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RESEARCH ARTICLE

Differential expression and hypoxia-mediated regulation of the N-myc downstream regulated gene family

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Abstract

Many organisms rely on oxygen to generate cellular energy (adenosine triphosphate or ATP). During severe hypoxia, the production of ATP decreases, leading to cell damage or death. Conversely, excessive oxygen causes oxidative stress that is equally damaging to cells. To mitigate pathological outcomes, organisms have evolved mechanisms to adapt to fluctuations in oxygen levels. Zebrafish embryos are remarkably hypoxia-tolerant, surviving anoxia (zero oxygen) for hours in a hypometabolic, energy-conserving state. To begin to unravel underlying mechanisms, we analyze here the distribution of the N-myc Downstream Regulated Gene (*ndrg*) family, *ndrg1-4*, and their transcriptional response to hypoxia. These genes have been primarily studied in cancer cells and hence little is understood about their normal function and regulation. We show here using *in situ* hybridization that *ndrgs* are expressed in metabolically demanding organs of the zebrafish embryo, such as the brain, kidney, and heart. To investigate whether *ndrgs* are hypoxia-responsive, we exposed embryos to different durations and severity of hypoxia and analyzed transcript levels. We observed that *ndrgs* are differentially regulated by hypoxia and that *ndrg1a* has the most robust response, with a nine-fold increase following prolonged anoxia. We further show that this treatment resulted in *de novo* expression of *ndrg1a* in tissues where the transcript is not observed under normoxic conditions and changes in Ndr1a protein expression post-reoxygenation. These findings provide an entry point into understanding the role of this conserved gene family in the adaptation of normal cells to hypoxia and reoxygenation.

Abbreviations: AP-1/2, activator proteins 1 & 2; Cap43, calcium-associated protein 43 kDa; CREB, cAMP-response element binding protein; Drg1, downregulated gene 1; EPO, erythropoietin; HIF-1 α , hypoxia-inducible factor 1 alpha; HRE, hypoxia response element; Igfbp-1, insulin-like growth factor-binding protein 1; LAMP1, lysosomal-associated membrane protein 1; Myc, myelocytomatosis proto-oncogene, basic helix-loop-helix transcription factor; NDRG, N-myc downstream regulated gene; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; PHD2, prolyl hydroxylase domain protein 2; PROXY-1, protein regulated by oxygen 1; Rab4, Ras-related protein Rab-4A; Rit42, reduced in tumor, 42 kDa; RTP, reducing agents and tunicamycin-responsive protein; STAT, signal transducer and activator of transcription; TNF- α , tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau protein; WISH, wholemount *in situ* hybridization.

Nguyet Le and Timothy M. Hufford contributed equally to this study.

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gene expression, hypometabolism, hypoxia, NDRG, zebrafish

1 | INTRODUCTION

Earth's atmosphere is composed of approximately 21% oxygen (O₂). Aerobic organisms use this environmental O₂ to produce ATP during oxidative phosphorylation. Hence, fluctuations in O₂ levels (either up or down) can have very detrimental outcomes for aerobic organisms. Severe hypoxia causes a decrease in ATP production due to diminished activity of the electron transport chain. Given that ATP fuels energy-demanding processes in the cell, its reduction can lead to cellular damage or death.^{1–4} Thus, hypoxia, hypoxemia or ischemia, is a contributing cause to many disease states in humans, including pulmonary vascular disease, acute kidney injury, neurodegenerative disease, and stroke.^{5–10} Conversely, excessive O₂ is equally, if not more harmful as it causes oxidative stress due to reactive oxygen species production that is damaging to macromolecules, including lipids, proteins, and nucleic acid.^{11–13}

To mitigate these adverse consequences, aerobic organisms have evolved mechanisms to adapt to low O₂ and maintain homeostasis. Such adaptations optimize access to O₂ by increasing red blood cell count and angiogenesis and altering energy metabolism, in part by switching from oxidative phosphorylation to glycolysis.^{14,15} In addition, cells conserve energy when exposed to chronic and severe hypoxia by reducing their metabolic rate. The latter is accomplished via suppression or arrest of energetically demanding processes such as cell division, transcription and translation, and down-regulating the activity of the sodium-potassium ATPase pump.^{16–23} While metabolic suppression has primarily been studied in organisms considered anoxia-tolerant, including painted turtles, crucian carp, naked mole rats and hibernating ground squirrels,^{16,24} it is likely to also be utilized in other organisms as well, albeit to a lesser extent. Zebrafish (*Danio rerio*) embryos maintain homeostasis under anoxia (zero O₂) by entering into a hypometabolic state characterized by reversible developmental and physiological arrest, which enables them to survive for up to 50 h.^{25,26} This protective response is developmentally regulated, with older embryos being less tolerant to anoxia.²⁶

Despite the necessity to conserve energy via suppression of transcription and translation, genes that are vital for the hypoxia response are in fact transcriptionally up-regulated under hypoxia.^{27–30} Such up-regulation is mediated by several transcription factors, the best studied of which is the Hypoxia-Inducible Factor-1 α (HIF-1 α).^{31–33} Under normoxic conditions (normoxia), the HIF-1 α subunit is hydroxylated by prolyl hydroxylase domain protein 2 (PHD2), marking

it for degradation by the von Hippel-Lindau protein (VHL). However, when O₂ levels are reduced, PHD2 activity is inhibited and stabilized HIF-1 α binds to the HIF-1 β subunit and translocates to the nucleus to regulate transcription. Upon entry into the nucleus, HIF- α/β heterodimers bind the hypoxia-response element (HRE). Even though this sequence is abundant in the genome, fewer than 1% of potential HRE sites are bound by the HIF complex under hypoxia, suggesting the existence of another layer of regulation.^{34–37} HIFs directly activate genes that mediate metabolic reprogramming from oxidative phosphorylation to glycolysis^{38,39} and genes that increase the available O₂ supply, such as *EPO*, *VEGF*, and its receptors.⁴⁰ Other HIF targets are implicated in autophagy, apoptosis, redox homeostasis, inflammation and immunity, stemness and self-renewal, metastasis and invasion.^{35,39,41,42} In addition to HIFs, several other transcription factors are known to influence the hypoxia response, including CREB, Myc, NF- κ B, and STATs, which engage in cross-regulatory interactions with HIFs.³²

Members of the N-myc downstream regulated gene (NDRGs) family are also hypoxia-responsive. The mammalian family consists of four members, *NDRG1–4*, while the zebrafish genome with its third round of genome duplication, encodes 6 paralogues, *ndrg1a*, *1b*, *2*, *3a*, *3b*, and *4*.⁴³ NDRGs are highly conserved across metazoans and the sequence homology is in fact greater for specific members of the family across different species (>80%) than between NDRG family members of the same species, which share ~57%–65% amino acid identity.⁴⁴ NDRGs belong to the α/β -hydrolase family, however, they are thought to be enzymatically inactive, lacking a critical catalytic triad.⁴⁵ NDRG1 (formerly known as *Drg1*, *Cap43*, *Rit42*, *RTP*, and *PROXY-1*) contains three tandem repeats (GTRSRSHSTSE) near its C-terminal and a phosphopantetheine sequence, which are two unique features that make it distinct from other NDRG family members. NDRG1 is thought to function as a tumor suppressor.⁴⁶ However, the absence of cancer resultant from germline mutations in humans⁴⁷ and targeted knockout in mice,⁴⁸ suggests that *NDRG1* may rather be involved in cancer progression (metastasis) rather than initiation.^{49–54} Human NDRG1 interacts with numerous other proteins in human cancer and other cell lines, including actin, Clathrin, and associated proteins AP-1 and AP-2, Caveolin-1, Kinesin, LAMP1, Rab4, and 26S proteasome components,^{49,51,55,56} consistent with a possible role in regulating vesicle trafficking.^{56,57} *NDRG1* and *NDRG2* transcript levels increase under hypoxia, as these genes have HIF-1 α binding sites (hypoxia-response elements or HREs) in their promoters.^{58–61}

However, NDRG regulation under hypoxic conditions is complex and does not depend solely on HIF-1 α , as several other transcription factors^{62,63} and *NDRG1* long non-coding RNA itself^{64,65} have also been implicated. *NDRG4* is transcriptionally up-regulated under hypoxia in cancer cells, however, in a TNF- α /NF- κ B rather than a HIF-1 α -dependent manner.⁶⁶ In contrast, *NDRG3* is regulated post-translationally in hypoxic cancer cells, via lactate binding, which stabilizes the protein and promotes cell proliferation and angiogenesis.⁶⁷ These findings indicate that NDRGs are regulated at the transcriptional and post-translational levels in response to hypoxia and promote adaptation to low O₂.

To date, NDRGs have mostly been studied in cancer cells and far less is known about their normal role and regulation. However, significant insights into their function are likely to result from the recently solved crystal structures of *NDRG1*, 2, and 3.^{68–70} While all members of this family can be regulated in response to fluctuations in O₂ levels, it is unclear what range and duration of hypoxia they respond to. Lastly, even though the spatial distribution of *NDRG* family members has been analyzed in zebrafish^{71,72} and frog (*Xenopus laevis* and *tropicalis*) embryos^{43,73,74} and mammals,^{75–83} it is unclear whether their spatial distribution changes under low O₂. We report here on the spatial distribution of members of the zebrafish *Ndr*g family and their regulation in response to hypoxia.

2 | MATERIALS AND METHODS

2.1 | Zebrafish

Zebrafish (*Danio rerio*) were raised and housed at 27°C on a 14/10 hour light/dark cycle. Zebrafish used in this study were the wild-type AB strain. Embryos were obtained by breeding male/female pairs. Maintenance of zebrafish and experimental procedures on larvae and adult zebrafish were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland Baltimore County. Zebrafish embryos (raised in normoxia) were staged and sorted according to Kimmel et al.⁸⁴ See Figure S2 for staging of anoxia-treated embryos.

2.2 | Hypoxia and anoxia treatments

For wholemount in situ hybridization (WISH), 24 hour post-fertilization (24 hpf) zebrafish embryos were dechorionated and then placed in 100 mm petri dish (CellTreat, Pepperell, MA, USA, Cat# 229663) containing 0% or 3% O₂ system water in an O₂ control glove box (Plas-Labs, Lansing, MI, USA model # 856-HYPO) set at 0% or 3% O₂ and 27°C. Following hypoxia treatment, embryos were

placed into 1.5 ml microcentrifuge tubes (~20 embryos/tube) with excess water removed. Embryos in each microcentrifuge tube were removed from the chamber and fixed in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) (Thermo Scientific, Waltham, MA, USA, Cat# J19943-K2) at 4°C overnight. Fixed embryos were rinsed in absolute methanol (Thermo Fisher Scientific, Waltham, MA, USA, CAS# 67-56-1) for 10 min at room temperature and stored in absolute methanol at –20°C.

For real-time PCR (qPCR), stage-matched control embryos were placed in 100 mm petri dishes containing 0% or 3% O₂ system water, placed in the O₂ control glove box set at 0% or 3% O₂ and 27°C. Following 4 and 8 h of treatment, single embryos were placed into 1.5 ml microcentrifuge tubes (Stellar Scientific Ltd. Co., Albuquerque, NM, USA, Cat# T17-100) with excess water removed. Single embryos in the microcentrifuge tubes were taken out of the chamber, flash frozen in liquid nitrogen, and stored at –80°C for total RNA extraction. Embryos raised under normoxic conditions were used as stage-matched controls for 3% O₂ (27 hpf for 4 h and 30.5 hpf for 8 h) and anoxia (26 hpf for 4 h and 27 hpf for 8 h) treatments.

2.3 | Riboprobe synthesis

The PCR template was cDNA synthesized from total RNA extracted from a combination of 6, 24, and 48 hpf zebrafish embryos. RNA extraction was performed with the QuickRNA MicroPrep Kit (Zymo Research, Irvine, CA, USA, Cat#R1051) and cDNA synthesis was carried out using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA, Cat# 1708890) according to the manufacturers' instructions.

PCR reactions were prepared using 1 μ l of diluted (1:5) cDNA as template in a total volume of 50 μ l. Primer concentrations were 10 μ M for each oligonucleotide. PCR-fragments were produced using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA, Cat#1851148) and Phusion-Polymerase (Thermo Scientific, Vilnius, LT, F530S) (35 cycles and 57°C annealing temperature). PCR-fragments were gel-purified using Micro Bio-Spin P-30 Gel columns Tris Buffer (RNase-free)(Bio-Rad, Hercules, CA, USA, Cat#7326250) and subsequently, 300–500 ng were used as template DNA to synthesize antisense RNA probes using in vitro transcription with the mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific, Carlsbad, CA, USA, Cat#AM1344), incorporating digoxigenin (DIG)-UTP via a DIG-labeling kit (Roche, Mannheim, Germany, Cat#11277073910).

To avoid amplification of regions of homology between *ndrg* members, all oligonucleotide primer pairs were designed against the 3'UTR of each gene, with the exception of *ndrg2* for which the primer pairs targeted the coding region (spanning exons 11–16) as well as the 3'UTR, as

specified in Li et al.⁷¹ For antisense probes, a T7 promoter sequence (5'-TAATACGACTCACTATAG-3') was added to the 5' end of each reverse primer. The following primer sets were used to amplify cDNA for 35 cycles as follows:

ndrg1a forward: 5'-ACCAATCAGTTCTGACTGTGCTGC-3'

ndrg1a reverse: 5'-TAATACGACTCACTATAGCACTCCAAACATGGAAAACGCAGA-3'

ndrg1b forward: 5'-ACACGCCTCAGCAGTTTAATCTGG-3'

ndrg1b reverse: 5'-TAATACGACTCACTATAGCTCAC TGAAGTCTTGCACAACCAG-3'

ndrg2 forward: 5'-ACAACACGTTCAAATGCCCCG-3'

ndrg2 reverse: 5'-TAATACGACTCACTATAGGGAAGACATGAGCTGGCTGT-3'

ndrg3a forward: 5'-GGTCTTCCAAGTGGTTTGAGATGC-3'

ndrg3a reverse: 5'-TAATACGACTCACTATAGTGAGAACAGTGGACAGTGACACT-3'

ndrg3b forward: 5'-GCCAGAGAGTGCTGGTCTAATGAA-3'

ndrg3b reverse: 5'-TAATACGACTCACTATAGCCGAGACATGCTAATCAGTAGCTC-3'

ndrg4 forward: 5'-GACTTGCGTCAGGGATGATAACCT-3'

ndrg4 reverse: 5'-TAATACGACTCACTATAGGAATGAGTGAGAGCAAGGGCCGAT-3'

2.4 | Wholmount RNA in situ hybridization

Normoxic controls were fixed at desired stages (shield, 15 somites, 24 hpf, and 48 hpf) in 4% PFA in PBS (Thermo Scientific, Waltham, MA, USA, Cat# J19943-K2) at 4°C overnight. Anoxia-exposed 24 hpf embryos were treated as described in the anoxia treatment section above. To prevent pigmentation from masking the WISH signal, embryos fixed after 24 hpf were incubated in 0.003% 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich, Milwaukee, WI, USA, Cat# P7629-100G) at 24 hpf until the time of fixation. WISH was performed on both normoxic and anoxic embryos using DIG labeled antisense probes according to the specifications published by Thisse and Thisse.⁸⁵ Briefly, embryos were rinsed in PBS (137 mM NaCl, 2.7 mM KCl, 8.8 mM Na₂HPO₄) with 0.1% Tween-20 (Sigma-Aldrich, Milwaukee, WI, USA CAS# 9005-64-5). Proteinase K (10 µg/ml; Sigma-Aldrich, Milwaukee, WI, USA CAS# 39450-01-6) treatment was performed for 10 min (24 hpf), 12 min (27 hpf control and 24 hpf + anoxia-treated) and 30 min (48 hpf) embryos. Embryos were then hybridized with DIG labeled antisense probes (in situ hybridization mix with 5% Dextran Sulfate (EMD Millipore Corp., Billerica, MA, USA, Cat#S4030)) at 70°C overnight. Following hybridization, excess probe was removed by washing embryos in a saline-sodium citrate (SSC) series (Thermo Fisher Scientific, Waltham, MA, USA, Cat# AM9763). For probe detection, alkaline

phosphatase-conjugated antibody (Roche Diagnostics, Mannheim, Germany, Cat# 11093274910) diluted (1:5,000) in pre-incubation (PI) buffer (PBS, 0.1% Tween 20, 2% sheep serum, 2 mg/ml BSA) (Thermo Fisher Scientific, Waltham, MA, USA, CAS# 9048-46-8) was added and incubated at 4°C overnight. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche, Mannheim, Germany, Cat#11383221001) was used in conjunction with nitro blue tetrazolium (NBT) (Roche, Mannheim, Germany, Cat#11383213001) for the colorimetric detection of alkaline phosphatase activity. When the signal was optimal, the reaction was stopped by washing in PBS with 0.1% Tween-20 and rinsed in 4% PFA overnight.

2.5 | Vibratome sectioning, microscopy, and imaging

Following WISH, embryos were embedded in 4% low melt agarose (IBI Scientific, Peosta, IA, USA, Cat# IB70050) in PBS (100 g/ml) and sectioned using a vibratome (Vibratome, 1500) set at 40 µm thickness. Cross-sections were mounted on glass slides in 50% glycerol (Sigma-Aldrich, Milwaukee, WI, USA, Cat# G7757-1GA) under cover slips.

Zebrafish embryos were mounted for imaging from a lateral or dorsal view on slides, in a drop of 4% w/v methylcellulose (Sigma Aldrich, Milwaukee, WI, USA, Cat# 274437-500G) in 1× E3 medium (5 mM NaCl, 0.17 mM 9M KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) (for zebrafish embryo). Bright-field images were captured using an AxioCam HRC 503 CCD camera mounted on an Axioskop (Carl Zeiss, Oberkochen, Germany). Immunolabeled embryos were imaged on a TCS SP5 confocal microscope (Leica, Wetzlar, Germany). Images were corrected for brightness and contrast along the entire image, and for comparison of normoxia and anoxia treated embryos the images were adjusted equally.

2.6 | Real-time quantitative PCR

RNA extractions and cDNA synthesis were carried out using single embryos collected at the appropriate developmental stage for the stage-matched controls and immediately following treatment for anoxia- and 3% O₂-treated embryos. RNA extractions were performed using the QuickRNA MicroPrep Kit (Zymo Research, Irvine, CA, USA, Cat#R1051). cDNA was synthesized from 100 ng total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA, Cat# 1708890) according to the manufacturers' instructions. The cDNA samples were diluted 1:10 with nuclease-free water (Life Technologies Corp., Austin, TX, USA, Cat# AM9937). qPCR experiments were carried out with a CFX96 Touch Real-time qPCR Detection System (Bio-Rad, Hercules, CA, USA) using the

SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA, Cat# 172-5271).

We tested a panel of candidate reference genes for the q-PCR analysis, including *ef1a*, *β -actin*, *rpl0*, *rpl13a*, and *ube2a*, which were previously demonstrated to be stable across zebrafish developmental stages and/or following harsh chemical treatments.^{27,86–88} Among these, *rpl0*, *rpl13a*, and *ube2a* did not amplify sufficient cDNA in 40 cycles and were not pursued further. *ef1a* and *β -actin* have both been used as reference genes for zebrafish hypoxia studies specifically^{27,87} and their transcripts were sufficiently abundant at 24 hpf. We selected *ef1a* for all experiments described below. Oligonucleotide primer pairs spanned regions common to all *ndrg* splice variants, with the exception of *ndrg1b* and *ndrg2*, which only have one variant. The PCR primer efficiency of each primer pair was assessed using cDNA dilution curves and values of 96%–104% were obtained for all except *ndrg1b*, which did not amplify efficiently (consistent with the lack of gene expression at 24 hpf observed using WISH and reported by others⁸⁹). Amplification specificity was determined following each run as the presence of a single melt peak for each transcript. The following primer sets were designed using Primer-BLAST and used to amplify cDNA for 40 cycles:

ndrg1a forward: 5'-ATCATGCAGCACTTCGCTGT-3'
ndrg1a reverse: 5'-CAATAGCCATGCCGATCACA-3'
ndrg1b forward: 5'-CATGGGCTACATGCCCTCTG-3'
ndrg1b reverse: 5'-TGACCCGATGAACTGTGCTC-3'
ndrg2 forward: 5'-AGCTGGAAAGAAAGTGCGAGA-3'
ndrg2 reverse: 5'-TTTACGCCGTCCGCTTATGT-3'
ndrg3a forward: 5'-GGACTAGCAATCTTGTGGAC-3'
ndrg3a reverse: 5'-TCTCGATTCCGAGGTCTTGA-3'
ndrg3b forward: 5'-GTCAGGCTTGATGATGGATG-3'
ndrg3b reverse: 5'-CCCTCTCAAAGTCACATGAAGG-3'
ndrg4 forward: 5'-AGCCAGCTATTCTGACCTAC-3'
ndrg4 reverse: 5'-GATATCCTTGAGGCATCTGG-3'
ef1a forward: 5'-TACCCTCTCTTGGTCGCTT-3'
ef1a reverse: 5'-TGGAACGGTGTGATTGAGGG-3'

Reactions were run in triplicate with 7–8 biological replicates, using 1 μ l of diluted cDNA as template in a reaction volume of 20 μ l. For all *ndrg4* reactions, 2 μ l of stock cDNA was used as template. Primer stock concentrations were 10 μ M and working concentrations were 0.5 μ M for each oligonucleotide (Thermo Fisher Scientific, Waltham, MA, USA). The following annealing temperatures were used for each target gene primer set: 57.0°C for *ndrg1a*, 56.6°C for *ndrg2*, 53.0°C for *ndrg3a*, 55.0°C for *ndrg3b*, and 51.0°C for *ndrg4*. Evaluation of results was performed with the CFX96 Touch RT-PCR Detection System program (Bio-Rad, Hercules, CA, USA) and using GraphPad Prism 8 & 9 software (Prism, San Diego, CA, USA). The presence of outliers was assessed using both Grubbs' ($\alpha = 0.05$) and ROUT ($Q = 1\%$) methods. Outliers identified with

both Grubbs' ($\alpha = 0.05$) and ROUT ($Q = 1\%$) methods and were further inspected and handled as follows.^{90,91} Outliers with Ct values that could not be attributed to experimental error (improper dilution, amplification failure) for a particular group were included, as the variation could be attributed to biological variation. Outliers with Ct values over 40 (e.g., a technical replicate for which amplification did not occur properly) were not further analyzed, and any biological replicates for which two or more technical replicates of the reference or target gene failed to amplify were entirely excluded from the analysis.

2.7 | Immunolabeling

Embryos were fixed in 4% PFA at RT for 1 h and washed in 1 \times PBS for 30 min on a nutator. Fixed embryos were permeabilized with cooled acetone for 5 min at -20°C , followed by a 5 min wash in 1 \times PBS. Embryos were subsequently incubated in Inoue blocking solution⁹² (5% Normal Goat Serum (NGS)) (Abcam, cat# ab7481, lot# GR325285-5), 2% BSA (Fisher Scientific, cat# BP1600-100, lot# 196941), 1.25% Triton X-100 (Fisher Scientific, cat# BP151-500, lot# 172611) in 1 \times PBS for 1 h at room temperature (RT) on a rotating platform (80 RPM). Incubation in primary antibodies was performed in I-buffer solution (1% normal goat serum, 2% bovine serum albumin, 1.25% Triton X-100, in 1 \times PBS) for 2 days at RT on a rotating platform (80 RPM). Embryos were then washed three times, 30 min each, with 1 \times PBS at room temperature. Secondary antibodies, diluted in I-buffer, were applied for 1 day at RT on a rotating platform (80 RPM). After secondary antibody incubation, embryos were washed three times with 1 \times PBS for 30 min.

2.7.1 | Primary antibodies

Anti-NDRG1 (1:200–500) (Sigma Aldrich, catalog # HPA-006881, lot# A69409, rabbit polyclonal).

2.7.2 | Secondary antibodies

Goat anti-rabbit Alexa Fluor 594 (Abcam, cat# ab150080, lot# GR3373513-1).

2.8 | Fluorescence intensity measurements of the pronephric duct

Fluorescence intensity corresponding to NdrG1a protein levels in the anterior and posterior pronephric duct was

measured using lateral views of immunolabeled embryos. A single tight region of interest (ROI) of the pronephric duct was measured for mean intensity using ImageJ (FIJI) software.

2.9 | Fluorescence intensity measurements of ionocytes

Fluorescence intensity corresponding to *Ndrgl1a* protein levels in ionocytes was measured by drawing a tight ROI around individual ionocytes¹⁰ in the yolk ball and yolk extension using ImageJ (FIJI).

3 | RESULTS

3.1 | The spatial distribution of the *ndrg* family during early development

The zebrafish genome encodes six homologs of the *ndrg* family: *ndrg1a* (ENSDARG00000032849), *ndrg1b* (ENSDARG00000010420), *ndrg2* (ENSDARG00000011170), *ndrg3a* (ENSDARG00000013087), *ndrg3b* (ENSDARG00000010052), and *ndrg4* (ENSDARG000000103937). To characterize the members of the *ndrg* family, we began by determining their spatial distribution in early-stage zebrafish embryos, using WISH. Since several members of this family contain large overlapping coding sequences (53%–65% homology), riboprobes were designed that bind to non-conserved regions in the 3'UTR of all *ndrgs*, with the exception of *ndrg2*. Due to issues with the amplification of the *ndrg2* 3'UTR, we used instead a riboprobe complementary to the coding region and 3'UTR of this gene that has low homology with other *ndrg* members.⁷¹

At the shield stage (6 hpf), *ndrg1a* expression is ubiquitous (Figure 1A). During segmentation (15-somites), *ndrg1a* becomes restricted to the pronephric ducts (embryonic kidney) and ionocytes (also known as mucous cells; Figure 1B,C); these cell types serve the common function of maintaining osmotic homeostasis by filtering ions across the plasma membrane. *ndrg1a* is also observed in the yolk at this stage of development (Figure 1B). At 24 hpf *ndrg1a* is weakly expressed in the epiphysis (embryonic gland that produces melatonin), in addition to the pronephric duct, ionocytes, and caudal vein (Figure 1D,E) and by 48 hpf, *ndrg1a* is observed in corpuscles of Stannius (endocrine glands in the kidney), liver, intestinal bulb, retina, and other brain regions (Figure 1F,G). The expression of *ndrg1b* is very dynamic. At the shield stage, it is ubiquitously expressed (Figure 1H), while by 15-somites and 24 hpf, it is no longer detected (Figure 1I–L). In contrast, at 48 hpf, *ndrg1b* is strongly expressed in the retina (Figure

1M,N). The expression of *ndrg2* is ubiquitous at shield stage (Figure 2A) and remains broadly distributed by 15-somites, in the embryo proper and the yolk (Figure 2B,C). At 24 hpf, *ndrg2* is strongly expressed in the brain, retina, spinal cord, and intermediate cell mass of the mesoderm (where hematopoiesis occurs; Figure 2D,E). At 48 hpf, *ndrg2* expression expands to the pectoral fin buds, somites and the heart, with basal levels observed throughout the embryo (Figure 2F,G). *ndrg3a* is broadly expressed at the shield stage (Figure 3A) and is observed in the head region and pronephric ducts at 15-somites (Figure 3B,C). At 24 hpf (Figure 3D,E), *ndrg3a* is also seen in pharyngeal pouches, pectoral fin buds and somites. By 48 hpf, *ndrg3a* signal is detected in the brain, cranial placodes, and the spinal cord in addition to the pronephric ducts and associated corpuscles of Stannius (Figure 3F,G). *ndrg3b* signal is not detected between shield stage and 24 hpf (Figure 3H–L); however, at 48 hpf it is observed in the brain and, at lower levels, in pectoral fin buds (Figure 3K,L). At the shield stage, *ndrg4* is expressed ubiquitously (Figure 2H) but becomes enriched in somites by the 15-somites stage (Figure 2I,J). At 24 hpf and 48 hpf (Figure 2K–N), *ndrg4* transcript is detected in the brain, the heart, the cranial placodes, the somites, the spinal cord, the pectoral fin buds, the intermediate cell mass of the mesoderm, and proctodeum.

3.2 | Normoxic control groups that account for hypoxia-induced developmental delays

To gain an understanding of the transcriptional regulation of *ndrgs* in response to low O₂, we exposed 24 hpf embryos to two different hypoxic conditions (3% and 0% O₂) for 4 or 8 h and analyzed transcript levels using qPCR (results presented in section below). Given that O₂ deprivation delays or arrests zebrafish development, an important consideration for these experiments is the appropriate normoxic control group to use, which we have designated as: time zero, age-matched, and staged-matched normoxic controls. Time zero controls are embryos that are the same age as the experimental group at the onset of treatment (i.e., 24 hpf). Age-matched controls are the same age (hpf) as the experimental groups (28 hpf for embryos subjected to 4 h of hypoxia and 32 hpf for embryos exposed to 8 h of hypoxia). Stage-matched controls are embryos at the same developmental stage as the experimental groups exposed to 4 or 8 h of hypoxia; the stage of development varies depending on the severity of the treatment, as embryos arrest faster under anoxia.

With respect to anoxia, when using time zero controls, qPCR results revealed that transcript levels were significantly up-regulated for *ndrg1a* following 4 and 8 h of treatment (3 and 8-fold up-regulation, respectively), while

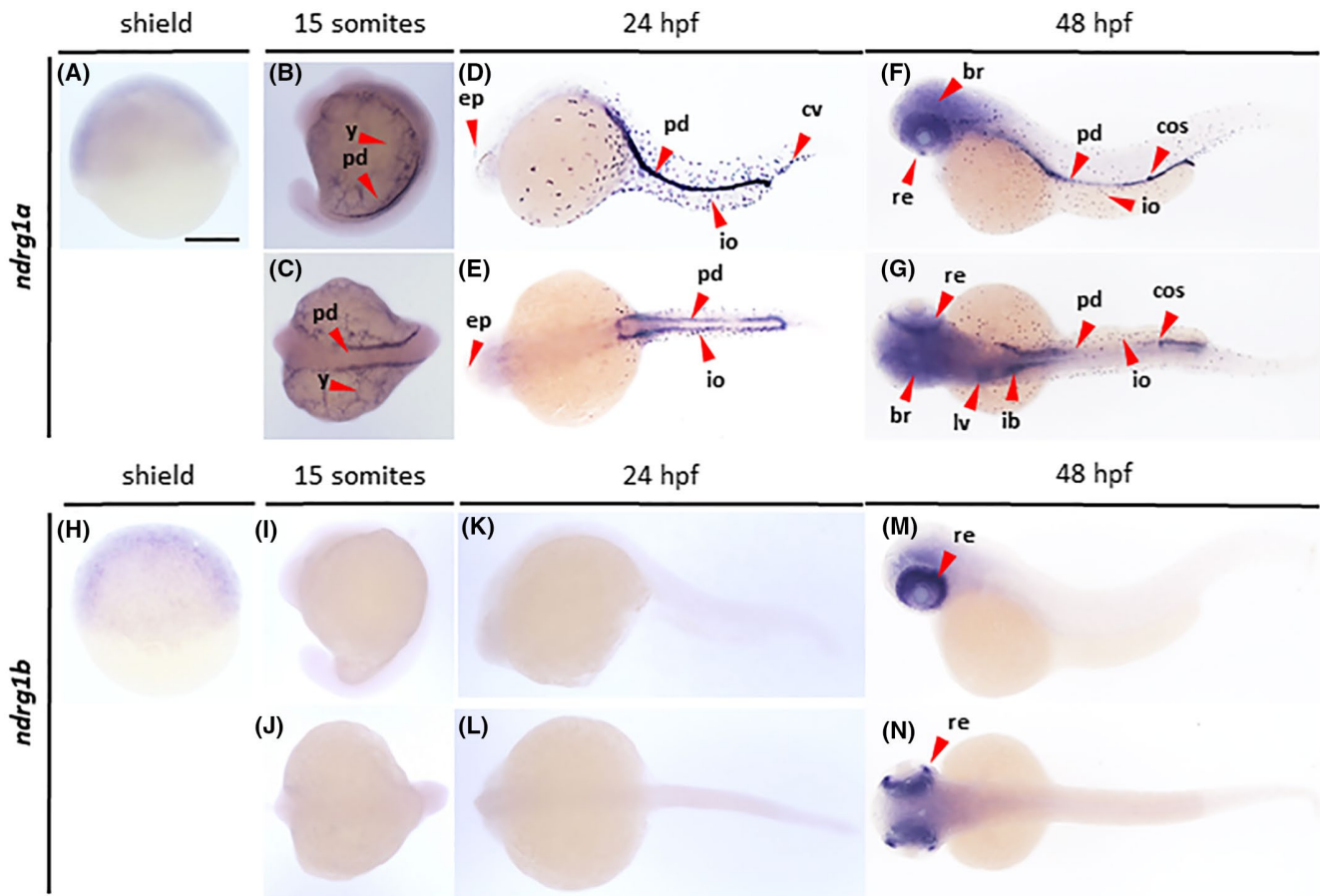


FIGURE 1 Gene expression analysis of *ndrg1*. Wholemount in situ hybridization analysis revealing the distribution of *ndrg1a* (A–G) and *ndrg1b* (H–N) transcripts in zebrafish embryos at shield (A, H), 15 somites (B, C, I, J), 24 hpf (D, E, K, L) and 48 hpf (F, G, M, N) stages, imaged from lateral (A, B, D, F, H, I, K, M) and dorsal (C, E, G, J, L, N) views. br, brain; cos, corpuscles of Stannius; cv, caudal vein; ep, epiphysis; ib, intestinal bulb; io, ionocyte; lv, liver; pd, pronephric ducts; re, retina; y, yolk. Scale bar, 250 μ m

other members of the *ndrg* family increased to a lesser extent (2-fold or less; Figure S1A). In contrast, the use of age-matched controls resulted in a different outcome, with *ndrg1a* and *3a* being significantly up-regulated following 4 and 8 h of anoxia while *ndrg2*, *3b* and *4* were down-regulated (Figure S1B). These differences in transcript levels using time zero and age-matched normoxic controls are most likely explained by dynamic gene expression during development, consistent with RNA Seq repository data (EMBL Zebrafish Expression Atlas)⁸⁹ showing that *ndrg* expression levels change significantly between 24 and 48 hpf. Based on these observations, the most appropriate normoxic control would be one that takes into account the developmental stage of the experimental group, that is, a stage-matched control.

Several criteria were used to match the developmental stages of experimental groups: the overall length of the embryo, the length of the yolk extension, head curvature, and level of pigmentation of the eye and the body (Figure S2). Based on these criteria, the following normoxic control groups were selected (where = indicates “best matched to”): 26 hpf normoxic control = 24 hpf

embryo exposed to 4 h of anoxia (or 24 hpf + 4 h anoxia; Figure S2A,B), 27 hpf normoxic control = 24 hpf + 8 h of anoxia (Figure S2C,D), 27 hpf normoxic control = 24 hpf + 4 h 3% O₂ (Figure S2E,F), 30.5 hpf normoxic control = 24 hpf + 8 h 3% O₂ (Figure S2G,H).

3.3 | Differential regulation of members of the *ndrg* family in response to low oxygen

Cells adapt in distinct manners to varying levels of O₂. Hypoxia (mild to severe) generally elicits metabolic re-programming via HIF-1 α -dependent transcriptional up-regulation of key genes that mediate the adaptive response.^{14,15} In contrast, anoxia-tolerance involves metabolic arrest, during which most ATP-demanding processes are suppressed, except for those that are essential for survival.^{16,24} To gain an understanding of the range of hypoxia conditions that elicit *ndrg* up-regulation and identify the members of this family that may promote

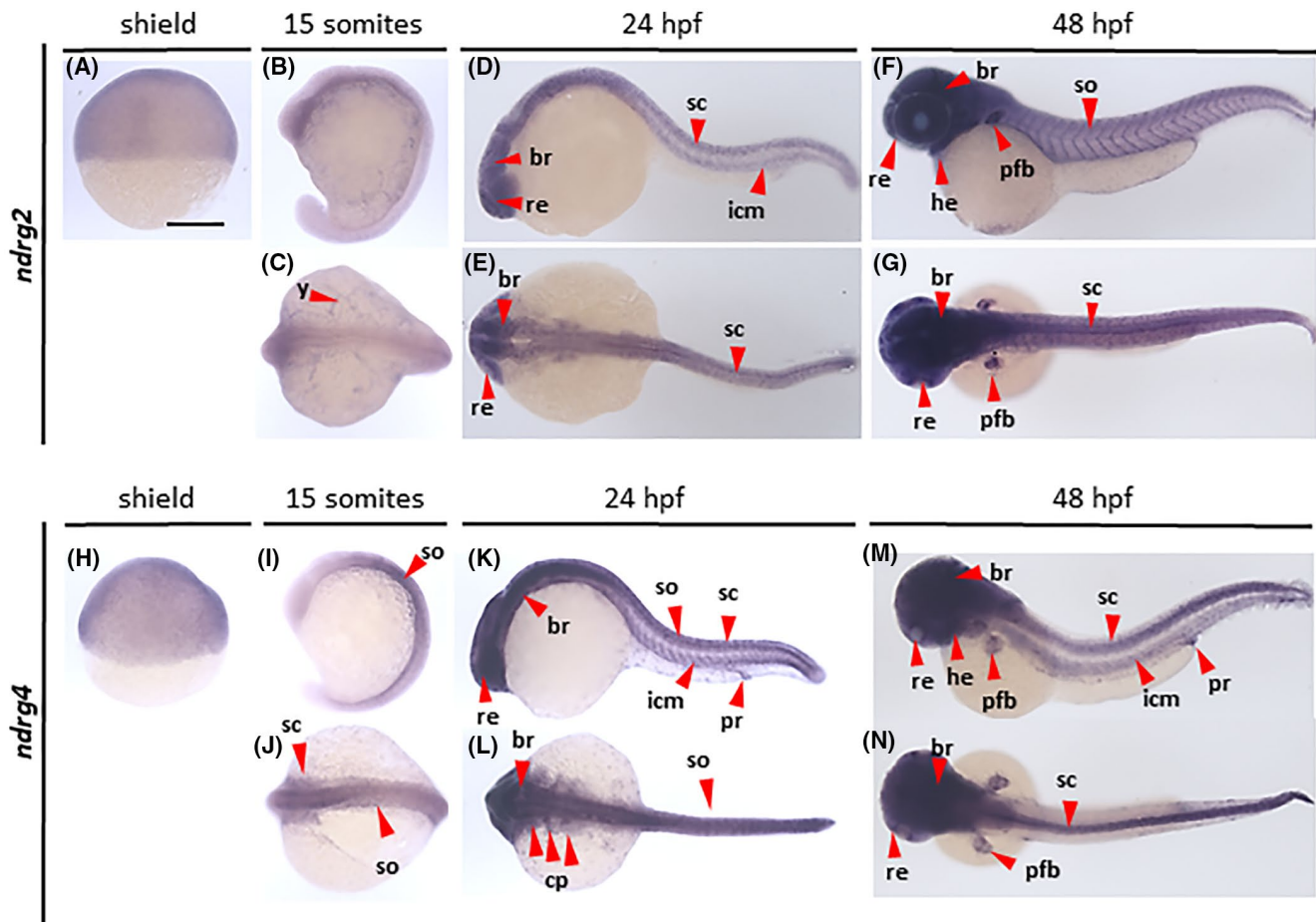


FIGURE 2 Gene expression analysis of *ndrg2* and *ndrg4*. Wholemount in situ hybridization analysis revealing the distribution of *ndrg2* (A–G) and *ndrg4* (H–N) transcripts in zebrafish embryos at shield (A, H), 15 somites (B, C, I, J), 24 hpf (D, E, K, L, J) and 48 hpf (F, G, M, N) stages, imaged from lateral (A, B, D, F, H, I, K, M) and dorsal (C, E, G, J, L, N) views. br, brain; cp, cranial placodes; he, heart; icm, intermediate cell mass of mesoderm; pfb, pectoral fin buds; pr, proctodeum; re, retina; sc, spinal cord; so, somites. Scale bar, 250 μ m

hypoxia adaptation, we subjected embryos to hypoxia (3% O_2) or anoxia (0% O_2) for 4 or 8 h.

In response to 4 h of 3% O_2 , the transcript levels of none of the *ndrg* family members were significantly altered (Figure 4A). After 8 h of 3% O_2 , *ndrg1a* was moderately up-regulated (1.9-fold). The expression of other members was not significantly altered (Figure 4B). These data trends are plotted in Figure 4C.

Following 4 h of anoxia, *ndrg1a* and *ndrg3a* were up-regulated (1.7-fold and 1.5-fold, respectively). In contrast, *ndrg2* was significantly down-regulated (–0.5 fold, respectively), while *ndrg3b* and *ndrg4* expression were not significantly altered (Figure 5A). By 8 h of anoxia, *ndrg1a* was further up-regulated 9.3-fold, and we also observed a slight up-regulation of *ndrg3a* (1.9-fold) (Figure 5B). *ndrg2* was down-regulated (–0.5-fold), and *ndrg3b* and *ndrg4* did not significantly change following 8 h of anoxia (Figure 5B). These data trends are plotted in Figure 5C.

Overall, these data reveal that the *ndrg* family is differentially regulated in response to low O_2 . *ndrg1a* is the most hypoxia-responsive member of the family during

early development and is transcriptionally up-regulated in response to severe and prolonged O_2 deprivation. This finding is consistent with a previous study using cancer cells revealing that members of the NDRG family mediate long-term adaptation to hypoxia.⁶⁷ Despite the lack of reported HIF-1 α binding sites in its promoter region, *ndrg3a* transcript levels also appear to be up-regulated under anoxia. *ndrg1b* levels were extremely low at the stages used in this qPCR analysis (Figure 1K,L) and were not further analyzed. Other members of the *ndrg* family may not be hypoxia-responsive at 24 hpf, at least not at the transcriptional level.

3.4 | The spatial distribution of *ndrg1a* changes in response to anoxia

To confirm that *ndrg1a* is indeed hypoxia-responsive and determine if any changes in its spatial distribution occur following the most stringent hypoxia treatment, we performed WISH using 24 hpf embryos that were exposed

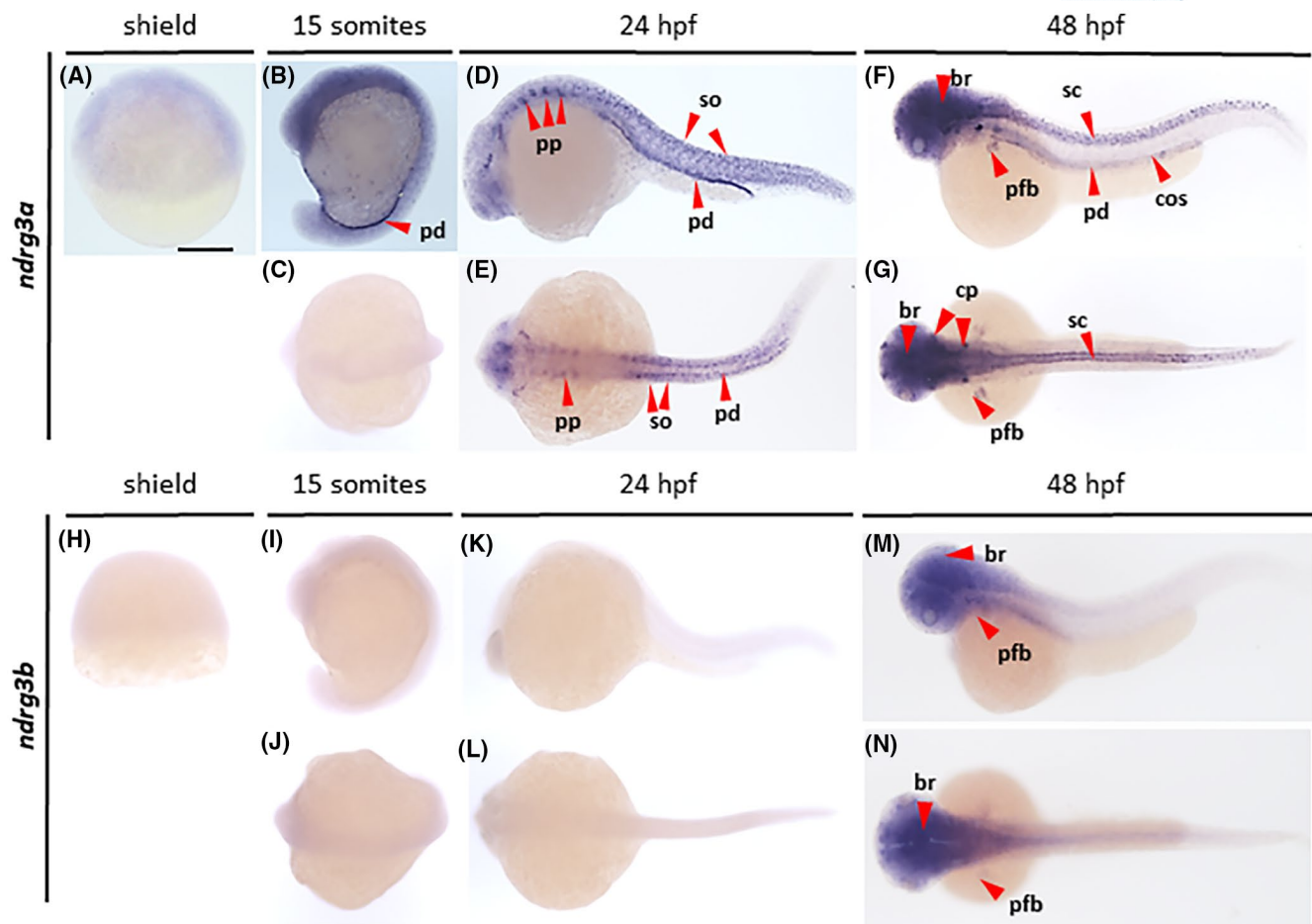


FIGURE 3 Gene expression analysis of *ndrg3*. Wholemount in situ hybridization analysis revealing the distribution of *ndrg3a* (A–G) and *ndrg3b* (H–N) transcripts in zebrafish embryos at shield (A, H), 15 somites (B, C, I, J), 24 hpf (D, E, K, L) and 48 hpf (F, G, M, N) stages, imaged from lateral (A, B, D, F, H, I, K, M) and dorsal (C, E, G, J, L, N) views. br, brain; cos, corpuscles of Stannius; cp, cranial placodes; pfb, pectoral fin buds; pp, pharyngeal pouches; pd, pronephric ducts; sc, spinal cord; so, somites. Scale bar, 250 μ m

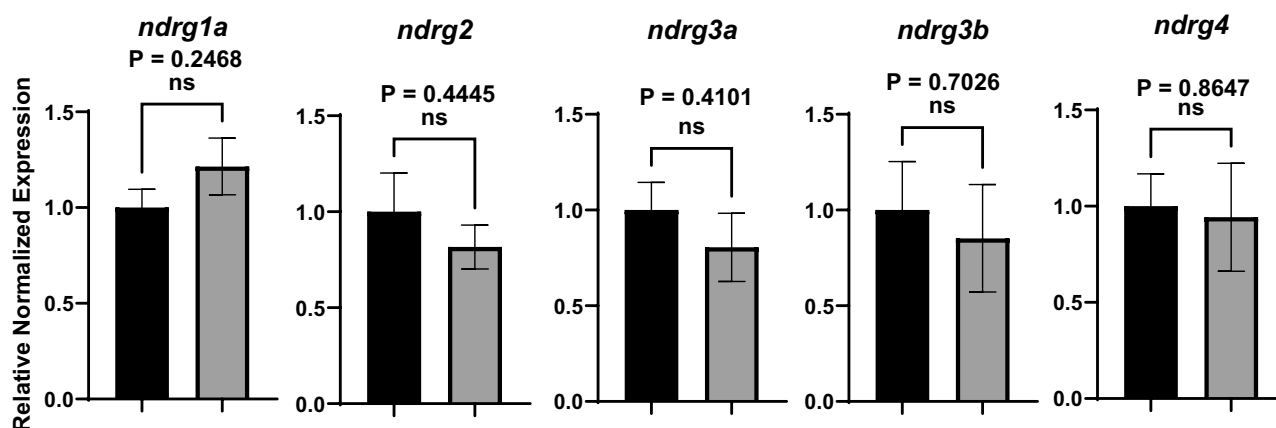
to 8 h of anoxia. Even though WISH is not a quantitative method to assess gene expression levels, we reasoned that the amount of transcript can at least be directly compared if control (stage-matched) and experimental (anoxia-treated) embryos are processed simultaneously during the color reaction step of WISH.

Following prolonged anoxia, *ndrg1a* transcript was maintained, possibly enhanced, in the pronephric duct, ionocytes, endodermal organs (liver, intestine) and epiphysis (Figure 6B,B',B'',C,D,F,F'). Interestingly, *ndrg1a* signal was also observed in tissues where this gene is not normally expressed (or expressed at levels that are below the detection limit) under normoxic conditions. Among these tissues, *ndrg1a* was prominently expressed in the inner ear (otic vesicle) (Figure 6B,B',C,D,F,F'). In addition, *ndrg1a* was observed in the head vasculature in anoxia-treated embryos, namely: the primordial mid-brain channel (pmbc), the dorsal aorta (da), the mid-cerebral vein (mcev), and the aortic arch (aa) (Figure 6B,B',F,F'). Although variable in levels between embryos

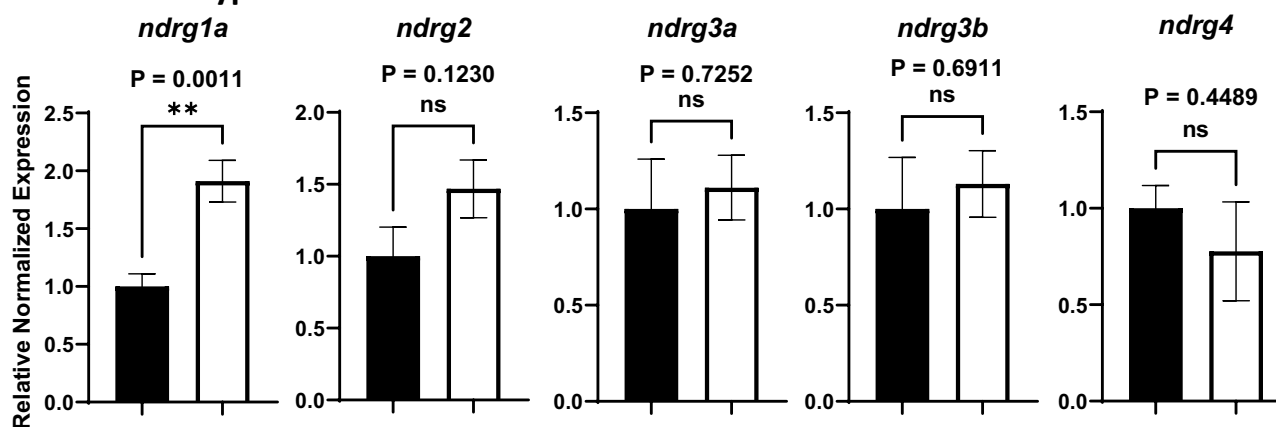
and experiments (possibly correlating with the duration of the color reaction), *ndrg1a* transcript was also seen in a segmentally-repeated pattern in the trunk (Figure 6A'',B'',C',D',E'',F'') that may correspond to somites. The mesoderm-expanded expression explains the thickened anterior-posteriorly oriented stripes of *ndrg1a* label observed from a dorsal view (Figure 6E,F). Furthermore, in samples where the labeling was generally stronger, *ndrg1a* transcript also became apparent in the hatching gland and lateral line primordium, a migrating epithelial placode that deposits a series of mechanosensory hair cell organ progenitors along the flank of the embryo (Figure 6C'). These observations were categorized into mild (Figure 6C-C'), moderate (Figure 6B-B'',F-F'',H, J), and severe (Figure 6D-D') transcriptional response patterns.

Cross-sections of control and anoxia-treated embryos confirmed the anoxia-induced expression of *ndrg1a* in otic vesicles (Figure 6G,H) and at basal levels throughout the somites, with some puncta of more intense label

(A) 4 Hours Hypoxia



(B) 8 Hours Hypoxia



(C)

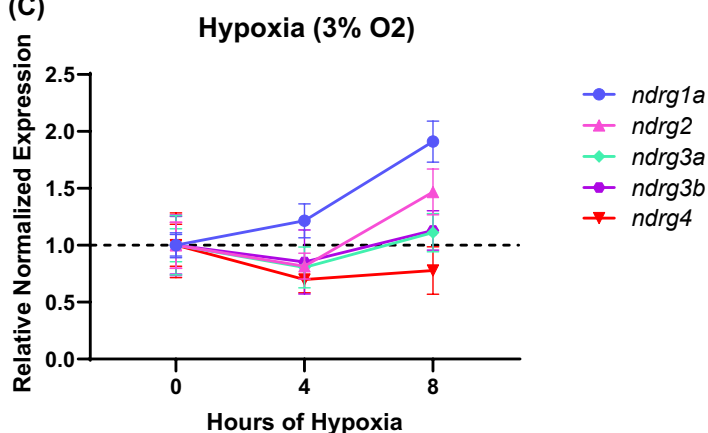


FIGURE 4 Changes in *ndrg* transcript levels in response to hypoxia (3% oxygen). (A, B) Real-time qPCR analysis of 24 hpf zebrafish embryos exposed to 4 h (A, grey bars) or 8 h (B, white bars) of hypoxia relative to normoxic (stage-matched) controls (A, B, black bars) normalized to *ef1a*. (C) Plotted graphical summary of qPCR results. The y-axis in the graphs represents the relative normalized expression of each gene. All fold changes were derived using the formula, $2^{-(\Delta\Delta CT)}$, error bars represent standard error of the mean. Significance was obtained using the unpaired, two-tailed *t*-test with Welch's Correction. ***p* < .01. Reactions were run in triplicate with 7–8 biological replicates (*n* = 7–8).

(Figure 6I,J). In addition, the sections revealed elevated expression of *ndrg1a* in the caudal aorta and vein (Figure 6I,J).

To determine whether these changes in *ndrg1a* mRNA expression are also observed at the protein level, we performed immunolabeling using anti-human NDRG1. The

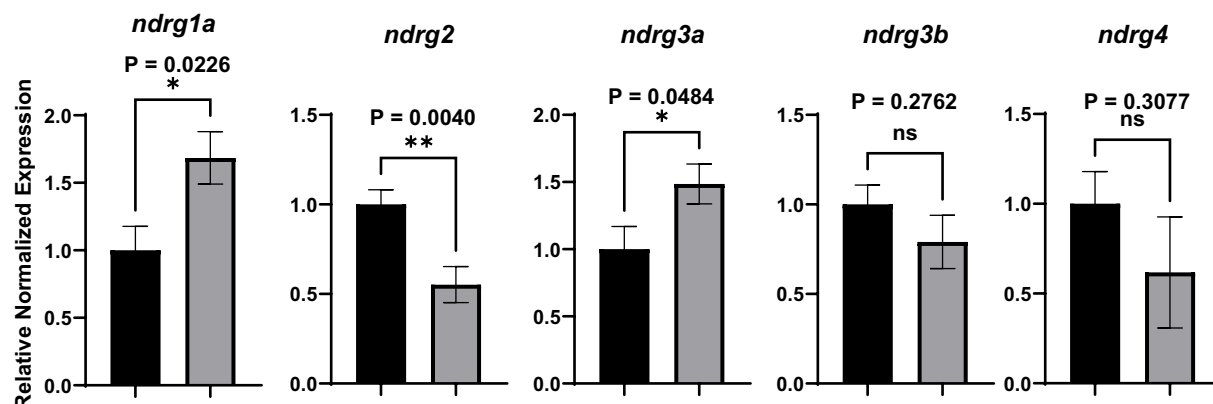
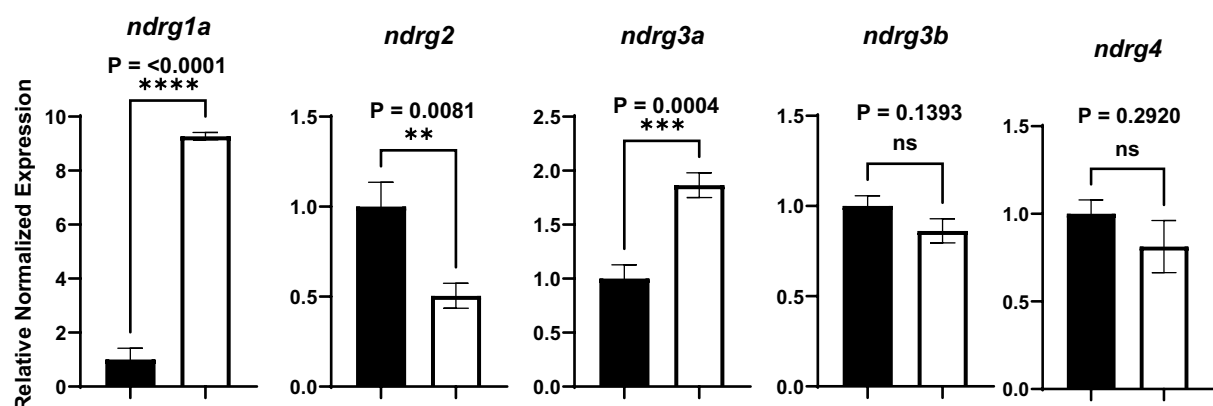
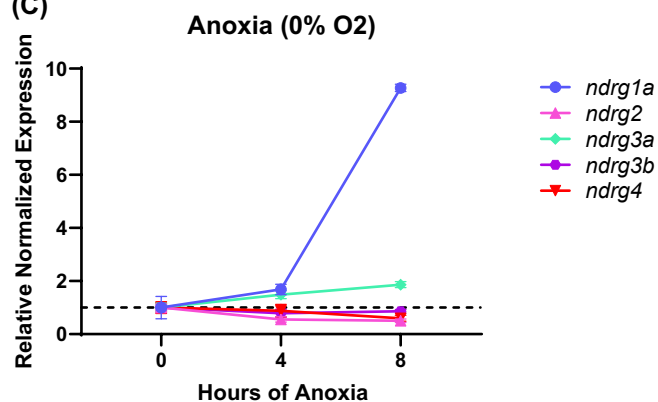
(A) 4 Hours Anoxia**(B) 8 Hours Anoxia****(C)**

FIGURE 5 Changes in *ndrg* transcript levels in response to anoxia. Real-time qPCR analysis of 24 hpf zebrafish embryos exposed to 4 h (A, grey bars) or 8 h (B, white bars) of anoxia relative to normoxic (stage-matched) controls (A, B, black bars) normalized to *ef1a*. (C) Plotted graphical summary of qPCR results. The y-axis in the graphs represents the relative normalized expression of each gene. All fold changes were derived using the formula, $2^{-(\Delta\Delta CT)}$, error bars represent standard error of the mean. Significance was obtained using the unpaired, two-tailed *t*-test with Welch's Correction. * $p < .05$, ** $p < .01$, *** $p < .0005$, **** $p < .0001$. Reactions were run in triplicate with 7–8 biological replicates ($n = 7$ –8)

duration of anoxia was extended beyond 8 h to include 12 h of treatment \pm a period of reoxygenation post-anoxia, anticipating that the mRNA transcribed *de novo* may not be

translated immediately or at all under anoxia. Following 8 and 12 h of anoxia (no reoxygenation), Ndr1a protein level was not noticeably elevated in the pronephric duct

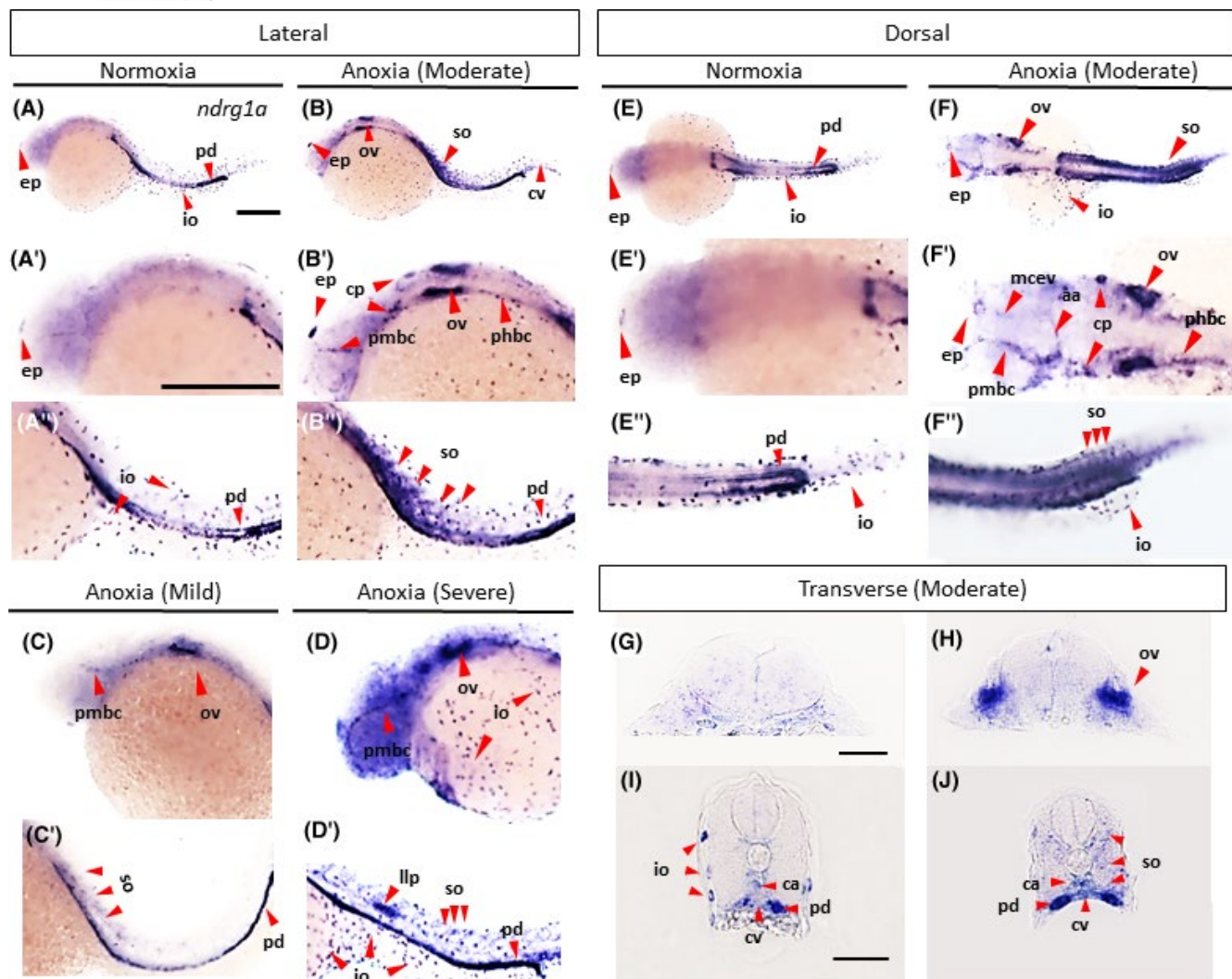


FIGURE 6 Analysis of *ndrg1a* expression in zebrafish embryos following 8 h of anoxia. Wholemount in situ hybridization analysis revealing the distribution of *ndrg1a* transcript in 24 hpf zebrafish embryo exposed to 8 h of anoxia (B–B', C–C', D–D', F–F', H, J) relative to normoxic (stage-matched controls) (A–A', E–E', G, I) imaged from a lateral view (A–D'), a dorsal view (E–F') and cross-sectional (G–J) views through the otic vesicles (G,H) and the trunk (I,J). (A'–B', E–F' are magnified views of (A,B,E,F). The mild (C,C') and severe (D,D') transcriptional responses are included to compare with the moderate response (B–B', F–F'). aa, aortic arch; cp, cranial placodes; cv, caudal vein; da, dorsal aorta; ep, epiphysis; hgc, hatching gland cells; io, ionocyte; llp, lateral line primordium; mcev, mid-cerebral vein; ov, otic vesicle; pcv, posterior cardinal vein; pmhc, primordial midbrain channel; pd, pronephric ducts; so, somites. The experiment was repeated in triplicate, with eight embryos representative of the group imaged. Scale bar in A,A', 250 μ m. Scale bar in G,I, 50 μ m

or ionocytes relative to stage-matched normoxic controls (Figure S3A–C',E), although quantification of fluorescence intensity revealed a slight increase in ionocytes (Figure S3C,F). Furthermore, with the exception of the otic vesicle, in which a weak signal was detected (Figure S3B',C'), we did not observe Ndr1a protein in the tissues in which *ndrg1a* transcript increased under anoxia. Interestingly, the addition of 3 h of reoxygenation post-anoxia (12 h) resulted in a striking increase in Ndr1a protein in the hatching gland, and, to a lesser extent, throughout the head region,

the pineal gland, the otic vesicle and the vasculature (posterior cardinal vein and dorsal aorta).

In summary, this analysis revealed that, following prolonged anoxia, *ndrg1a* transcript is maintained or enhanced in tissues in which it is present under normoxic conditions (pronephric duct, ionocytes, and epiphysis) and expanded to additional tissues (vasculature, otic vesicles, and somites). The overall increase in *ndrg1a* across multiple tissues accounts for the dramatic 9-fold up-regulation in transcript observed using qPCR (Figure 5B).

This increase in transcript levels was confirmed at the protein level in some tissues (the hatching gland most prominently), but only following a period of reoxygenation. These findings reveal hypoxia-dependent transcriptional regulation of *ndrg1a* in an intact, developing organism and identify tissues in which *ndrg1a* and other members of this family may play a protective role following hypoxia or upon return to normoxic conditions.

4 | DISCUSSION

4.1 | Expression of *ndrg* family members during early development

WISH analysis of the *ndrg* family shows that during early development (shield), *ndrgs* are broadly expressed, with the exception of *ndrg3b* that is below detection levels (shield to 24 hpf). The overlapping expression of *ndrg1a*, *1b*, *2*, *3a*, and *4* suggests that these genes may be functionally redundant. From mid-somitogenesis onward, *ndrgs* acquire more distinct spatial distribution patterns, consistent with previous studies revealing expression of members of this family in different cell types in the mouse brain⁷⁵ and organs/tissues of *Xenopus laevis* and *tropicalis* embryos.^{43,73,74}

The distribution of *ndrgs* is quite similar in fish and amphibian (*Xenopus tropicalis*) embryos. *ndrg1a* is observed in the eye, pronephric duct, intestinal bulb, and liver of zebrafish and *Xenopus* embryos.^{43,73,74} However, *ndrg1a* distribution in *Xenopus* appears broader than that of zebrafish, as it is also reported in the frog notochord, branchial arches, and pancreas. Similar to the expression pattern of zebrafish *ndrg2*, the distribution of *Xenopus ndrg2* is enriched in the nervous system; although there are also clear differences between these organisms since zebrafish *ndrg2* is prominent in the heart and somites while *Xenopus ndrg2* is found throughout the epidermis.⁴³ Zebrafish *ndrg3a* and *Xenopus ndrg3* are both present in cranial placodes and spinal cord, but the former also localizes in the pharyngeal pouches, pronephric duct, and somites while the latter is enriched in the heart and otic vesicles.⁴³ *ndrg4* is expressed throughout the nervous system in both zebrafish and *Xenopus*, but only observed in the zebrafish intermediate cell mass and proctodeum, and in the *Xenopus* pronephric duct.⁴³ Overall, these expression patterns suggest that the function of *Ndrgs* is at least partially conserved between fish and amphibians.

4.2 | *ndrgs* respond differentially to hypoxia

While transcription is an energy-demanding process that is suppressed under severe hypoxia,¹⁷ genes that mediate

hypoxia adaptation are generally up-regulated.^{14,15} Our qPCR data reveal that among the *ndrg* family members, *ndrg1a* is the most hypoxia-responsive and that prolonged (8 h) anoxia elicits the strongest increase in transcript levels. These findings corroborate with data from previous studies revealing that zebrafish and mammalian *NDRG1* have HREs in their promoter region and are up-regulated in a HIF-1 α -dependent manner in response to hypoxia.^{59,93} *ndrg3a* is also up-regulated under anoxic conditions, albeit to a lesser extent than *ndrg1a* and does not have confirmed HREs, suggesting that other transcription factors contribute to its up-regulation. We observed very high Ct values for *ndrg1b* in our qPCR experiments (data not shown), suggesting low levels of transcript. The low abundance of *ndrg1b* and *ndrg4* at 24 hpf was also reported in an RNA-seq analysis of zebrafish across developmental stages.⁸⁹ Our in situ hybridization results for *ndrg1b* also suggest low expression levels around 24 hpf (Figure 1K,L), with expression becoming first noticeable by 48 hpf in the retina. This spatio-temporal expression profile of *ndrg1b* corroborates with previously published data.⁹⁴

Our qPCR data also revealed that *ndrg2*, *3b*, and *4* are either unchanged or down-regulated, which can be explained in several ways. Unchanged values may reflect that the transcripts are stabilized, as previously reported for other genes,⁹⁵ or that the rates of synthesis and degradation are equally matched. Down-regulation could be due to mRNA decay exceeding the rate of synthesis or active repression of gene expression to conserve ATP.^{16,17,95–97} However, repression seems unlikely, as it is generally reserved for genes whose protein products are required for energetically demanding processes (e.g., *elongation factor 5A* that mediates translation).¹⁷ Given that HREs have been reported in zebrafish *ndrg1a*, *1b* and human *NDRG2* regulatory regions,^{58,93} it is surprising that our qPCR analysis revealed that transcript levels of *ndrg2* are either unchanged or decreased under low O₂. It is also possible that a milder hypoxia treatment may be required to elicit up-regulation of *ndrg2*. Surprisingly, we also did not observe significant changes in *ndrg4* transcript levels. Zebrafish *ndrg4* plays essential roles in regulating cardiomyocyte growth and proliferation,⁷² processes that should be suppressed under anoxia. *NDRG4* may be regulated by other hypoxia-responsive transcription factors⁹⁸ and may also be responsive to milder conditions than those studied here.^{99,100} Indeed, a previous study revealed that hypoxia (5%) but not anoxia exposure of 24 hpf zebrafish embryos, caused the up-regulation of *igfbp-1*, *epo*, and *vegf*.⁸⁷ Another explanation is that hypoxia-induced transcriptional regulation is dynamic and up-regulation of these genes may occur at later developmental stages, as was previously shown for *igfbp-1* and *vegf* that are up-regulated in hypoxia-exposed 36 hpf, but not 24 hpf embryos.⁸⁷

4.3 | *ndrg1a* is up-regulated in metabolically demanding tissues following prolonged anoxia

Previous studies using human cancer cells^{59,60} or homogeneous cell lines (trophoblasts)¹⁰¹ have revealed that *NDRG1* is up-regulated in response to hypoxia. However, little is known about how this response is orchestrated across multiple tissues of a whole organism. Using WISH and immunolabeling, we investigated the distribution of *NdrG1a* in 24 hpf zebrafish embryos exposed to prolonged anoxia, a treatment that elicits the most robust increase in *ndrg1a* transcript. Given that these conditions are very stringent, we reasoned that any tissue/organ in which *NdrG1a* levels are significantly increased must require the activity of this protein to adapt to low O₂.

Our study revealed that, following anoxia, *ndrg1a* is up-regulated in the epiphysis and possibly the pronephric duct, ionocytes, and endodermal organs (although the WISH procedure was not sensitive enough to detect an increase relative to the already high normoxic levels of *ndrg1a* in these cells). The epiphysis, also known as the pineal gland, receives information about the light-dark cycle from the environment and produces the hormone melatonin in response to this information. Melatonin has multiple cellular functions, including reducing oxidative stress,¹⁰² which is elevated under hypoxia and can cause cell death.¹⁰³ In addition to responding to light-dark stimuli, the pineal is also hypoxia-responsive, as stabilized Hif-1 α modulates clock gene expression in zebrafish pineal cells.^{104,105} Given that *ndrg1a* is a Hif-1 α target, it is possible that *NdrG1a* is implicated in the regulation of clock genes and melatonin production under low O₂.¹⁰⁶ The liver is quite effective at taking up O₂ and is normally well-supplied by the bloodstream; nevertheless, it is susceptible to hypoxic injury and associated complications.¹⁰⁷ In contrast, the intestine normally experiences wide fluctuations in O₂ throughout the day with some regions becoming hypoxic. Genes that aid in the maintenance of the hypoxic intestine are HIF-1 α -regulated, providing a potential explanation for the expression of *ndrg1a* in this tissue.^{108,109} The function of *ndrg1a* in the pronephric duct and ionocytes is unclear, but these cells rely on the metabolically demanding sodium-potassium ATPase pump to maintain ionic gradients and hence are likely to be sensitive to O₂ depletion.¹¹⁰

In addition to enhanced expression of *ndrg1a* in the epiphysis, we also observed expansion of *ndrg1a* distribution to tissues/organs where it is not present under normoxic conditions (or expressed at low levels), namely the inner ear (otic vesicles), head vasculature, and somites. Previous studies have demonstrated that mutations in *NDRG1* are associated with Charcot-Marie-Tooth disease type 4D (CMT4D),⁸³ a demyelinating neuropathy that

causes hearing loss in humans. Furthermore, hypoxia can cause hearing loss^{111–114}; thus it is possible that *NdrG1a* protects the inner ear or/and connected auditory nerve fibers from hypoxia-induced damage. Vascular sprouting is a well-documented hypoxic response to maximize O₂ delivery.¹¹⁵ *NDRG1* was previously shown to mediate endothelial cell migration under intermittent hypoxia,¹¹⁶ raising the question of whether its up-regulation under anoxia serves a similar purpose in head vasculature. Somites give rise to skeletal muscle cells, which experience cellular hypoxia and lactic acidosis during exercise that is further exacerbated by environmental hypoxia.¹¹⁷ It is possible that *NdrG1a* protects muscle cells from acidosis or promotes hypometabolism in these cells. The hatching gland is a transient organ that releases enzymes from cytoplasmic granules that mediate hatching of the embryo from its surrounding chorion. Hypoxia is known to induce precocious hatching in zebrafish in a matrix metalloproteinase 13-dependent manner.¹¹⁸

In contrast with *ndrg1a* transcript, protein levels did not increase significantly following prolonged exposure to anoxia. Rather, elevated protein levels were observed post-reoxygenation (in the hatching gland most prominently). It is possible that the discrepancy between RNA and protein expression following prolonged hypoxia is due to lack of recognition of a post-translationally modified form of *NdrG1a* in some tissues. Alternatively, the *ndrg1a* transcript that is synthesized under hypoxia may mostly be translated post-reoxygenation, possibly to protect cells from oxidative stress or activate other post-hypoxia adaptive responses.¹¹⁹

Even though other members of the *ndrg* family are not transcriptionally up-regulated under anoxia (or at least not as significantly as *ndrg1a*), there is evidence that they can be post-translationally modified in response to hypoxia.^{67,120–122} In this regard, it is interesting that *ndrg2*, *3a*, *3b*, and *4* are expressed in the pectoral fin buds, which are known to play a respiratory role in fish.^{123,124} Furthermore, these genes are expressed in several metabolically demanding tissues, including the brain, spinal cord, heart, and kidney.

In summary, we have shown that *ndrgs* are distributed across a range of hypoxia-sensitive/responsive tissues and that the levels of *ndrg1a* and *3a* are selectively increased following prolonged exposure to anoxia. Future studies will address whether members of this family promote hypoxia adaptation of the tissues and organs in which they are expressed.

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DISCLOSURES

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHOR CONTRIBUTIONS

N. Le executed the WISH and qPCR experiments and analyzed the data; T. Hufford executed qPCR experiments and validation, plotted and analyzed the qPCR data; J. Park optimized and validated immunolabeling reagents, performed immunolabeling experiments, and analyzed the data; and R. Brewster planned and oversaw the project and analyzed the data; N. Le wrote the first draft of the manuscript, T. Hufford and R. Brewster edited the manuscript.

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