造をしている。この両視細胞の膜構造の違いは *N, N'* -didansyl cystine (DDC)による蛍光染色によって光学顕微鏡下で区別することが可能であり、錐体外節のみが非常に強く蛍光を発する。そこで、NDRG1a-1およびNDRG1b強制発現個体から視細胞を単離、染色し、観察した。その結果、どちらの系統でも錐体外節からは強い蛍光が観察される一方で、円錐状の桿体外節からは蛍光は観察されなかった。よって、NDRG1a-1 およびNDRG1b強制発現桿体で観察される円錐状の外節は、元々桿体が有する外節膜構造を保ったまま、円錐状の外節へと変性したことが示唆される。

以上の結果より、ゼブラフィッシュにはNDRG1a-1, 2・NDRG1b蛋白質の合計3種類の蛋白質が存在し、そのうちNDRG1a-1・NDRG1b蛋白質が視細胞形成過程において正常な外節形成に必須であることと、また、錐体外節の形態形成または保持にも関与することが示唆される。加えて、錐体の巨視的な外節の形状と微視的な膜構造には直接的な関係がないことが示唆される。

Abstract

Rods and cones are functionally and morphologically distinct. In our lab, N-myc downstream-regulated gene 1b (*ndrg1b*) was previously identified as a cone-specific gene in carp. In this thesis, I show that NDRG1b and its paralog, NDRG1a-1, contribute to photoreceptor outer segment (OS) formation in zebrafish. First, the localization of the three NDRG1 homologs, NDRG1a-1 and NDRG1a-2, the two splice variants of NDRG1a, and NDRG1b was examined in adult and developing zebrafish retina. In adult zebrafish retina, all of the homologs were expressed mainly in photoreceptors. NDRG1a-1 was localized to the entire cone plasma membranes, and also in rod plasma membranes except at its outer segment (OS). NDRG1a-2 was expressed in the thin process in cones, and NDRG1b was expressed only in cones and localized to the entire plasma membranes. To understand the functional role(s) of NDRG1 family proteins, the effects of (1) knockdown of two of the dominant homologs, NDRG1a-1 and NDRG1b, at early developmental stages, and then (2) overexpression or ectopic expression of these NDRG1a-1, NDRG1a-2 and NDRG1b proteins in zebrafish rods were examined. Knockdown of NDRG1a-1 or NDRG1b protein with morpholino(s) induced significant reduction of the OS volume in both rods and cones. Overexpression of NDRG1a-1 in rods and ectopic expression of NDRG1b in rods under the control of *rhodopsin* promoter both induced macroscopic rod OS morphological alterations from cylindrical to tapered shape, which suggested that NDRG1a-1 and NDRG1b are involved in normal rod and cone OS development. Taper-shaped OSs freshly isolated from these rods were not stained with *N,N'*-didansyl cystine, a fluorescent probe, that specifically labels infolded membrane structure of cone OS. The result showed that rod OS membrane structure is preserved in these taper-shaped OSs and therefore, suggests that tapered OS shape is not directly related to the formation of the infolded membrane structure in cone OS. No apparent alteration was observed in rods where NDRG1a-2 was ectopically expressed. Because NDRG1a-1 and NDRG1a-2 are different in the amino acid sequence only in their N-terminal regions, this region in NDRG1a-1 seems to be important to cause the macroscopic rod OS morphological alterations.

Contents

Introduction

In the vertebrate retina, there are two types of photoreceptors, rods and cones. They are distinct in function and morphology[1,2]. Rods are more sensitive to light than cones, so that rods govern scotopic vision and cones mediate photopic vision including color discrimination. In addition, briefer responses allow cones to detect light stimuli with higher time resolution. It has been known for years that the structure of the outer segment (OS) is also remarkably different from each other. Rod OS is macroscopically cylindrical and consists of a stack of disk membranes surrounded by a plasma membrane. Cone OS is macroscopically conical or tapered, and consists of topologically continuous infolded plasma membrane.

In the previous study in our lab using purified carp rods and cones[3], Shimauchi-Matsukawa *et al.* found genes specifically expressed in cones including N-myc downstream-regulated gene-like (formerly *ndrg1l,* now *ndrg1b*). NDRG protein family consists of four protein subtypes; NDRG1, NDRG2, NDRG3 and NDRG4[4]. Among them, *NDRG1* mRNA is ubiquitously expressed in most human tissues, but its expression in the mammalian retina has not been studied. NDRG1 protein seems to be localized to various intracellular compartments and be involved in many biological functions in an organ- or tissue-specific manner[5]. For example, NDRG1 protein is identified as a metastasis suppressor in a variety of cancers[5, 6]. In neurons, *NDRG1* gene is identified as a responsible gene for Charcot-Marie-Tooth disease type 4D which is characterized by Schwann-cell dysfunction of peripheral nervous system[7]. Recent study showed that the lack of this gene induces demyelination in mice, a phenocopy of this human disease[8, 9]. However, functional roles of NDRG1 proteins or their functional mechanisms have not been clearly understood. It is partly because studies made under *in vitro* conditions do not always agree with those made under *in vivo* conditions[10].

In the present study, I made efforts to understand the role of NDRG1 family proteins in zebrafish retina. In teleost, there are two homologs of *ndrg1* gene, *ndrg1a* (consisting of two variants, *ndrg1a-1* and *ndrg1a-2*) and *ndrg1b*. Zebrafish *ndrg1a* gene is known to be expressed ubiquitously including in the eye (http://zfin.org/action/figure/all-figure-view/ZDB-PUB-040907-1?probeZdbID=ZDB-CDNA-040425-2588)[11]. However, it is not known whether *ndrg1a* is *ndrg1a-1* and/or *ndrg1a-2*, because two splice variants, *ndrgia-1* and *ndrg1a-2*, were not distinguished in that study. Zebrafish *ndrg1b* gene is preferentially expressed in the eye (http://zfin.org/action/figure/all-figure-view/ZDB-PUB-040907-1?probeZdbID=ZDB-CDNA-040425-2588)[11]. Therefore, I cloned mRNAs of zebrafish NDRG1 family proteins, *ndrg1a-1*, *ndrg1a-2* and *ndrg1b*, and examined their protein localization in

zebrafish rods and cones. My initial study using morpholinos seemed to suggest involvement of *ndrg1a-1* and *ndrg1b* in normal development of rods and cones. Therefore, *ndrg1a-1*, which is expressed in both zebrafish rods and cones, was overexpressed in rods, and *ndrg1b*, which is specifically expressed in zebrafish cones similarly as in carp, was ectopically expressed in zebrafish rods. Additionally, *ndrg1a-2* was also ectopically expressed in rods. Results presented in this thesis suggest that NDRG1a-1 and NDRG1b proteins are involved in forming a normal OS shape in zebrafish photoreceptors.

Materials and Methods

Animals

All experiments with zebrafish (*Danio rerio*) in this study were performed in accordance with the institutional guidelines and all experimental protocols were approved by Osaka University Graduate School of Frontier Biosciences (approval number FBS-14-006). *Tupfel long fin* strain was used and the fish were kept under 14 hr light/10 hr dark cycle at 28.5°C. When necessary, the fish were anesthetized by putting them in ice-chilled water and decapitated during the light period.

Identification of carp *ndrg1b* and three zebrafish *ndrg1* family mRNAs

For carp *ndrg1b*, full-length carp (*Cyprinus carpio*) *ndrg1l* (now, *ndrg1b*) cDNA was identified and obtained by screening a carp retinal cDNA library[3] with a DIG-labeled (Roche) partial coding sequence fragment found previously. Positive clones were sequence-verified.

To detect the expression of zebrafish *ndrg1* family mRNAs, coding sequence of each of the known zebrafish orthologs (NM_001128353.1 for *ndrg1a-1*, NM_213348.3 for *ndrg1a-2*, and NM_200692.2 for *ndrg1b*) was amplified by reverse transcription polymerase chain reaction (RT-PCR). First-strand cDNA was synthesized from zebrafish adult eye total RNA by reverse transcription using SuperScript III reverse transcriptase (Thermo Fisher Scientific) with an oligo $(dT)_{18}$ primer. Primer sets used for PCR to obtain full-length *ndrg1a* family genes are listed in Table 1.

The PCR was performed with Ex Taq (TaKaRa, Ohtsu, Japan) and the PCR products were cloned into pGEM-T-Easy vector (Promega) and amplified. The inserts were verified by DNA sequencing.

Generation of antisera

Partial peptides or a whole protein (Fig. 2a) was used to raise antisera for this study. To raise the antisera against zebrafish NDRG1a-1 and NDRG1a-2, their partial peptides,

Table 1. Primer sequence for cloning of zebrafish NDRG1 family proteins.

Primer	Sequence (5'-3')
ndrg1a-1, Forward	GCGCACACAAGAGCTTTACTT
ndrg1a-2, Forward	CACGCGTCCGGTAGTCAATTGTCTTC
ndrg1a-1 and ndrg1a-2, Reverse	CGATGTCTCTGTGCTGCATTGC
ndrg1b, Forward	GCGCCACACTGTAAACAAGAGG
ndrg1b, Reverse	CTCCCCCAGGCATTCATTCACA

Met1-Ala17 in NDRG1a-1 for anti-NDRG1a-1, Met1-Lys30 in NDRG1a-2 for anti-NDRG1a-2, the sole different position in their sequences were used. Besides, common C-terminal 54 amino acids in NDRG1a-1 and NDRG1a-2 for anti-NDRG1a# antiserum to detect both NDRG1a-1 and NDRG1a-2 were also prepared. To raise anti-NDRG1b antiserum, whole protein was used for NDRG1b. The corresponding positions are underlined in Fig. 2a.

I found polymorphism in NDRG1 family proteins in zebrafish (see Results), but amino acid sequences in the partial peptides utilized for generation of anti-NDRG1a-1 and anti-NDRG1a-2 antisera are common in all of the NDRG1a-1 and NDRG1a-2 variants. In the NDRG1b variants, 1 or scattered 6 amino acid substitutions out of 359 total amino acids were present in the variants compared with the amino acid sequence in the NDRG1b protein (NDRG1b_1) used for generation of anti-NDRG1b antiserum. I believe that these substitutions do not affect significantly the activity of anti-NDRG1b antiserum to recognize this protein.

A DNA sequence corresponding to each peptide was PCR-amplified with a primer set containing EcoRI site in the forward primer and SalI site in the reverse primer, subcloned into a pGEM-T-Easy vector and amplified in *E. coli* XL1 Blue strain. After the sequence was verified, the vector was cut with EcoRI and SalI. Then the insert was ligated to the corresponding sites of pGEX-5X-1 (GE Healthcare) to obtain an N-terminally Glutathione S-transferase (GST)-fused product.

To raise antiserum against NDRG1b protein, I prepared N-terminally GST-fused NDRG1b whole protein similarly as the above peptides for the expression of these peptides. In case when a DNA used for expression of a partial peptide or NDRG1b protein contains a restriction site for NcoI, XhoI or SalI, I replaced the corresponding triplet with that coding the same amino acid but consisting of a different set of a triplet (silent mutation) prior to the construction to obtain a proper product. Each plasmid was transformed into *E. coli* BL21 (DE3) strain for protein expression.

A suspension of *E. coli* expressing the GST-fused partial peptides or a whole protein was sonicated, and the supernatant was collected and purified according to the manufacturer's instruction. A peptide or a protein was mixed with Freund's Adjuvant (Sigma-Aldrich) and about 100-200 μg of each peptide or a protein was used to immunize ddY mice.

To examine specificity of each of the antisera, I obtained each of whole proteins of NDRG1 family proteins (LC093848.1 as NDRG1a-1, LC093852.1 as NDRG1a-2 and LC093857.1 as NDRG1b) that were fused with Maltose-binding protein (MBP) at their N-termini (abbreviated as MBP-NDRG1a-1, for example). For this, each of cDNA of

NDRG1 family proteins was ligated to EcoRI/SalI sites of pMAL-C2E (New England Biolabs). As a control, I also obtained MBP by introducing a stop codon immediate downstream of DDDDK, a linker for the protein to be expressed. All of the proteins were expressed in *E. coli* BL21 (DE3) strain and purified according to the manufacturer's protocol. These proteins were then subjected to size-fractionation and the largest bands were collected and stored at -80°C until use.

In the preliminary survey using crude anti-NDRG1a-1 antiserum and crude anti-NDRG1b antiserum, anti-NDRG1a-1 antiserum reacted to MBP-NDRG1b, and anti-NDRG1b antiserum reacted to recombinant NDRG1a-2. To obtain specific antiserum against NDRG1a-1, therefore, anti-NDRG1a-1 antiserum was adsorbed by MBP-NDRG1b and anti-NDRG1b antiserum was adsorbed by recombinant NDRG1a-2 to obtain specific antiserum against NDRG1b.

In the purification of anti-NDRG1b antiserum, we used NDRG1a-2 whole protein for adsorption. NDRG1a-2 was prepared by amplifying the cDNA by PCR containing NcoI and XhoI sites, inserted into the corresponding sites in pET-16b (Novagen). Subsequently this protein was expressed in *E. coli* BL21 (DE3) strain. After sonication and centrifugation (15,000 rpm, 4°C, 10 min) the supernatant was used for the source of NDRG1a-2. Both antisera were purified and used for the subsequent experiments. Anti-NDRG1a-2 antiserum specifically reacted to MBP-NDRG1a-2 and therefore, anti-NDRG1a-2 antiserum was used without purification.

Selectivity of each of antiserum used was examined with immunoblot as shown in Fig. 2b. In addition to this, the selectivity of anti-NDRG1a-1 antiserum and that of anti-NDRG1b antiserum were also confirmed immunohistochemically by checking the selective reduction of the corresponding protein expression level in fish injected with morpholino(s) against NDRG1a-1 and NDRG1b, respectively. Selectivity of NDRG1a-2 antiserum was also confirmed from the fact that it did not show the signal in wildtype rods (Fig. 3b and Figs 4c and d) but did show it in rods where NDRG1a-2 was forced to be expressed (Fig. 12d).

To examine the expression of NDRG1 family proteins in the zebrafish retina with immunoblot, typically ~20 sheets of adult zebrafish retinas were collected. The fish had been dark-adapted overnight, anesthetized and sacrificed in ice-chilled water in the dark. Then, both eyes were detached and retinas were enucleated under a stereomicroscope in dim light. The retinas were solubilized by homogenization in sample buffer (50 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [w/v] glycerol, 0.0025% bromophenol blue, 1.25% [v/v] β-mercaptoethanol), heated for 10 mins at 95°C, chilled on ice, snap-frozen and stored at -80°C until use. Immunoblot analysis was made as described previously[12].

Immunohistochemistry and immunocytochemistry

Expression of NDRG1 family proteins in the retina and their subcellular localization were examined by immunohistochemistry basically according to the method reported previously[13]. Light-adapted zebrafish adult eyes were fixed with a solution containing 4% paraformaldehyde in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.3) for an hour at 4°C. Then the solution was replaced sequentially with PBS containing 10% sucrose (10% sucrose/PBS), 20% sucrose/PBS for at least 2 hrs each and subsequently 30% sucrose/PBS overnight. Finally the tissues were immersed and embedded overnight in a mixture of OCT compound (Tissue tech) and 30% sucrose/PBS at a 1:1 ratio, and stored at -80°C until use. For larvae, eyes were prepared in the same way as adult eyes except that larval whole bodies at 48 hours postfertilization (hpf), 54 hpf, 60 hpf and 72 hpf were fixed for 13.5 hrs, while at 96 hpf and 6 days postfertilization (dpf) they were fixed for 18 hrs and 24 hrs, respectively.

The fixed eyes cryosectioned at 10 μm thickness were placed on a MAS- or FRONTIER-coated slide (Matsunami, Osaka, Japan) and dried for 2.5 hrs. After washing out the resin with PBS containing 0.5% Triton X-100 (PBST) three times (5 mins x 3), the sections were treated overnight at 4°C with a blocking reagent (5% normal goat serum in PBST). After the sections were washed with PBST three times, the samples were incubated overnight at 4°C with antiserum we prepared and when necessary, with commercially available antibody, in the blocking reagent.

In addition to the antisera raised as above, I used other primary antisera and all of the antibodies used in this study are as follows: specific anti-NDRG1a-1 antiserum (1:250 dilution), anti-NDRG1a-2 antiserum (1:200) and anti-NDRG1b antiserum (1:500); anti-Gt1α (1:500) and anti-Tom20 (1:500) antibodies both from Santa Cruz Biotechnology (sc-389 and sc-11415, respectively); anti-rhodopsin antiserum (1:2000), anti-red/green opsin antibody reacting to both red- and green-sensitive opsins (1:500), anti-blue opsin antiserum (1:500), anti-UV opsin antibody (1:300), a mixture of anti-cArr1 (cone arrestin 1) antiserum (1:200) and anti-cArr2 (cone arrestin 2) antiserum (1:200) all as described previously[13].To detect immunoreactivities, Alexa Fluor 488 anti-mouse, Alexa Fluor 488 anti-rabbit and Alexa Fluor 568 anti-mouse antibodies (Abcam) were used at a dilution of 1:500.

Expression of each NDRG1 protein in single isolated cells was examined basically according to the method reported previously[14]. Rods and cones isolated from the retina (see below) were attached to a glass slide by centrifugation (300 rpm, 2 min; Cytopro, ELITech). The cells were fixed with 100% methanol for 2.5 min at room temperature

and quickly treated for another 1 min with Ringer's solution (119.9 mM NaCl, 2.6 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM $MgSO_4$, 1 mM $NaHCO_3$, 16 mM glucose, 0.5 mM NaH_2PO_4, 4 mM HEPES, pH 7.5) containing 0.005% Triton X-100 and that containing 5% normal goat serum for permeabilization and blocking, respectively. Other procedures were the same as the immunostaining of the retina in the eye as stated above except that Ringer's solution was used instead of PBST.

Anti-rhodopsin antiserum, and anti-red and green (red/green) opsin antibody reacting to both red- and green-sensitive opsins[13] were used to identify the rod and the cone OS, respectively. When temporal cone opsin expression was examined (Fig. 6c), a mixture of anti-red/green-sensitive opsin antibody, anti-blue-sensitive opsin antiserum and anti-UV-sensitive opsin antibody[13] was used. In addition, to observe the cell morphology of a rod, anti-rod transducin antibody (sc-389; Santa Cruz Biotechnology) was used. To observe the cone morphology, a mixture of anti-cone arrestin 1 (cArr1) antiserum and anti-cone arrestin 2 (cArr2) antiserum[13] were used. Anti-Tom20 antibody (sc-11415; Santa Cruz Biotechnology) was used to identify mitochondria.

The eye sections or isolated cells were observed using an inverted confocal microscope (LSM 510 META, Zeiss) with plan neofluar 20x/NA 0.5 and C-APO 40x/NA 1.2 water immersion objective lenses.

Analysis of temporal expression patterns of NDRG1 family genes and proteins

For temporal analysis of the expression of mRNA of a gene, forty zebrafish larvae each were collected at ~12 hour intervals from 1 hpf to 96 hpf. Forty unfertilized eggs were also used. The samples were snap-frozen in liquid nitrogen and stored at -80°C until use. Frozen larvae were homogenized with 250 μL of TRI Reagent (Sigma-Aldrich) and total RNA was isolated according to the manufacture's instruction. A total of 1 μg of RNA was primed with an oligo $(dT)_{18}$ primer and reverse-transcribed with SuperScript III RTase (Thermo Fisher Scientific) or water as a negative control at 42°C for an hour and cDNAs were stored at -80°C until use. RT-PCR was performed using 30 ng of cDNA templates at 94°C for 30 sec, then at 58°C for 30 sec and finally at 72°C for 40 sec. The reactions were repeated for 35 cycles. Primers used for the RT-PCR were listed in Table 2.

Expression of a protein during development was quantified using spread and non-spread criterion (Fig. 6d). When a protein is expressed in a layer or layers throughout the retina continuously, the expression of that protein is regarded as "spread". When a protein is expressed in a small area in a layer or layers, or expressed intermittently with gaps horizontally, the expression was regarded as "non-spread".

Table 2. Primer sequence for spatio-temporal expression pattern.

Primer	Sequence (5'-3')
ndrg1a-2, Forward	TGAGGGAGGCTGTCCAGAAA
ndrg1a, Reverse	GGTGACGGTACCTGCCAATG
ndrg1b, Forward	CAGCACACCAGTCTTTTACAGCA
ndrg1b, Reverse	AGGACCATTGGTAGGCTCTCAGA
opn1lw1, Forward	GGGAAGCAATGTTCACATATACC
opn1lw1, Reverse	CATCTTTCCCAAGAGATGACAGT
gnat2, Forward	GAGGATAAGGAAATGGCCAAGAA
gnat2, Reverse	GAGGTTCTGGAGCTTTTGACTA
β-actin, Forward	CATCGGCAATGAGCGTTTCCGTT
β-actin, Reverse	GTTCGAGAGTTTAGGTTGGTCG

"Spread expression" was defined by the ratio of the number of retinas showing "spread" to the total number of retinas examined.

Morpholino-Mediated Knockdown of NDRG1a-1 and NDRG1b at Early Stages

One- to two-cell-stage zebrafish embryos were injected with 3 ng of morpholinos to knockdown NDRG1a-1 or NDRG1b. (Knockdown does not always mean that the expression of the corresponding gene is inhibited by 100 %. It is difficult to determine the degree of inhibition individually because the effects of morpholinos are transient and different individually.) Expression of NDRG1a-2 protein in adult photoreceptors seemed to be minimal (Fig. 4) and its expression in the retina was not detected at early developmental stages from 48 hpf to 6 dpf (data not shown), so that knockdown of NDRG1a-2 was not conducted in this study. Morpholinos were designed and synthesized by Gene Tools (Philomath, OR) and for NDRG1a-1, $MO_{1a\text{-}1}$ (5'-CCATATCGGAGTCTTCCAGAACCAT-3') was used to inhibit the translation. For NDRG1b, two types of morpholinos were used: MO_{1b}-T (5'-ATATCGTCCATTCTCAACGAGGACA-3') to inhibit translation and MO_{1b}-S (5'-TCCTCCTACAAAGGAAACATTATGG-3') to inhibit splicing of NDRG1b pre-mRNA. The peptide (aberrant NDRG1b protein) produced in the presence of MO_{1b}-S should be MVLEDSESSVFELDITIRAVLSQCFITKTCMRSCNTSLCAMLMPRDSRRVPARSPLTIPTLQWISFLRAYQWSSIILV, in which the amino acid residues underlined are mutated due to the inhibition of normal splicing. When a mixture of MO_{1b}-T and MO_{1b}-S was used, the amount of MO_{1b}-T and MO_{1b}-S were set to 1.5 ng each so that the total amount was unchanged in the morpholino study. A standard control oligo (3 ng, Gene tools) was used as a negative control.

Microinjection of morpholinos was performed using a glass micropipette made

from a glass capillary (G-100; Narishige, Tokyo, Japan) with a pipette puller (P-97 IVF; Sutter, Novato, CA), and by giving N_2 pressure with an electric microinjector (custom-designed IM-30; Narishige). Morpholino-injected larvae were raised in Raising Medium consisting of 0.03% REI-SEA marine salt (Iwaki, Tokyo, Japan) and 106.6 μg/L methylene blue. At 96 hpf, they were collected for phenotypic analysis.

Generation of transgenic zebrafish

Silent mutations were introduced by site-directed mutagenesis prior to construction of an expression vector if each gene had NcoI, XhoI, NotI and/or SalI restriction site in its coding sequence. To identify cones readily, mCherry was expressed in cones in the membrane-bound and prenylated form in some of the studies. The construct, mCherry-HrasCAAX, where CAAX is a prenylation sequence, was produced by the Tol2kit[15]. First, mCherry sequence was amplified from pmCherry-N1 (Clontech) by the primer sets in which HrasCAAX sequence was contained in the reverse primer and mCherry-HrasCAAX was subcloned into pGEM-T-Easy vector for amplification in bacteria. The insert was excised with NcoI and NotI, and ligated to the downstream of a vector containing a promotor region of cone specific transducin α subunit (TαCP)[16].

NDRG1 family proteins were overexpressed (NDRG1a-1) or ectopically expressed (NDRG1a-2 and NDRG1b) in zebrafish rods. As a control, zebrafish expressing unmodified mCherry in rods was produced. To establish these transgenic lines, coding sequence of each protein was amplified by PCR with the primer sets containing NcoI site in a forward primer and XhoI site in a reverse primer, and subcloned into pGEM-T-Easy vector to amplify. The inserts were then excised with NcoI and NotI, and ligated to the downstream next to the rhodopsin promoter in a pCR2.1-TOPO vector (Thermo Fisher Scientific)[17]. Based on a finding by Kwan *et al.*[15], I generated a construct of rhodopsin promoter-NDRG1 family protein (or mCherry in a control)-IRES-mSEGFP-KrasCAAX-6x SV40 late polyA, where IRES stands for slightly modified internal ribosome entry site (Clontech), and mSEGFP, monomeric Super Enhanced GFP[18]. The resultant vectors were introduced into *E. coli* DH5α (DE3) strain to amplify, and they were purified by QIAGEN Plasmid Midi Kit (QIAGEN). The plasmids were digested and linearized by SalI, purified by phenol-chloroform extraction, precipitated by ethanol, and dissolved in distilled water. The concentration of linearized plasmids was quantified spectrophotometrically and stored at -20º C until use.

The SalI-linearlized plasmid (20 ng/μL) in 2-3 nL of 0.15 M KCl solution containing 0.05% phenol red was injected into the one-cell stage of zebrafish egg.

Zebrafish were raised in Raising Medium supplemented with 200 μM 1-phenyl-2-thiourea (nacalai, Kyoto, Japan). At 3 dpf, larvae in which many proportions of rods are positive for mSEGFP were selected with a wide-field epifluorescence microscope (BX51, Olympus) and raised to adult. These adult F_0 fish were crossed with wildtype zebrafish and eggs were raised in Raising Medium containing 1-phenyl-2-thiourea, and those larvae (F_1) carrying the transgene heterologously (+/-) were selected at 3 dpf and further raised until adult. Transgenic zebrafish lines were maintained by selecting these heterologous fish after crossing them with wildtype zebrafish.

Dissociation of rods and cones

Adult zebrafish were dark-adapted overnight, anesthetized in ice-chilled water and sacrificed in the dark. Then, both eyes were detached and the retinas were enucleated under a stereomicroscope in dim light. Retinas were treated with Ringer's solution (119.9 mM NaCl, 2.6 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM $MgSO_4$, 1 mM $NaHCO_3$, 16 mM glucose, 0.5 mM NaH_2PO_4, 4 mM HEPES, pH 7.5) containing 1 mg/mL hyaluronidase[19] (Sigma-Aldrich) for 2.5 mins followed by gentle pipetting to obtain isolated rods and cones.

***N, N'*-Didansyl cystine staining**

To examine whether rods with tapered OS show the OS similar to that of a cone, rods and cones were tested with *N,N'*-didansyl cystine (DDC, Sigma-Aldrich) which has been shown to label cone OS consisting of infolded plasma membrane much more effectively than rod OS[20]. For this purpose, retinas were dissected and photoreceptors were isolated as described above and a few drops of a suspension of rods and cones were gently mixed with 100 μL of Ringer's solution containing 0.25% (w/v) agarose (nacalai) preincubated at 38°C. A portion of the mixture (~50 μL) was placed on a glass-bottom dish (Matsunami) and then the dish was placed on ice for about a minute to gel the mixture. Then 200 μL of 20 μM DDC dissolved in Ringer's solution was added to the gel for 5 mins at room temperature, and the gel was washed once with Ringer's solution. The dish was filled with Ringer's solution and observed under a confocal microscope (LSM 780, Zeiss) with C-APO 40x/NA 1.2 water immersion objective lens. DDC was excited at 405 nm and all the fluorescence longer than 410 nm was collected.

Filipin III staining

Freshly dissociated photoreceptors were stained for 5 min with 10 μg/mL of filipin III dissolved in Ringer's solution at room temperature, embedded in 0.25 % agarose and fluorescence was observed for both wildtype and NDRG1a-1 overexpressing or NDRG1b ectopically expressing rod OSs under confocal microscope (LSM 780, Zeiss) excited at 405 nm. All the fluorescence longer than 410 nm was collected.

Image analysis and statistical analysis

All of the acquired images in this study were analysed with ImageJ (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD). Signal intensity of each pixel in the image was converted to an 8-bit representation. Immunopositive area was surrounded with a line manually by eyes to determine the size of the area and also the total signal intensity in this area. To determine the concentration of the pigment, the average intensity of an opsin signal was obtained with dividing the total signal intensity by corresponding immunopositive area. Data are presented as mean ± standard deviation, and levels of significance (P value, Student's t-test) are indicated in the figure legends.

Results

Identification of Three NDRG1 Family Proteins in Zebrafish

In the previous study in carp[3], a partial cDNA sequence (*ndrg1l*) possibly encoding NDRG1b protein was found as a gene specifically expressed in cones. To ascertain that putative carp *ndrg1l* is really a carp ortholog of zebrafish *ndrg1b*, full-length cDNA clones containing the partial sequence found previously were isolated from a carp retinal cDNA library and verified by DNA sequencing (DDBJ accession number, LC102483.1).

The deduced amino acid sequence identity is 88 % between carp NDRG1L and zebrafish NDRG1b (*Dr*NDRG1b) (Fig. 1a). Phylogenetic tree was constructed using determined carp NDRG1L amino acid sequence and those of other NDRG family proteins in the database including NDRG1, NDRG2, NDRG3 and NDRG4 in other animals (Fig. 1b). All of these results supported the notion that carp NDRG1L (*Cc*NDRG1L, hereafter I call it *Cc*NDRG1b) was the ortholog of *Dr*NDRG1b, which in turn suggested that *Dr*NDRG1b is also specifically expressed in cones.

Therefore, I switched the experimental animal from carp to zebrafish, because genetic study is more practical in zebrafish than in carp. In zebrafish, two splice variants are known to be present for *ndrg1a* gene: *ndrg1a-1* and *ndrg1a-2.* They are different only at their N-terminal amino acid sequences, Met1 - Ala17 in NDRG1a-1 and Met1-Lys30 in NDRG1a-2 (Fig. 2a, underlined in magenta and blue, respectively). I cloned all of these three genes, *ndrg1a-1*, *ndrg1a-2* and *ndrg1b* in zebrafish.

a

	*Cc*NDRG1b	*Dr*NDRG1a-1	*Dr*NDRG1a-2
*Dr*NDRG1a-1	66.1 (78.1)	—	—
*Dr*NDRG1a-2	62.4 (74.4)	93.6 (95.2)	—
*Dr*NDRG1b	87.6 (92.3)	65.7 (78.1)	62.0 (74.2)

Dr, zebrafish; *Cc*, carp.

b

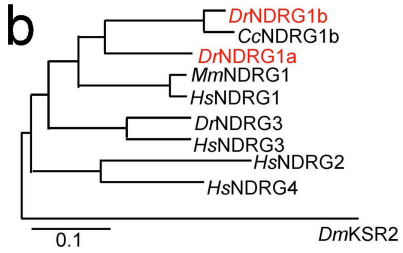

Figure 1. Carp and zebrafish NDRG1 family proteins. (**a**) Amino acid identities (similarities in parentheses) among carp NDRG1b (*Cc*NDRG1b, LC102483.1), zebrafish (*Dr*) NDRG1a-1 (*Dr*NDRG1a-1, NP_001121825.1), *Dr*NDRG1a-2 (NP_998513.2), and *Dr*NDRG1b (NP_956986.1). (**b**) Phylogenetic tree of NDRG family proteins. *Dr*, zebrafish; *Cc*, carp; *Mm*, mouse; *Hs*, human; *Dm*, fruit fly. Phylogenetic tree was obtained by Neighbor-joining method using the full-length of the identified carp NDRG1b protein sequence (LC102483.1) and NDRG protein sequences in zebrafish (*Dr*NDRG1a-1, NP_001121825.1; *Dr*NDRG1b, NP_956986; *Dr*NDRG3a, NP_955811.1), in human (*Hs*NDRG1 isoform1, NP_006087.2; *Hs*NDRG2 isoform a, NP_963293.1; *Hs*NDRG3 isoform a, NP_114402.1; *Hs*NDRG4 isoform1, NP_075061) and in mouse (*Mm*NDRG1, NP_032707.2).

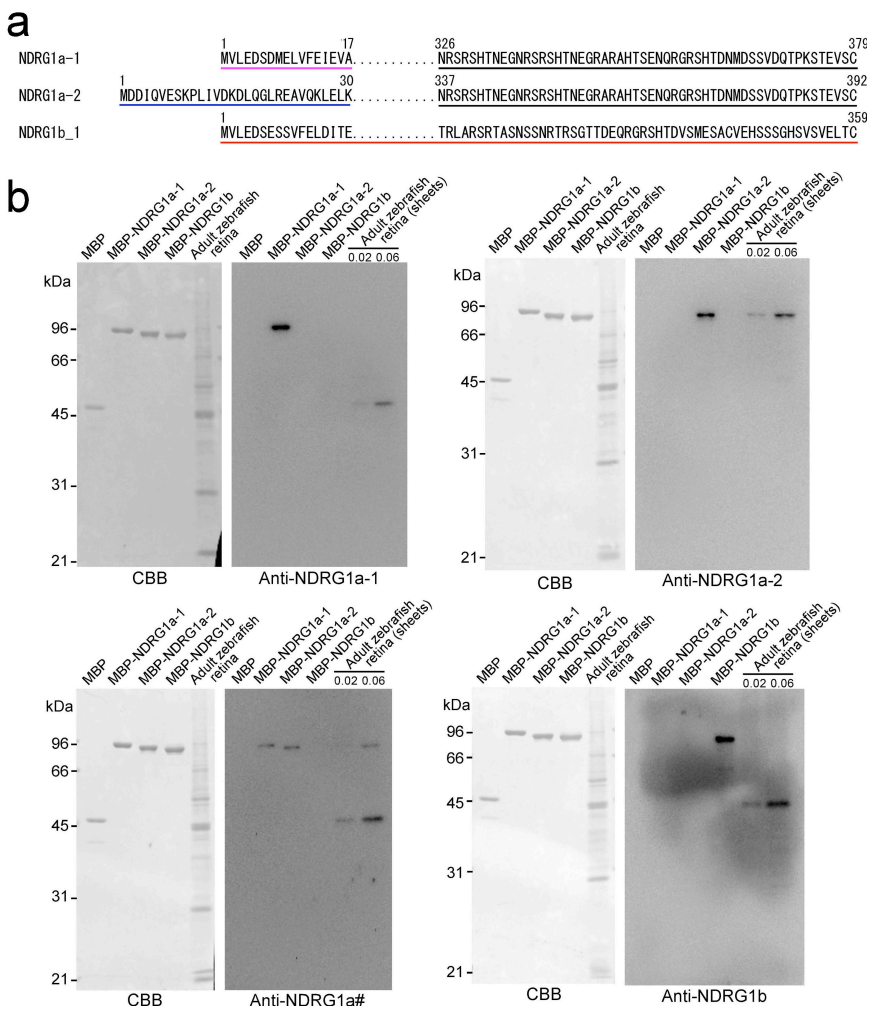

Figure 2. Specificity of antiserum raised against NDRG1a-1, NDRG1a-2 or NDRG1b. (**a**) Amino acid sequence used for raising each antiserum (underlined with different colors). There were several polymorphic variants in each of the zebrafish proteins (see text), but for NDRG1a-1 and NDRG1a-2, the amino acid sequences in the regions shown were the same in all of these variants. For NDRG1b, the sequence of one representative (NDRG1b_1) is shown. Note that the N-terminal sequences of several amino acids are similar between NDRG1a-1 and NDRG1b_1 while it is different in NDRG1a-2, and that the rest of the sequence other than Met1-Ala17 in NDRG1a-1 and Met1-Lys30 in NDRG1a-2 is the same between NDRG1a-1 and NDRG1a-2. (**b**) Specificity of each purified antiserum against NDRG1a-1 (upper left), NDRG1a-2 (upper right), both NDRG1a-1 and NDRG1a-2 (lower left), and NDRG1b (lower right). In each pair of the panels, Coomassie Brilliant Blue (*CBB*)-staining (left) and immunoblot staining (right) are shown. Proteins used for detecting specificity were all N-terminally MBP-fused NDRG1 family proteins (see Materials and Methods). Each NDRG1 family protein was also detected in adult zebrafish retina. The amount of the retina probed is indicated as portions of a retina (*sheets*). In these sheets, single bands corresponding to the calculated molecular masses were detected for NDRG1a-1 (upper left) and NDRG1b (lower right). Anti-NDRG1a-2 recognized a single band of ~90 kDa (upper right), which was also detected with anti-NDRG1a# (lower left). The results suggest that the ~90 kDa band is a homodimer of NDRG1a-2.

There were many single nucleotide polymorphisms in these homologs (LC093846 - LC093851 for *ndrg1a-1*; LC093852 - LC093855 for *ndrg1a-2*; LC093856, LC093857 and LC102484 for *ndrg1b*). As the representative of each NDRG1 family protein for functional analysis hereafter, I selected the variant that shows minimum amino acid substitutions compared with the amino acid sequence of an NDRG1 family protein in the database (NP_001121825.1 for *Dr*NDRG1a-1, NP_998513.2 for *Dr*NDRG1a-2 and NP_956986.1 for *Dr*NDRG1b). The representatives in this study were NDRG1a-1_1 (LC093848.1), NDRG1a-2_1 (LC093852.1) and NDRG1b_1 (LC093857.1). As for NDRG1a-1_1 and NDRG1a-2_1, amino acid sequences were the same as those in the database. There were 4 amino acid substitutions in NDRG1b_1: Cys was substituted for Arg (at position 39), Gly for Ser (142), Leu for Met (148) and Ser for Asn (200). Compared with these representatives, in 6 NDRG1a-1 variants, 1 or 2 amino acid substitutions were found in 3 variants, while in all of 4 NDRG1a-2 variants, amino acid sequences were the same. In 3 NDRG1b variants, one variant showed one amino acid substitution, and the other showed 6 substitutions but the substitutions were scattered.

Generation of Antibodies

To determine their localization in photoreceptors, anti-NDRG1a-1, anti-NDRG1a-2 antisera which target the non-overlapping N-termini of NDRG1a proteins, and anti-NDRG1a# antiserum targeting their common C-terminus were raised for NDRG1a. NDRG1b whole protein was used to generate anti-NDRG1b antiserum (Fig. 2a). Specificity of these antisera was proven by immunoblot showing that only corresponding MBP-tagged NDRG1 family protein is recognized by the antisera (Fig. 2b). In zebrafish retinas, single band of ~47kDa, which corresponds to NDRG1a-1, was detected with anti-NDRG1a-1 antiserum (Fig. 2b). Anti-NDRG1a-2 antiserum recognized a single band of ~90kDa. Anti-NDRG1a# antiserum detected two bands corresponding to ~47kDa and ~90kDa. These results suggest that NDRG1a-2 forms the homodimer of ~90 kDa. NDRG1b antiserum recognized single band of about 43kDa. Detected bands for both NDRG1a-1 and NDRG1b were slightly larger than the calculated molecular weights, which is the characteristic for NDRG1 protein[21, 22].

Subcellular Localization of NDRG1 Family Proteins in Adult Zebrafish Rods and Cones

Localization of each protein was immunoprobed in adult zebrafish eye (Fig. 3) and at the photoreceptor layer (Fig. 4). The results revealed that NDRG1a-1 is expressed specifically in the photoreceptor cell layer (Fig. 3a), and at the photoreceptor layer, it is

expressed in rods plus all four types of cones (green signals in Figs. 4a and 4b; positions of the ellipsoid and the nucleus are indicated by vertical red and blue bars, respectively, in rods and all types of cones in Fig. 4a). In rods, entire cell membranes other than those of OS seem to be immunopositive (green signals in Fig. 4a and in upper panels in Fig. 4b; see also green signal surrounding red mitochondrial signal indicated by an arrowhead in Fig. 4a). At the basal part of rod OS, immunopositive thin processes are extended from the inner segment to the OS (Fig. 4b, upper panels), and they could be the calycal processes. Staining of each rod with anti-NDRG1a-1 antiserum may not be obvious in Fig. 4a and in Fig. 3a, but immunocytochemistry for isolated rods similar to that shown in the right-most panel in Fig. 4b revealed that 100% of rods were immunopositive for NDRG1a-1 in 100 rods examined. Although the reactivity was relatively difficult to detect at the nuclear region in rods in Fig. 4a, staining with antiserum against the C-terminal region NDRG1a-1 (anti-NDRG1a# antiserum) gave

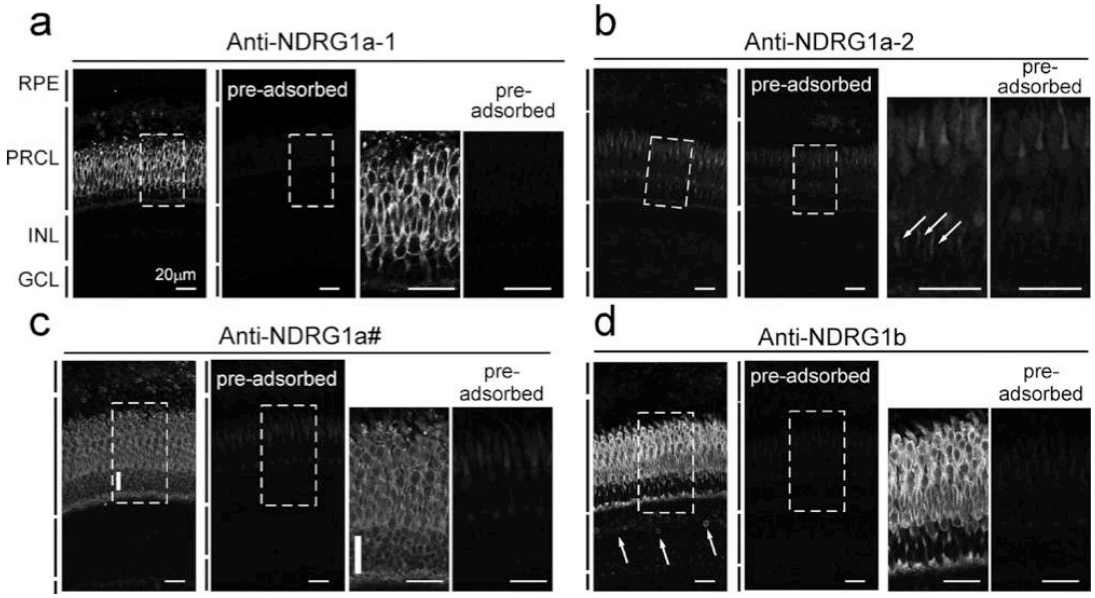

Figure 3. Localization of NDRG1a-1, NDRG1a-2 and NDRG1b proteins in adult zebrafish retina. Retinal sections were immunoprobed with anti-NDRG1a-1, anti-NDRG1a-2, anti-NDRG1a#, or anti-NDRG1b antiserum (leftmost panel in each of **a** - **d**). In (**a**) - (**d**), control measurement was made with each antiserum pre-adsorbed by each corresponding NDRG1a family protein (second left panels in **a** - **d**). The area surrounded by a dotted rectangular was magnified and is shown in the right two panels in (**a**) - (**d**). Arrows in (**b**) indicate the thin cone process detected by anti-NDRG1a-2 antiserum. Thick vertical white bars in (**c**) indicate the immunostaining of NDRG1a-1 in the plasma membranes surrounding the rod nucleus (see text). Arrows in (**d**) indicate NDRG1b positive cells in the inner nuclear layer. *RPE*, retinal pigment epithelium; *PRCL*, photoreceptor layer; *INL*, inner nuclear layer; *GCL*, ganglion cell layer. Scale bars indicate 20 μm throughout this figure.

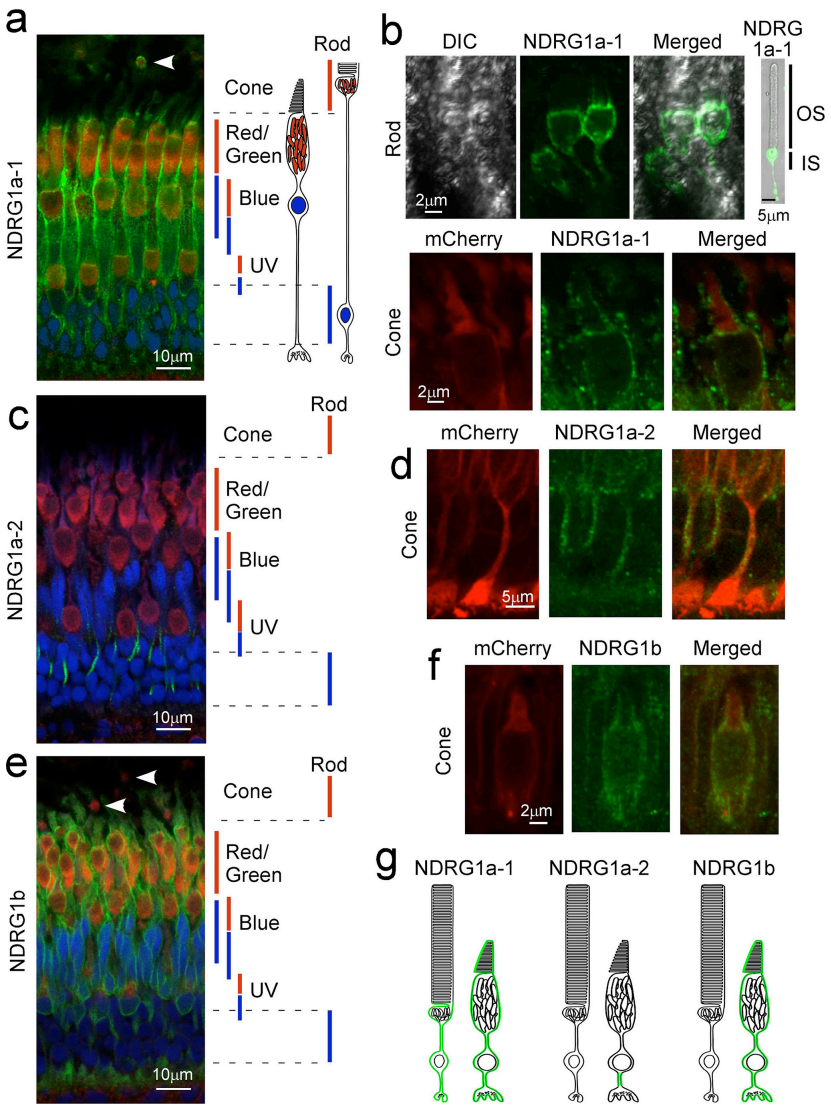

Figure 4. Subcellular localization of NDRG1 family proteins in zebrafish photoreceptors. Adult zebrafish retinas were immunostained with specific anti-NDRG1a-1 (**a** and **b)**, anti-NDRG1a-2 (**c** and **d**) or anti-NDRG1b (**e** and **f**) antiserum (all with green signals). Mitochondria and nucleus were counterstained with anti-Tom20 antibody (red signals) and Hoechst 33342 (blue signals), respectively (**a**, **c** and **e**). In **a**, **c** and **e**, approximate positions of ellipsoid containing mitochondria (red bars) and nucleus (blue bars) for each cone type (red/green-, blue- and UV-sensitive cones) and for rods are indicated. NDRG1a-1 was found to be expressed in the ellipsoid but not in the OS in rods (*arrowhead* in **a,** and upper panels in **b**). In cones, NDRG1a-1 was found both in the OS and ellipsoid (**a** and lower panels in **b**). NDRG1a-2 was found in the thin process of a cone (**c** and **d**). NDRG1b was found in the entire region of a cone (**e** and **f**) but not in rod ellipsoid (*arrowheads* in **e**). In **a**, **c** and **e**, wildtype zebrafish retinas were used, and in the fine study in **b**, **d** and **f**, the retinas consisting of cones expressing mCherry-HrasCAAX were used to readily identify cones. (**g**) Schematic representation of localization of NDRG1 family proteins in adult zebrafish photoreceptors (shown in green). DIC, differential interference contrast image.

clear immunopositive signals at the rod nuclear region (vertical white bars in Fig. 3c). (Anti-NDRG1a# antiserum also recognizes NDRG1a-2, but localization of NDRG1a-2 is different from that of NDRG1a-1 and NDRG1a-2 is not expressed in rods, see below). In cones, the entire cell membranes (green signals in Fig. 4a) including those of OS seem to be NDRG1a-1 immunopositive (lower panels in Fig. 4b). Immunopositive signals were observed at the basal part of cone OS, which could be due to the staining of the calycal processes. In addition, positive signals were also seen at the apical part of the OS, and it appears that NDRG1a-1 is expressed in the whole OS. NDRG1a-1 immunopositive signals seem to be stronger in cones than in rods when signal intensities were compared at the rod nuclear layer (vertical white bars in Fig. 3c) and at the cone nuclear layer (the layer above the vertical white bars in Fig. 3c).

NDRG1a-2 is expressed only in cones and localized to the thin process of cones (green signals in Figs. 4c and 4d), although the signal itself (arrows in Fig. 3b) is not so strong compared with the background. NDRG1b is expressed only in cones and in all four types of cones (green signals in Figs. 4e and 4f), but not in the rod ellipsoid region (arrowheads in Fig. 4e) or rod nuclear region (Fig. 3d) in good agreement with the previous study in carp[3] in our lab. In addition, some cells in the inner nuclear layer were also NDRG1b immunopositive (arrows in Fig. 3d). Fig. 4g shows the schematic representation of localization of each NDRG1 family protein in rods and cones in zebrafish. None of NDRG1 family proteins is localized in rod OS.

Expression of NDRG1 family mRNAs and proteins at early developmental stages

Temporal expression patterns of *ndrg1a-1*, *ndrg1a-2* and *ndrg1b* mRNAs at early developmental stages were examined using total RNAs derived from whole body (Fig. 5). *De novo* mRNA expression of *ndrg1a-1* gene and that of *ndrg1b* gene were greatly increased around 48 hpf, which is correlated well with the expression of mRNAs of red-sensitive cone opsin (*opn1lw1*) and cone transducin α-subunit (*gnat2*), markers of the development of cone photoreceptors, with one notable exception that *ndrg1b* is also expressed in unfertilized eggs. The reason for the quick disappearance of *ndrg1b* just after fertilization was not known. On the other hand, expression pattern of *ndrg1a-2* remarkably differed from the other two genes. Namely, comparable level of *ndrg1a-2* expression was observed throughout the stages examined and also in unfertilized eggs. In a previous study, *ndrg1a* (*nbdrg1a-1* and/or *ndrg1a-2*, see above) mRNA expression was observed throughout the early developmental stages (10 hpf - 60 hpf) in many tissues[11]. My result, therefore, suggests that *ndrg1a* mRNA detected in the previous study would be mainly *ndrg1a-2* mRNA.

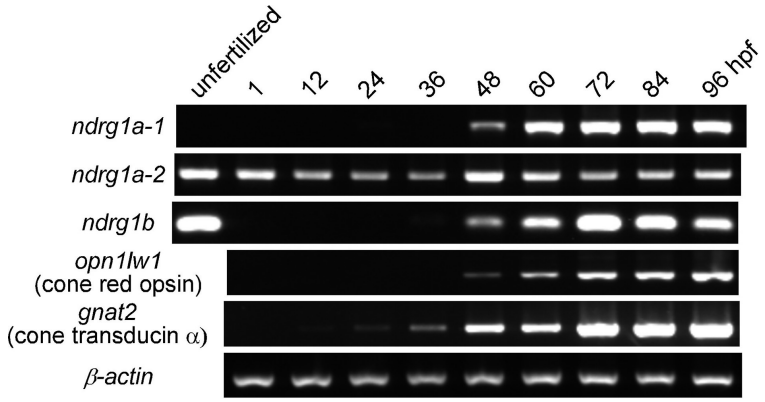

Figure 5. Temporal mRNA expression patterns of NDRG1 family proteins at early developmental stages. Each result shown is from one of two independent studies that gave similar expression patterns. *hpf*, hours postfertilization; *opn1lw1*, red-sensitive cone opsin; *gnat2*, cone transducin α-subunit.

Spatiotemporal expression patterns of these three NDRG1 family proteins in a developing retina were examined using specific antisera and they were different among the proteins (Fig. 6). Expression of NDRG1a-1 was restricted to the photoreceptor layer (PRCL) at any developmental stages examined (Fig. 6a). It was first detected around 48 hpf in a ventronasal patch (VN in Fig. 6a, left) and then throughout the PRCL at later stages, 72 hpf (3 dpf) and 6dpf (Fig. 6a, middle and right, respectively). Expression of NDRG1b was detected in PRCL around 48 hpf similar to NDRG1a-1, however, at this stage, relatively strong NDRG1b expression was also observed in inner nuclear layer (INL). (Fig. 6b, left). At later developmental stages, expression region of NDRG1b became restricted mainly in PRCL (Fig. 6b, right) but weak expression was also observed in INL at adult stage (Fig. 3d). Detectable level of NDRG1a-2 protein expression was not observed in the retina at any early developmental stages examined.

Temporal expression pattern of NDRG1 family proteins was further compared with that of total cone opsins including red/green-, blue- and UV-sensitive pigments. Based on a spread/non-spread criterion (Fig. 6c, see Materials and Methods), it was found that the onset of expression of NDRG1a and NDRG1b was earlier than that of cone opsins (Fig. 6d).

Effects of Knockdown of NDRG1a-1 or NDRG1b on Photoreceptor Formation at Early Developmental Stages

In adult zebrafish, NDRG1 family proteins are mainly localized to photoreceptors (Figs. 3 and 4). In the course of the studies on possible roles of NDRG1 family proteins in photoreceptors, I first knocked down these family proteins with morpholinos to

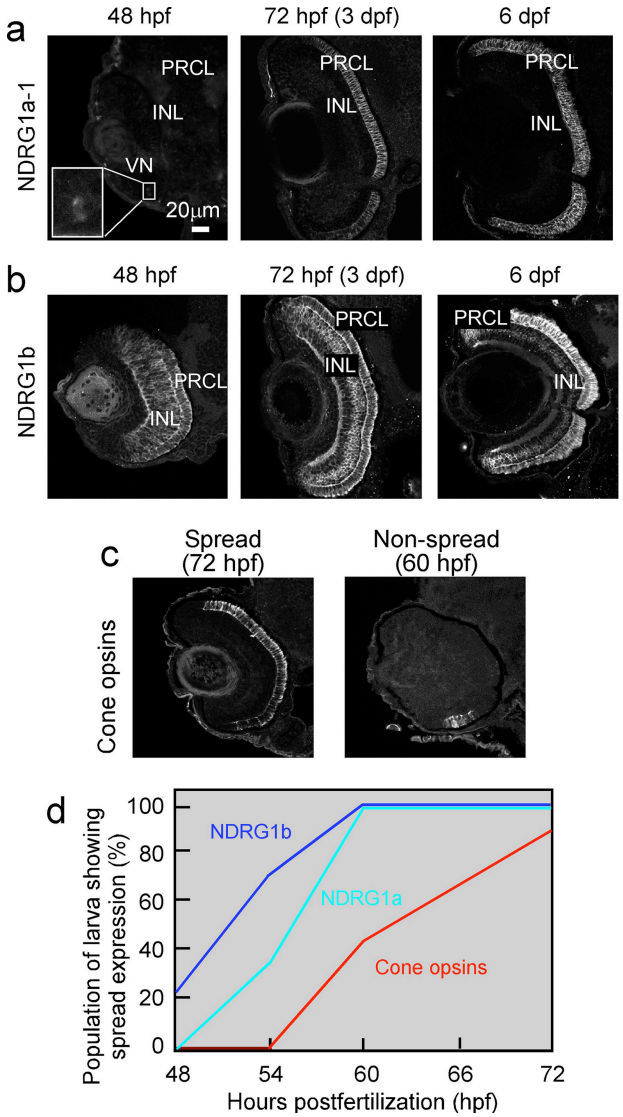

Figure 6. Spatiotemporal expression patterns of NDRG1a-1 and NDRG1b proteins. NDRG1a-1 (**a**) and NDRG1b (**b**) were immunostained with the corresponding specific antisera at the three developmental stages indicated. In (**a**) at 48 hpf, NDRG1a-1 was detected only in the region of ventronasal patch (*VN*), and subsequently it was detected in the photoreceptor layer (*PRCL*) but not in the inner nuclear layer (*INL*). In (**b**), NDRG1b was detected in both the PRCL and INL at 48 and 72 hpf, and its distribution was enriched to PRCL at 6 days postfertilization (*dpf*). The expression level of each protein at indicated time was estimated with spread/non-spread criterion (**c**, see Materials and Methods), and the result is shown together with that obtained for total cone opsins (**d**). In (**d**), data point is the percentage of the number of the retinas showing "spread" image in the total number of the retinas examined (n = 8-10) at each time point (48, 54, 60 and 72 hpf). Magnifications are the same in (**a**) - (**c**) (scale bar, 20 μm).

examine possible roles of these proteins in photoreceptors because of their convenient usage. As stated above, NDRG1a-2 was not detected immunohistochemically during

early developmental stages, the period in which morpholinos are effective to knockdown the genes. For this reason, I focused on *ndrg1a-1* and *ndrg1b* genes in the knockdown studies here.

A morpholino against NDRG1a-1 protein targeting the translation start site was designed ($MO_{1a\text{-}1}$, see Materials and Methods). Although the nucleotide sequence of this site of *ndrg1a-1* is 76 % identical to that of *ndrg1b* (19 nucleotides out of 25 nucleotides, Fig. 7), this morpholino specifically inhibited expression of NDRG1a-1 (compare Figs. 7b and 7c). At larval stages, knockdown of *ndrg1a-1*, which is expressed in both rods and cones (Fig. 4), caused reduction of rhodopsin and red/green-sensitive opsin signals (Figs. 8a, 8b, respectively), and therefore, proper photoreceptor OS formation was not seen at 96 hpf. However, macroscopic structure of the photoreceptor layer was not affected significantly ($MO_{1a\text{-}1}$ and Control, respectively, in Figs. 8b and 8c). Rod OS area was determined from rhodopsin-immunopositive area and the number of rod cells was counted by the number of rod transducin (Gt1α)-immunopositive cells (Fig. 8a). The rod OS area per rod was remarkably

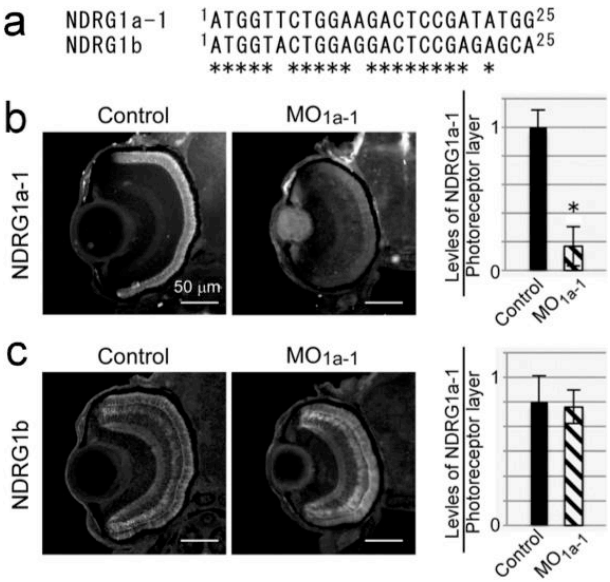

Figure 7. Reduction of NDRG1a-1 protein levels at 96 hpf caused by $MO_{1a\text{-}1}$. (**a**) Similarity of nucleotide sequences at the translation initiation site of NDRG1a-1 and that of NDRG1b. (**b**) NDRG1a-1 was detected with purified anti-NDRG1a-1 antiserum in the fish injected with control morpholino (*Control*) and that injected with $MO_{1a\text{-}1}$ ($MO_{1a\text{-}1}$). Immunofluorescent signals in the photoreceptor layer were quantified to estimate the expression levels of NDRG1a-1 (right bar graph, n = 11 for both *Control* and $MO_{1a\text{-}1}$-injected fish; *P < 0.000005). (**b**) NDRG1b was detected with anti-NDRG1b antiserum in the *Control* and $MO_{1a\text{-}1}$-injected fish. Immunofluorescent signals were quantified to estimate the expression levels of NDRG1b (right bar graph, n = 11 for *Control* fish and n = 12 for $MO_{1a\text{-}1}$-injected fish). Scale bar is 50 μm throughout this figure.

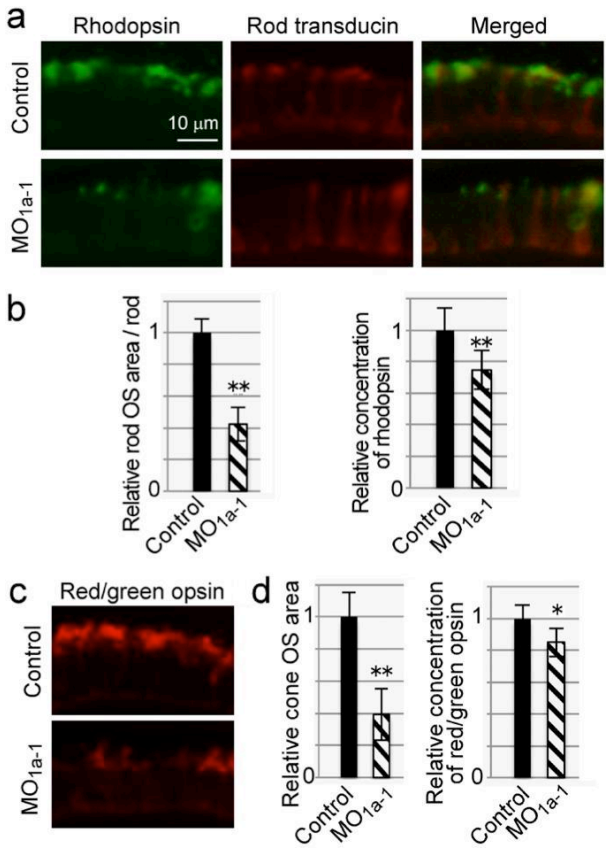

Figure 8. Effects of NDRG1a-1 knockdown with a morpholino against *ndrg1a-1* (MO_{1a-1}). (**a**) At 96 hpf, effects of MO_{1a-1} on photoreceptors were examined. In the retinas of control morpholino-injected (*Control*) and MO_{1a-1}-injected fish (*MO_{1a-1}*), expression of rhodopsin and rod transducin were immunodetected. (**b**) From studies as shown in (**a**), rod OS area was determined from rhodopsin-immunopositive region (left panels in **a**) and the number of rods was determined from rod transducin-immunopositive cells (middle panels in **a**). From these determination, relative rod OS area per rod (left) and relative rhodopsin concentration (right) were estimated for the retinas of control and MO_{1a-1}-injected fish (n = 11 for both *Control* and *MO_{1a-1}*). (**c**) In the retina from MO_{1a-1}-injected fish, red/green-sensitive opsin was immunodetected. (**d**) Relative cone OS area (left) and relative concentration of red/green-sensitive opsin (right) were estimated in the retinas of control and MO_{1a-1}-injected fish as in (**b**) (n = 11 for *Control* and n = 9 for *MO_{1a-1}*). Magnifications are the same in (**a**) and (**c**) (scale bar, 10 μm). *P < 0.005, **P < 0.0000005.

reduced in the knockdown fish (Fig. 8b, left). Similarly, rod transducin signals were also reduced in the OS (compare middle two panels in Fig. 8a). When the signal intensity of rhodopsin was divided by the signal-positive area to estimate the concentration of rhodopsin, it was slightly reduced in the knockdown fish but not so much different between MO_{1a-1}-injected and control morpholino-injected fish (Fig. 8b, right). This result indicated that at the photoreceptor layer, MO_{1a-1} reduced the rod OS volume without significantly affecting the rhodopsin concentration in the OS. For cones

(Fig. 8c), similarly immunopositive areas for red/green opsin were reduced (Fig. 8d, left), and the concentration of red/green-sensitive opsin was slightly reduced in the knockdown fish but its level was not so much different from that in the control (Fig. 8d, right), which indicated that the total cone OS volume was reduced but the concentration of red/green cone opsin was not affected. In both types of photoreceptors, therefore, inhibition of expression of NDRG1a-1 protein largely affected the OS volume and slightly, if any, the opsin concentration in the OS.

Likewise, the effects of knockdown of *ndrg1b*, which is expressed only in cones, were examined at 96 hpf using two morpholinos aiming to block translation of the protein (MO_{1b}-T) and/or pre-mRNA splicing (MO_{1b}-S). Knockdown of *ndrg1b* reduced the expression of NDRG1b protein (Fig. 9), and led to the inhibition of cone OS formation (Fig. 10): cone OS area estimated from red/green-sensitive opsin signals was smaller in MO_{1b}-T-, MO_{1b}-S- and a mixture of MO_{1b}-T and MO_{1b}-S-injected fish than that of control fish (left panels in Fig. 10a, and left in Fig. 10b). However, red/green opsin concentration (middle in Fig. 10b) or expression of other proteins such as cArr1 plus cArr2 (middle panels in Fig. 10a, and right in Fig. 10b) was not affected significantly. The effects of NDRG1b morpholino on expression of red/green-sensitive

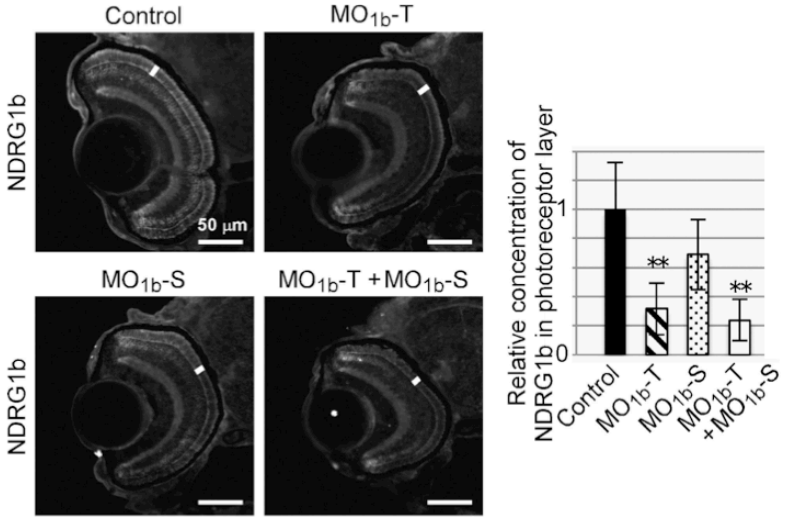

Figure 9. Reduction of NDRG1b protein levels with injection of MO_{1b}-T and/or MO_{1b}-S at 96 hpf. NDRG1b was immunodetected in fish injected with control morpholino (*Control*), MO_{1b}-T (MO_{1b}-T), MO_{1b}-S (MO_{1b}-S) or MO_{1b}-T plus MO_{1b}-S (MO_{1b}-T+MO_{1b}-S). Relative concentration of NDRG1b obtained after dividing the total signal by the area of the photoreceptor layer (thick white bar in each of the four fluorescent images) was determined and is shown in the bar graph (n = 5 for *Control*, n = 9 for both *MO_{1b}-T* and *MO_{1b}-S*, and n = 3 for *MO_{1b}-T+MO_{1b}-S*). In the case of MO1b-S, a mutated peptide of which N-terminal several amino acid sequence is the same as that of the wildtype NDRG1b should be expressed in cones, which would account for why the detected NDRG1b level was relatively higher in the MO_{1b}-S-injected fish. Scale bar is 50 μm throughout this figure. **P < 0.01.

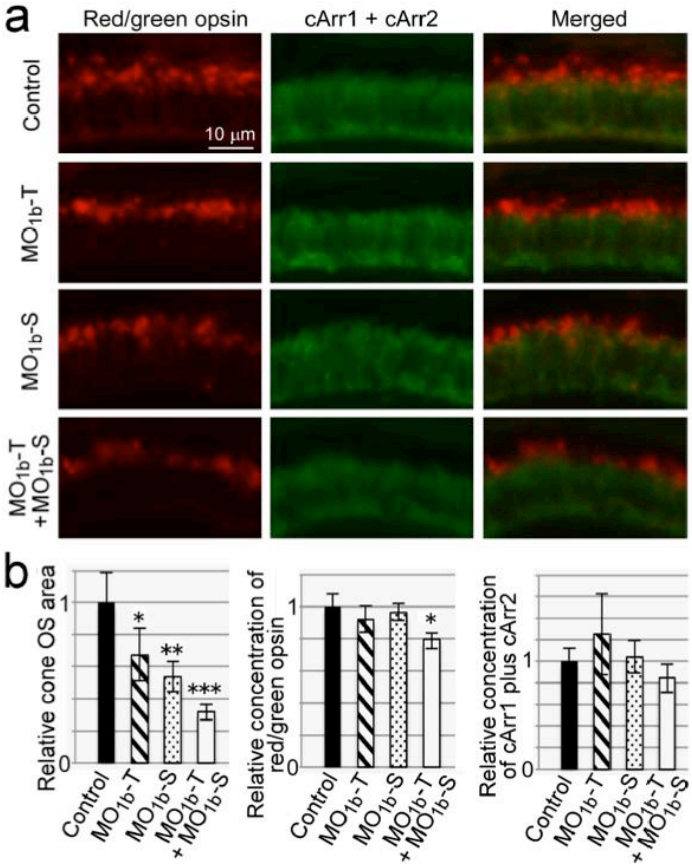

Figure 10. Effects of NDRG1b knockdown with morpholinos. (**a**) At 96 hpf, effects of morpholinos against NDRG1b (NDRG1b morpholinos) on cone photoreceptors were examined. In the retinas of control morpholino-injected (*Control*) and each of the NDRG1b morpholino-injected fish (*MO1b-T*, *MO1b-S* and *MO1b-T*+*MO1b-S*), expression of red/green-sensitive opsin and that of cArr1 plus cArr2 were immunodetected. (**b**) From studies as shown in (**a**), relative cone OS area (left) and relative concentrations of red/green-sensitive opsin (middle) and cArr1 plus cArr2 (right) were estimated for the retinas of control and NDRG1b morpholino-injected fish (n = 5 for *Control*, n = 9 for *MO1b-T*, n = 9 for *MO1b-S* and n = 3 for *MO1b-T*+*MO1b-S*. Magnifications are the same in (**a**) (scale bar, 10 μm). *P < 0.05, **P < 0.001, ***P < 0.0005.

opsin were very similar to those observed in the $MO_{1a\text{-}1}$-injected fish.

Although *ndrg1b* is specifically expressed in cones, reduction of NDRG1b showed effects on rods. The two NDRG1b morpholinos individually and additively reduced both the rod OS volume per rod and rhodopsin concentration slightly (Fig. 11). It would be due to the altered retinogenesis caused by altered cone photoreceptor development, which begins prior to the rod development[23].

Effects of forced-expression of NDRG1 family proteins in rods

In the study with morpholinos, however, potentially there could be off-target

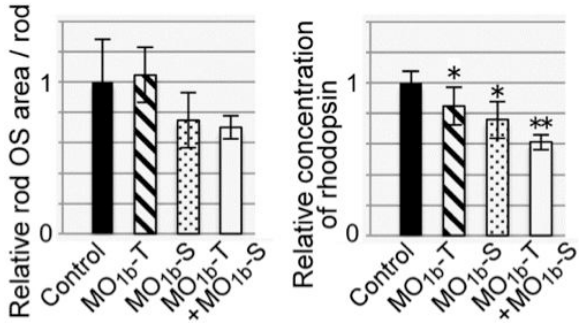

Figure 11. Effects of NDRG1b morpholinos on rods. Rod OS area per rod (left) and relative rhodopsin concentration (right) were determined in separate studies under similar conditions as in (A), but with use of anti-rhodopsin antiserum and anti-Gt1α antibody as in Figs. 10a and b (n = 4 for *Control*, n = 9 for MO_{1b}-T, n = 9 for MO_{1b}-S, and n = 3 for *MO1b-T+MO1b-S*). *P < 0.05, **P < 0.01.

effects[24]. Therefore, to understand the phenotype of the genetically modified animals more straightforwardly, NDRG1 family proteins were overexpressed (NDRG1a-1) or ectopically expressed (NDRG1a-2 and NDRG1b) in rods under the control of rhodopsion promoter, and morphological and immunohistochemical analyses were made at adult stages in the transgenic strains exogenously and stably expressing one of each protein to uncover phenotypes in rods. In the followings, I abbreviate the transgenic fish, NDRG1a-1-overexpressing fish as NDRG1a-1-Oe, NDRG1a-2-ectopically-expressing fish as NDRG1a-2-Ee and NDRG1b-ectopically-expressing fish as NDRG1b-Ee.

Fig. 12a shows immunohistochemical studies carried on rod transducin (Gt1α) in transgenic fish. One noticeable point in these studies was that the thickness of the photoreceptor layer, from rod OS to the outer limiting membrane (OLM), was thinner in these transgenic fish than in the wildtype (Fig. 12a). The retinal thickness other than that of the photoreceptor layer and the eye size were not significantly affected in these transgenic fish. The effect was much larger in NDRG1a-1-Oe and NDRG1b-Ee than in NDRG1a-2-Ee. Similarly, rod transducin signal was much reduced in NDRG1a-1-Oe and NDRG1b-Ee.

The above observations prompted me to observe rod OS directly in these transgenic fish by isolating transgenic rods because NDRG1 family proteins are localized to cone OS but not to rod OS (Fig. 4) and in *Xenopus* retina, it has been shown that at early stages of the development, rod OS appears cone-like shape[25]. In control adult zebrafish expressing mCherry, most rods had a cylindrical normal OS (Fig. 12b, left) and sometimes rods having tapered OS were observed (Fig. 12b, right) at a very low population (<2 %, Fig. 12c, left). In NDRG1a-1-Oe and NDRG1b-Ee, this population increased dramatically (30-40 % of total rods), while it did not change

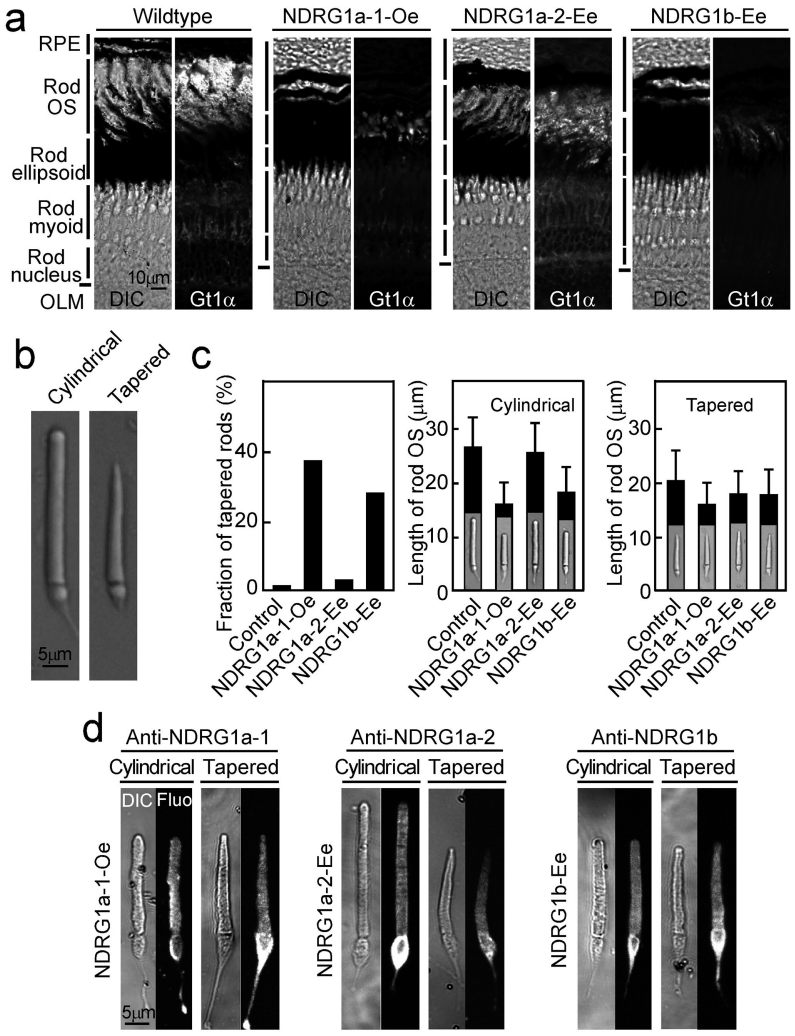

Figure 12. Effects of overexpression of NDRG1a-1, ectopic expression of NDRG1a-2 or NDRG1b in adult zebrafish rods. (**a**) NDRG1a-1 was overexpressed (*NDRG1a-1-Oe*), NDRG1a-2 was ectopically expressed (*NDRG1a-2-Ee*) or NDRG1b was ectopically expressed (*NDRG1b-Ee*) in zebrafish rods. Retinas from each transgenic fish were examined at adult stages. Each retina including that of wildtype (*Wildtype*) was sectioned and viewed with differential interference contrast (*DIC*) or immunoprobed with anti-rod transducin α-subunit (*Gt1α*). Magnifications are the same throughout the images in (**a**) (scale bar, 10 μm). In NDRG1a-1-Oe and 1b-Ee, rod outer segment layer (*Rod OS*) was much thinner than those in the wildtype and NDRG1a-2-Ee. Vertical bars from top to bottom in the left of each pair of images show a part of a layer of the retinal pigment epithelium (*RPE*), outer segment, ellipsoid, myoid and nucleus layers, respectively, and horizontal bars show the outer limiting membrane (*OLM*). The rod outer segment layer can be identified with anti-Gt1α-positive signals (right image in each pair). (**b**) A rod with normal cylindrical OS (left) and that with tapered OS (right). Magnifications are the same in (**b**) (scale bar, 5 μm). (**c**) Rods in mCherry-expressing control (*Control*), NDRG1a-1-Oe, NDRG1a-2-Ee or NDRG1b-Ee were isolated, and the fraction of tapered rods was determined (left). The number of rods with tapered OS were 2.0 % in Control (217 rods with tapered OS and 10429 rods with cylindrical OS in 5 fish), 38 % in NDRG1a-1-Oe (539 tapered and 892 cylindrical in 5 fish), 3.8 % in

NDRG1a-2-Ee (278 tapered and 7011 cylindrical in 4 fish) and 28 % in rods in NDRG1b-Ee (1258 tapered and 3155 cylindrical in 4 fish). In addition, the length of the rods showing a cylindrical shape (middle) and the length of the rods showing tapered shape (right) were measured in the population indicated above. Each result shows mean ± SD (middle and right). (d) Expression of NDRG1a-1 (left 4 panels), NDRG1a-2 (middle 4 panels) and NDRG1b (right 4 panels) were immunodetected with corresponding specific antiserum in isolated rods showing cylindrical OS (left 2 panels in each set of panels) and those showing tapered OS (right 2 panels in each set) in each transgenic fish. Each rod was viewed with DIC (*DIC*) or immunofluorescently (*Fluo*). Magnifications are the same in (d) (scale bar, 5 μm).

significantly in NDRG1a-2-Ee (Fig. 12c, left). Similarly, mean OS length of the normal cylindrical rods was shortened significantly (60-70 % of the control) in NDRG1a-1-Oe and NDRG1b-Ee, while in NDRG1a-2-Ee, it was similar to that of control rods (Fig. 12c, middle). The mean OS length of tapered rods was not different significantly among the control and the transgenic fish (Fig. 12c, right). Interestingly, overexpressed or ectopically expressed NDRG1 family proteins were found in the rod OS in addition to the inner segment in all strains no matter whether the OS shape was cylindrical or tapered (Fig. 12d), which is in contrast to the subcellular localization of these proteins in the wildtype rods summarized in Fig. 4g.

Examination of microscopic membrane structure of tapered rod OS

Macroscopic morphological changes from cylindrical to tapered OS shape observed in rods in NDRG1a-1-Oe and NDRG1b-Ee suggested the possibility that the OS of these rods is made of the infolded plasma membranes similar to the OS of cones. To test this possibility, rods in NDRG1a-1-Oe and NDRG1b-Ee were freshly isolated and stained with *N,N'*-didansyl cystine (DDC), a fluorescent reagent that stains the cone OS surrounded by infolded plasma membranes much more effectively than the rod OS enfolded by cylindrical plasma membrane (Fig. 13a)[20]. Rods with tapered OS in NDRG1a-1-Oe and NDRG1b-Ee did not show fluorescence while the OS of a cone present in the same visual field was fluorescent (Fig. 13b), which showed that the rods with tapered OS in either strain do not contain infolded membranes and microscopic membrane structure is preserved in the OS.

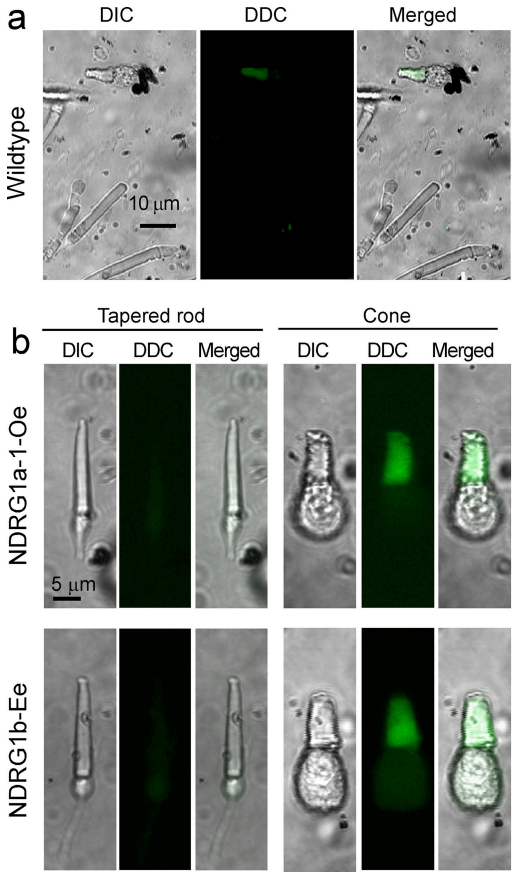

Figure 13. DDC staining of rods showing taper-shaped OS. (**a**) Typical DDC staining of wildtype (*Wildtype*) rods and cones. Only cone OS was stained. (**b**) Rods with tapered OS obtained from NDRG1a-1-Oe (*NDRG1a-1-Oe*) and NDRG1b-Ee (*NDRG1b-Ee*) was negative for DDC staining (upper and lower left 3 panels, respectively). Cone OSs in NDRG1a-1-Oe and NDRG1b-Ee were positively stained (upper and lower right 3 panels, respectively). Cells in (**a**) and (**b**) were viewed with DIC (*DIC*) and under fluorescent microscope to detect the fluorescence of DDC (*DDC*). Scale bar is 10 μm in (**a**). Magnifications are the same in all of the panels in (**b**) (scale bar, 5 μm).

Discussion

Difference in the Expression Patterns among the Three NDRG1 Family Proteins, NDRG1a-1, NDRG1a-2 and NDRG1b

In the present study, I showed that NDRG1 family proteins are expressed in rod and cone photoreceptors in adult zebrafish, although their subcellular localizations are different among the three. NDRG1a-1 is expressed in both rods and cones. However, in rods, NDRG1a-1 is present in the ellipsoid region plus other part except the OS while, in cones, it is found in the entire region including the OS (Figs. 4a, 4b, 4g). The expression level of NDRG1a-1 seems to be lower in rods than in cones (Figs. 4a and 3a). NDRG1a-2 is found only in the thin process in cones (Figs. 4c, 4d, 4g). The amino acid sequence is different only at their N-terminal regions between NDRG1a-1 and NDRG1a-2 (see Results, Fig. 2a), and this difference in the sequence should be responsible for this localization difference between the two splice variants. NDRG1b is expressed only in cones, which is consistent with the previous study in carp cones[3] in our lab, and it is localized in the entire region including the OS (Figs. 4e - 4g). The difference in the subcellular localization among these family proteins would mean that the role of each of the family proteins is probably slightly different. This notion is supported with the findings that during early development, the temporal expression pattern of mRNA (Fig. 5) is different among these family proteins. Spatial expression pattern of the protein is also different. NDRG1a-2 was not detected during early development of the retina from 48 hpf through 6 dpf, although its mRNA was present throughout the period from 1 hpf to 4 dpf (Fig. 5). Therefore, NDRG1a-2 seems to be expressed in tissues other than the retina during early development and NDRG1a-1 expression was restricted in the photoreceptor layer (Fig. 6a). NDRG1b was first expressed throughout the retina and then its expression was restricted to the photoreceptor layer, most probably to cones (Fig. 6b). The broader spatial expression pattern of NDRG1b together with maternal expression of NDRG1b mRNA (Fig. 5) and early expression of NDRG1b protein (Fig. 6d) suggest more general and important role(s) of NDRG1b than NDRG1a-1 in the retinal development in zebrafish.

Possible Roles of NDRG Family Proteins

In this study, it was shown that knockdown of NDRG1a-1 with a morpholino reduced the volume of both rod OS and cone OS, and that of NDRG1b caused similar effects on cone OS (Figs. 8 and 10). Overexpression of NDRG1a-1 or ectopic expression of NDRG1b in rods both induced the increase of the population of tapered rod OS together with the increase of rods with shortened OS (Fig. 12). These apparently

confusing results that all reduced the OS size (see Figs. 8, 10 and 12) could be explained by involvement of NDRG1 protein family in cholesterol uptake.

Although the mechanism is not known, the length of the rod OS is shortened in an animal model of Smith-Lemli-Opitz syndrome which accompanies reduction of cholesterol levels[26]. In addition, it is known that cholesterol content is regulated and different at different positions in the rod OS[27]: cholesterol concentration is high in the rod plasma membrane plus disk membranes at the base of the OS, but it is low at the apical tip. These lines of evidence suggest that cholesterol is one of the important lipid components in rod OS and it is possible that the shape of the OS is altered when the content of cholesterol is different from the normal levels. In cultured human cells, Pietiäinen *et al*[28]. reported that NDRG1 depletion reduced the content of low-density lipoprotein (LDL) receptor that uptakes free and esterified cholesterol from LDL, a protein-lipid particle containing free and esterified cholesterol in addition to phospholipids, and also reduces free cholesterol content at the plasma membrane and the total cholesterol content in the cell. It is presumably because reduced LDL receptor levels caused reduction of uptake of cholesterol supplied by LDL. It is, therefore, possible that knockdown of NDRG1a-1 or NDRG1b with morpholino(s) reduced the supply of cholesterol to photoreceptors and therefore the cholesterol available to form OS, which would result in the decrease of the OS size. In this case, the pigment concentration itself would not be affected and not altered significantly from that in the wildtype, which is in agreement with what I observed in this study (Figs. 8 and 10).

On the other hand, when NDRG1a-1 is overexpressed or NDRG1b is ectopically expressed in rods, probably cholesterol uptake is increased in these rods. Because the cholesterol content is different positionally in the rod OS[27], the increased cholesterol levels in these rods could cause a change in the lipid composition to possibly alter the shape of the OS from a long cylindrical to a shorter cylindrical or a tapered OS.

Currently, it is not known whether NDRG1 family proteins in zebrafish regulate the expression of LDL receptor. However, LDL receptor is known to be expressed in the photoreceptor inner segment[29] which is the major site of the endocytosis in photoreceptors[30]. NDRG1a-1 in rods and cones, and NDRG1b in cones are both expressed in the inner segment ellipsoid region where LDL receptor is probably present, which supports the notion that NDRG1a-1 and NDRG1b have some roles in the LDL receptor expression in the ellipsoid plasma membrane.

Based on the consideration above, I tried to examine whether cholesterol level in taper-shaped rod OSs in the transgenic fish is higher than in wildtype rod OSs by using filipin III, a fluorescent polyene macrolide antibiotic that can be used for detection of

cholesterol levels[28]. The result was in agreement with my above speculation, and the signal intensity of filipin III was 14-24 % higher as a mean in tapered rod OSs than in wildtype rod OSs (124 ± 29% (mean ±SD), n = 18 from three fish, p = 0.00647 in NDRG1a-1-Oe; 114 ± 27%, n = 9 from one fish, p = 0.138, in NDRG1b-Ee). However, signal intensity varied noticeably even in control wildtype rod OSs (roughly ± 50% from the mean, n = 21 from three fish), and I am not fully confident on the values indicated above. For reliable measurements, therefore, I need to improve the method and/or use other methods such as quantitative biochemical measurement of cholesterol. Obviously, future studies are needed to elucidate the exact functional mechanisms of the effects observed in this study.

In Fig. 12, it was shown that overexpressed NDRG1a-1 or ectopically expressed NDRG1b induced the formation of rods with tapered OS. It should be mentioned that ectopically expressed NDRG1a-2 did not show this effect. Because the amino acid sequence of NDRG1a-1 is different from that of NDRG1a-2 only at the N-terminal region, the N-terminal region in NDRG1a-1 is responsible to form tapered rod OS. It is interesting that similar amino acid sequence is present at the N-terminus in NDRG1b (Fig. 2a) which caused similar effects as NDRG1a-1. When NDRG1a-1 was overexpressed or NDRG1b ectopically expressed, these proteins were present in both the cylindrical and tapered rod OS (Fig. 12d). This result indicates that expression of NDRG1a-1 or NDRG1b in the OS is independently of OS shape or not associated directly with the OS morphology. This notion is supported with the finding that rods with tapered OSs in the wildtype fish did not show clear immunoreactivity to NDRG1a-1(data not shown). Evidently, further studies are required to understand the mechanism of the OS morphological changes induced by the overexpression of NDRG1a-1 and ectopic expression of NDRG1b in rods.

Structure of Tapered Rod OS

At early developmental stages in *Xenopus*, it is known that rods with “conical” OS but containing disks separated from the plasma membrane are present[25]. In zebrafish, I confirmed that more than 70% of the rod OSs shows similar "conical" or short tapered shape in 6 pdf zebrafish. It is evident that the shape of the OS of a rod changes from “conical” to “cylindrical” during development. From the findings that the tapered OS of a rod does not show DDC staining (Fig. 13) and that the shape of the rod OS is conical at early developmental stages, it is evident that the mechanism responsible for the formation of conical or tapered OS is different from the mechanism of the infold of the plasma membrane characteristic to the cone OS. Expression level of NDRG1a-1 seems

to be higher in cones than in rods (Fig. 4a), and NDRG1b is additionally expressed in cones. This fact indicates that the total expression level of NDRG1 family proteins is fairly higher in cones than in rods in zebrafish. It is known that rods are evolved from cones[31]. Therefore it could be the case that during evolution, rods obtained the ability to form a cylindrical OS in the adult by losing NDRG1b and reducing NDRG1a-1 expression levels.

Achievements

Publications

Peer-Reviewed Original Research Articles

1. Takita S., Wada Y., Kawamura S. Effects of NDRG1 family proteins on photoreceptor outer segment morphology in zebrafish. *Sci. Rep.* 6, 36590 (2016).
2. Hayakawa Y., Takita S., Kikuchi K., Yoshida A., Kobayashi M. Involvement of olfaction in spawning success of medaka *Oryzias latipes*. *Japan. J. Ichthyol.* **59**(2), 111-124 (2012).

Conference Presentations

Oral:

1. 瀧田真平・和田恭高・河村　悟. 哺乳類 NDRG1 のゼブラフィッシュ相同蛋白質の視細胞における機能解析.　日本動物学会近畿支部　2014 年 5 月　(姫路)
2. 瀧田真平・和田恭高・河村　悟. ゼブラフィッシュ視細胞における NDRG1·NDRG1L 蛋白質の機能解析.　第 16 回　視覚科学フォーラム　2012 年 8 月 (毛呂山)

Poster:

1. Takita S., Wada Y., Kawamura S. Effects of NDRG1 family proteins on photoreceptor outer segment morphology in zebrafish. XXII Biennial Meeting of the International Scociety for Eye Research, Sep, 2016 (Tokyo)
2. Takita S., Wada Y., Kawamura S. Functional analysis of zebrafish orthologues of mammalian NDRG1 protein in photoreceptors. 52th Biophysical Society of Japan. Sep, 2014 (札幌)
3. Takita S., Wada Y., Kawamura S. Functional analysis of zebrafish orthologues of mammalian NDRG1 protein in photoreceptors.　51th Biophysical Society of Japan. Oct, 2013 (京都)

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Abbreviation

DDC, N,N'-didansyl cystine
dpf, days post-fertilizartion
hpf, hours post-fertilizartion
gnat2, cone transducin α-subunit
GCL, ganglion cell layer
GST, glutathione S-transferase
Gt1α, rod transducin
INL, inner nuclear layer
IS, inner segment
MBP, maltose-binding protein
ndrg1a-1, N-myc downstream regulated gene 1a variant 1
NDRG1a-1-Oe, NDRG1a-1-overexpressing
ndrg1a-2, N-myc downstream regulated gene 1a variant 2
NDRG1a-2-Ee, NDRG1a-2-ectopically-expressing
ndrg1b, N-myc downstream regulated gene 1a variant 1
NDRG1b-Ee, NDRG1b-ectopically-expressing
MO, morpholino
OLM, outer limiting membrane
Opn1lw1, opsin 1 long wavelength 1
OS, outer segment
PRCL, photoreceptor layer
RPE, retinal pigment epithelium
RT, reverse transcription

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