N-myc Downstream Regulated Gene 1 (NDRG1) functions as a molecular switch for cellular adaptation to hypoxia

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ABSTRACT

Lack of oxygen (hypoxia and anoxia) is detrimental to cell function and survival and underlies many disease conditions. Hence, metazoans have evolved mechanisms to adapt to low oxygen. One such mechanism, metabolic suppression, decreases the cellular demand for oxygen by downregulating ATP-demanding processes. However, the molecular mechanisms underlying this adaptation are poorly understood. Here, we report on the role of *ndrg1a* in hypoxia adaptation of the anoxia-tolerant zebrafish embryo. *ndrg1a* is expressed in the kidney and ionocytes, cell types that use large amounts of ATP to maintain ion homeostasis. *ndrg1a* mutants are viable and develop normally when raised under normal oxygen. However, their survival and kidney function is reduced relative to WT embryos following exposure to prolonged anoxia. We further demonstrate that Ndrg1a binds to the energy-demanding sodium-potassium ATPase (NKA) pump under anoxia and is required for its degradation. Consequently, *ndrg1a* mutants that fail to downregulate NKA, have reduced ATP levels compared to WT embryos. Lastly, we show that sodium azide treatment, which increased lactate levels, was sufficient to trigger NKA degradation in an Ndrg1a-dependent manner. These findings support a model whereby Ndrg1a functions as a molecular switch for long term adaptation to hypoxia via metabolic suppression.

INTRODUCTION

Hypoxia contributes to multiple disease conditions, including acute kidney injury (Shu et al., 2019), pulmonary hypertension (Bosc, Resta, Walker, & Kanagy, 2010), obstructive sleep apnea (Douglas et al., 2010), neurodegenerative disease (Peers et al., 2009), and ischemia/stroke (Won, Kim, & Gwag, 2002). Despite these hypoxia-related pathological outcomes, low oxygen is encountered in many environments, such as at high altitude, overwintering in frozen ponds, embryonic development in utero and in the center of solid tumors. Hence, metazoans have evolved mechanisms to adapt to hypoxia and maintain homeostasis. These adaptations differ among cell types and species and the degree of protection they confer, however, they generally involve enhanced oxygen delivery, autophagy to recycle cellular constituents, and metabolic reprogramming (switch from oxidative phosphorylation to glycolysis) (Bellot et al., 2009; Guillemin & Krasnow, 1997; Semenza, 2001; Xie & Simon, 2017). Important mediators of such responses include hypoxia inducible factor 1α (Hif1α), a transcription factor that is stabilized by low oxygen (Semenza, 2012) and AMP-activated kinase (AMPK), whose activation is triggered by an increase in AMP or reactive oxygen species levels (Dengler, 2020). In addition, cells adapt to chronic and severe hypoxia by reducing their metabolic rate via suppression of ATP-consuming processes (Hochachka & Lutz, 2001). This regulation allows cells to maintain

steady ATP levels even though ATP production is decreased. As ion pumps are major utilizers of cellular ATP, a key aspect of metabolic suppression is reduction in ion pumping via downregulation of NKA (Bogdanova, Petrushanko, Hernansanz-Agustin, & Martinez-Ruiz, 2016; Buck & Hochachka, 1993; Gusarova et al., 2011). In alveolar cells, this process depends on activation of AMPK (Gusarova et al., 2011).

The NDRG family consists of four members (NDRG 1-4) that belong to the α−β hydrolase superfamily, however, they lack the catalytic domain to be enzymatically active (Shaw, McCue, Lawrence, & Dordick, 2002). Several members of this family have altered expression in cancer cells and tumor suppressor or oncogenic functions that impact cell proliferation, differentiation and metastasis (Melotte et al., 2010). NDRGs are also implicated in stress response and some members are transcriptionally up-regulated, in a Hif1α -dependent manner, following exposure to hypoxia (Angst et al., 2006; Cangul, 2004; Chen, Nelson, & Sadovsky, 2006; Melotte et al., 2010; Park et al., 2000). In hypoxic cancer cells, NDRG3 is stabilized by lactate and promotes angiogenesis and cell growth via activation of the Raf-ERK pathway (Lee et al., 2015). NDRG1 (formerly known as Drg1, Cap43, Rit42, RTP, and PROXY-1) protects human trophoblasts from hypoxic injury by triggering an anti-apoptotic response (Chen et al., 2006; Choi, Oh, Kim, Sadovsky, & Roh, 2007; Roh,

Budhraja, Kim, Nelson, & Sadovsky, 2005). While NDRGs are clearly implicated in the stress response, their physiological roles in hypoxia adaptation of normal cells are mostly unknown.

The zebrafish genome encodes six *ndrg* family members that are highly homologous to mammalian *NDRGs* (Melotte et al., 2010). We have previously shown that *ndrg1a* mRNA is up-regulated nine fold in zebrafish embryos exposed to prolonged anoxia (Le et al., under revision), despite a general suppression of transcription under severe hypoxia (Storey & Storey, 2007). These observations suggest that *ndrg1a* plays an essential role in hypoxia adaptation. *ndrg1a* is primarily expressed in the embryonic kidney (pronephric duct or PD) and ionocytes (Le et al., under revision), cell types that maintain ion homeostasis via the activity of membrane pumps and ion transporters (Hwang & Chou, 2013).

We report here on the function of *ndrg1a* in the anoxia-tolerant zebrafish embryo, which can survive up to 50 hours of anoxia in a state of reversible arrest (Mendelsohn, Kassebaum, & Gitlin, 2008; Padilla & Roth, 2001). While *ndrg1a* is expressed in the PD and ionocytes, it is not required for the differentiation or function of these cells under normal conditions (normoxia). We rather found that *ndrg1a* promotes organismal survival and protects the kidney from hypoxic injury following exposure to prolonged anoxia. We further show that, under normoxic conditions, Ndrg1a spatially overlaps with NKA in the PD and ionocytes and that

NKA degradation is accelerated in response to prolonged anoxia, in an Ndrg1a-dependent manner. Given that *ndrg1a* mutants have reduced ATP levels relative to WT embryos post-anoxia, we propose that activation of Ndrg1a contributes to metabolic suppression. Lastly, we show that an increase in intracellular lactate (the end product of glycolysis) may be sufficient to trigger NKA degradation, a response mediated by Ndrg1a. These findings support a model whereby Ndrg1a functions as a molecular switch for long term adaptation to hypoxia via metabolic suppression.

METHODS

Zebrafish husbandry and use

Wild type (WT) zebrafish (*Danio rerio*) of the AB strain were reared and manipulated using protocols approved by the Institutional Animal Care and Use Committee at the University of Maryland Baltimore County. Fish were maintained in UV-irradiated, filtered running water and exposed to a 12:12 light/dark cycle. Male and female fish were separated by a partition that was removed after the first light to initiate spawning. Embryos were collected and staged according to previously described methods (Kimmel et al., 1995). Both age and stage-matched controls were used for hypoxia/anoxia -treated embryos. The sex of the embryos used is unknown.

CRISPR/Cas9 mutagenesis to generate *ndrg1a* mutants

Target Selection. The online bioinformatics tool CHOPCHOP (https://chopchop.cbu.uib.no/) was used to identify three unique CRISPR targets for *ndrg1a* (NM_001128353, isoform 1; GRCz10). Each guide RNA (gRNA) target sequence was chosen based on homology to other sequences in the published zebrafish genome, predicted off-target effects, and self-complementation. Two gRNAs were used to target the intron-exon splice

junctions of *ndrg1a* exons 4 (gRNA1) and 5 (gRNA2), and a third gRNA was complementary to coding sequence in exon 7 (gRNA3) (Supplemental Fig. 1A). The CRISPR target sequences are as follows:

gRNA1*: 5'-CCACGTCATGTTCCTACACGGGG-3'*

gRNA2*: 5'-AGGCAATGTAACTCACCCAGTGG-3'*

gRNA3*: 5'-CTTAGAAAGGATGTAAGCACCGG-3'*

Generation of insertion-deletions (indels). Embryos were mutagenized using active ribonucleoproteins (RNPs) consisting of the Alt-R CRISPR/Cas9 high fidelity nuclear-targeted Cas9 protein (HF-nCas9) and synthetic gRNAs according to the manufacturer's recommendations (Integrated DNA Technologies; IDT). RNPs were co-injected in one-cell stage embryos in the animal pole for multiplexed editing of *ndrg1a*.

CRISPR mutant identification and characterization of the ndrg1a^{mbc1} allele. Genomic DNA sequencing of one of the F2 females identified 1-9 bp deletions in the vicinity of each of the gRNAs (Supplemental Fig. 1B). Sequencing of the corresponding transcript revealed a large deletion spanning exons 4-15 (likely caused by gRNA1) (Supplemental Fig. 1C), which resulted in a truncated protein product that lacks the α−β hydrolase fold in addition to the C-terminal three

tandem repeats and the phosphopantetheine sequence which are characteristic of NDRG1 (14) (Supplemental Fig. 1D). A homozygous mutant line was established for this allele, *ndrg1a*^{mbc1} (designated as *ndrg1a* $^{-/-}$). Immunolabeling and western blotting using anti-NDRG1 (directed against AA 82-203 of human NDRG1; UniProt accession no. Q92597) did not detect any signal in *ndrg1a*$^{-/-}$ mutants (Supplemental Fig. 1E, G). In contrast, wholemount *in situ* hybridization (riboprobe targeting the 3' end of the gene and 3'UTR) revealed that the transcript is expressed at normal levels (Supplemental Fig. 1F). The failure to detect protein is therefore likely to be a consequence of protein truncation.

Morpholino-mediated Ndrg1a knockdown

Morpholinos (MOs) targeting the translational start site of *ndrg1a* (Supplemental Fig. 1A) and the human β-globin gene (standard, control MO) (Schmajuk, Sierakowska, & Kole, 1999) were commercially obtained (Gene Tools, LLC) and administered at 2nl/embryo using 0.1mM working solution. Each MO was injected at the 1–4 cell stages into the yolk margin just underneath the embryo proper. The MO sequences are as follows:

ndrg1a (MO1): 5'- CGTCCATTCTCAACGAGGACACCCG -3'

Standard control MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'

Specificity of the *ndrg1a* MO1 was confirmed using immunolabeling and western blotting, which revealed that Ndrg1a protein was absent or greatly reduced in MO-injected embryos (Supplemental Fig 1E, G). As expected, *ndrg1a* transcript levels, detected using whole-mount *in situ* hybridization, appeared normal in *ndrg1a* MO-injected embryos Supplemental Fig 1F).

Whole-mount *in situ* hybridization

Embryos were fixed in 4% paraformaldehyde (PFA) at the desired stages and processed for *in situ* hybridization as previously described (Thisse and Thisse, 2008), using riboprobes against zebrafish *atp1a1a1.2, kcnj1a.1, ndrg1a, slc12a3, slc20a1a*. Primers used for probe design were as follows: The underlined font indicates the T7 RNA polymerase binding site.

atp1a1a1.2:

FWD: 5'-CTTATGGAATCCAGGTGGCATC-3'

REV: 5'-<u>TAATACGACTCACTATAG</u>GCAGTACCTTCAACACAGTTGG-3'

kcnj1a.1:

FWD: 5'-CCTGGAGGTGTGGCTTTGAT-3'

REV: 5'-<u>TAATACGACTCACTATAG</u>AGCCATGCCATCGAGGAAAA-3'

ndrg1a:

FWD: 5'-AGCTCACACCTCCGAAAACC-3'

REV: 5'-TAATACGACTCACTATAGACGATGTCTCTGTGCTGCAT-3'

slc12a3:

FWD: 5'-ATGGGAATCCAAGGCCCAAG-3'

REV: 3'-TAATACGACTCACTATAGACACAGGAGCCAATGGTAGC-3'

slc20a1a:

FWD: 5'-ACCATCTTTGAGACAGTGGGTG-3'

REV: 5'-TAATACGACTCACTATAGCAGCCCAGTGAGATTAGCAGGG-3'

Immunolabeling and other labeling procedures

Immunolabeling. Embryos were fixed in 4% paraformaldehyde (PFA) at 4°C overnight and washed in 1x PBS for 30 min. Fixed embryos were permeabilized with cooled acetone for 5 min at -20°C, followed by a 5 min wash in 1x PBS. Embryos were subsequently incubated in Inoue blocking solution (Inoue & Wittbrodt, 2011) (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 1x PBS for 1 hour 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 1x PBS) for 2 days at 4°C on a rotating platform (80 RPM). Embryos were then washed three times, 30 min

each, with 1x PBS at room temperature. Secondary antibodies, diluted in I-buffer, were applied for 1 day at 4°C on a rotating platform (80 RPM). After secondary antibody incubation, embryos were washed three times with 1x PBS for 30 min. Primary antibodies: anti-NDRG1 @ 1:500 (Sigma Aldrich, catalog # HPA006881, lot# A69409, rabbit polyclonal), anti-ATP1A1A @ 1:500 (DHSB, catalog # a5s, lot# 10/17/19, mouse monoclonal), anti-GFP @ 1:500 (Invitrogen, cat# A21311, lot# 2207528, rabbit polyclonal, Alexa Fluor 488 conjugated), anti-ZO-1 @ 1:500 (Invitrogen, cat#40-220D*, lot# UB280595, rabbit polyclonal). Secondary antibodies: Goat anti-rabbit Alexa Fluor 594 (Abcam, cat# ab150080, lot# GR3373513-1), Goat anti-mouse Alexa Fluor 488 @ 1:500 (Abcam, catalog # ab150113, Lot#GR3373409-1). Other labels added after secondary antibodies. Phalloidin-Alexa 594 (Invitrogen, cat#A12381, lot# 2256805), Phalloidin-Alexa 405 (Invitrogen, cat#A30104, lot# 2277734) and DAPI (Thermo Scientific, cat# 62248, lot# WD3246961) were used according to the manufacturer's instructions.

Whole-mount proximity ligation assay (PLA). To identify *in situ* protein-protein interactions, PLA was performed using the PLA Duolink kit (Sigma Aldrich, catalog# DUO92101). The protocol was performed according to the manufacturer's instructions with the following modifications: All steps were performed in 96-well plates, 40 mM final volume, at RT, rotating (80 RPM).

Embryos were incubated in primary antibody solution at RT overnight. Embryos were then treated with Duolink PLA probes at RT overnight, followed by incubation in the ligation solution at RT overnight and an additional 1 hour at 37°C. Between primary antibody, Duolink PLA probe, and ligation solution incubations, samples were washed with 1x Wash Buffer A at RT for 3 x 20 min. Embryos were incubated in the amplification solution at RT overnight, followed by 1 hour at 37°C. Lastly, samples were washed in 1x Wash Buffer B at RT for 3 x 20 min, then in 0.01x Wash Buffer B at RT for 20 min.

Sectioning and microscopy

Sectioning embryos. Transverse sections (40 μm) of fixed and labeled embryos were obtained using a vibratome (Vibratome, 1500). Embryos were mounted in agarose (4 %) blocks prior to sectioning. Sections through the anterior and posterior pronephric duct were distinguished based on tissue morphology, as the distance between the two tubules is larger in the anterior region.

Compound microscopy. Live, dechorionated embryos were immobilized using the sedative tricaine methanesulfonate at 0.001% w/v in embryo medium E3 (5mM NaCl, 0.33mM CaCl2, 0.17mM KCl, 0.33mM MgSO4). Sedated or fixed embryos were mounted on depression slides in E3 medium. Lateral views of 24-

48 hpf embryos were viewed at 50X magnification using an Axioskop II compound microscope (Carl Zeiss, Axioscope II) and imaged with an AxioCam (Carl Zeiss, 504 CCD camera).

Confocal microscopy. For lateral views, 24-48 hpf embryos were mounted in E3 medium on glass-bottom culture dishes with size #1.5 coverslips (Mattek, cat# P50GC-1.5-14-F). Images were captured with a 5X air objective lens using an inverted SP5 laser scanning confocal microscope (Leica Microsystems). For imaging transverse sections of embryos, a 63X oil objective lens was used. Images were analyzed and processed using FIJI ImageJ (National Institutes of Health) and PowerPoint (Microsoft). When comparing protein levels between embryos in the same experimental repeat, all specimens were processed using identical settings, including gain.

Western blot Analysis

Embryos were dechorionated and mechanically lysed in precooled cOmplete Protease Inhibitor Cocktail (Millipore Sigma, catalog# 11836145001). The bicinchoninic acid (BCA) protein assay was used to determine the total protein concentration in embryo lysates. Western blot analysis was performed by loading 7.5 g of total protein per lane into 7.5% Tris-glycine SDS-PAGE gels (Bio-Rad,

catalog #4561026) and transferring to methanol-activated PVDF membranes (Bio-rad, cat# 1620177. Specific protein detection was performed using diluted primary antibodies as follows: anti-NDRG1 @ 1:5000 (Sigma-Aldrich, catalog# HPA006881, rabbit polyclonal); anti-GAPDH @1:5000 (GeneTex, catalog #GTX124503; batch: RRID:AB_11165273, rabbit polyclonal). The secondary antibody used is Goat anti-rabbit @ 1:10,000 (Cell Signaling Technology, catalog #7074; RRID:AB_2099233). Molecular weights were determined using pre-stained protein ladders (Thermo Fisher Scientific, catalog #26616; GE Healthcare, catalog #RPN800E). Protein abundance was detected on membranes using the SuperSignal West Femto enhanced chemiluminescence kit (Thermo Fisher Scientific, catalog #34095) and Blu-C high-contrast autoradiography film (Stellar Scientific, catalog #BLC-57-100).

Drug treatments

Sodium azide (NaN3) (catalog #S2002-5G) was used at 300 μM concentration in E3 embryo medium to increase endogenous lactate levels, as previously described (Oginuma et al., 2017). Since sodium azide arrests or delays development, age and stage-matched untreated controls were used.

Protease inhibitor MG-132 (EMD Millipore Corp, cat# 474791-5MG, lot# 3537249) and autophagy inhibitor Chloroquine (Sigma Aldrich, cat# C6628-50G, batch # 0000090940) were used at 100 μM and 1 mM, respectively in E3 embryo medium.

Hypoxia and anoxia treatments

For all hypoxia experiments, oxygen levels were controlled using a PLAS LABS' Controlled Environmental Chamber (model# 856-HYPO). A combination of pure nitrogen (N2) (Airgas) and room air was perfused into the chamber and resulting oxygen concentrations were measured using the PLAS LABS' built-in oxygen sensor. Additionally, a handheld portable oxygen meter (Yellow Springs Instruments (YSI) Item# 626972) was placed inside of the hypoxia chamber to directly monitor oxygen levels in E3 embryo medium. E3 medium was placed in the PLAS LABS chamber in a beaker and aerated using an air pump to accelerate equilibration of dissolved oxygen levels with the chamber's ambient oxygen level for 12 hours before experimentation.

Survival tests

Anoxia survival. Survival was assayed immediately following anoxia exposure. Embryos were examined for severe tissue necrosis and deterioration using bright field imaging.

Anoxia + re-oxygenation survival. Embryos that survived anoxia were transferred to normoxic water and examined after two days of re-oxygenation for signs of viability, including heartbeat and response to touch. The surviving embryos were further categorized into either mild or severe edema cases. All survival experiments were performed at least three times with > 25 embryos per experiment.

Kidney clearance assay

Kidney function was examined in WT and *ndrg1a*$^{-/-}$ mutants using the kidney clearance assay (KCA) (Christou-Savina, Beales, & Osborn, 2015).

KCA using embryos raised under normoxic conditions. WT embryos and *ndrg1a*$^{-/-}$ mutants were injected at 48 hpf in the pericardial cavity with rhodamine dextran (10,000 MW; Invitrogen, cat# D1824, lot# 2270252; working concentration of 5 mg/ml in distilled water). Fluorescence in the heart chamber of injected embryos was imaged and quantified 3 and 24 hours post-injection (hpi)

using an Axioskop II compound microscope (Carl Zeiss) and AxioCam 504 CCD camera (Carl Zeiss). Embryos were imaged at 50 X total magnification using identical exposure settings. Images were taken in an area of interest centered on the heart and analyzed and processed using ImageJ (National Institutes of Health).

KCA using embryos exposed to anoxia followed by re-oxygenation. Same procedure as described above but with the following modifications: 24 hpf WT embryos and *ndrg1a*$^{-/-}$ mutants were exposed to 12 h of anoxia, followed by injection of rhodamine dextran (under normal oxygen) and two days of re-oxygenation.

ATP assay

24 hpf WT embryos and *ndrg1a*$^{-/-}$ mutants were exposed to 0, 6, 12 h of anoxia and snap-frozen in liquid nitrogen immediately post-anoxia treatment and removal of E3 medium. Metabolites were extracted and ATP concentration (mM/embryo) measured using a luminescent ATP Detection Assay kit (Abcam, catalog # ab113849), according to the manufacturer's instructions.

Lactate assay

Lactate levels were measured in 24 hpf WT and *ndrg1a*$^{-/-}$ mutant embryos exposed to either anoxia or sodium azide. For each treatment group, 20 embryos were snap-frozen in liquid nitrogen after embryo medium removal and kept at - 80°C until further processing. The L-lactate fluorometric kit (BioVision, catalog #K607) was used according to the manufacturer's instructions for small biological samples. No-sample background control fluorescence was subtracted from the experimental samples' fluorescence to account for background fluorescence.

Metabolomic analysis

A metabolomic analysis was performed on 5 hpf WT embryos exposed to 1 hour of anoxia and age-matched normoxic controls (5 hpf). 400 µl of methanol and 400 µl of water were added as a polar solvent to decant the embryos over ice. After decanting, 400 µl of isopropanol was added as a nonpolar solvent. Samples were rocked at 4º C for 20 min and centrifuged at 13,000 g for 5 min. The water-soluble metabolites (top of phase separation) was extracted and stored at - 80ºC. Four biological replicates were obtained and 10 embryos were used per sample. Mass spectra were acquired using a Phenomenox Luna NH2 column connected to an Agilent 1200 HPLC and Agilent 6210 ESI-TOF mass spectrometer in a

negative ion mode. Data were analyzed with Agilent software packages (MassHunter, Mass Profiler, Profinder and Mass Profiler Professional) using 1,000 ion counts as an arbitrary cutoff. An extracted ion chromatograph (EIC) was then generated for each ion. Ions that did not show clear peaks were discarded from the subsequent analysis. Ions were identified by comparing molar mass against Mzed database (Draper et al., 2009). The lactate peak (observed: 89.0240, calculated mass for M-H ions is 89.0239) was extracted, and the area was used for comparison.

Alignment of NDRG Family Members

The protein sequences of several vertebrate NDRG family members (human: NDRG3, NP_114402.1; mouse: NDRG3, NP_038893.1, zebrafish: Ndrg1a, NP_998513.2, zebrafish: Ndrg1b: NP_956986.2, zebrafish Ndrg3a: NP_955811.1, zebrafish Ndrg3b: XP_005162043.1) were aligned using the Clustal Omega Multiple Sequence Alignment software (https://www.ebi.ac.uk/Tools/msa/clustalo/) to identify homologous regions of interest.

Measurements

Fluorescence intensity measurements of the pronephric duct. Fluorescence intensity corresponding to ATP1A1A protein levels in the anterior and posterior pronephric duct was measured using lateral views of immunolabeled embryos. Two regions of interest (ROI) centered along the anterior or posterior halves of the pronephric duct were delineated for intensity measurements using ImageJ (FIJI) software.

Fluorescence intensity measurements of ionocytes. Fluorescence intensity corresponding to ATP1A1A protein levels in ionocytes was measured by drawing a tight ROI around individual ionocytes (5-10) in the yolk ball and flank region demarcated by the distal ends of the yolk extension using ImageJ (FIJI).
Ionocyte length: Ionocyte length (micron) was measured by drawing a line along the longest axis of the cell.

Experimental design and statistical analysis

Graphing and statistical analysis were performed using Prism 9 (Graph Pad). Statistical significance was declared in circumstances where $p <$ or $= 0.05$. Experiments comparing three or more groups were analyzed using a two-way ANOVA test. An unpaired t-test was used when comparing means of two

independent groups. Data were reported as the mean +/- SEM unless otherwise indicated.

RESULTS

ndrg1a is not required for kidney and ionocyte differentiation or function

We have previously shown that *ndrg1a* is a hypoxia-responsive gene whose transcript levels increase proportionately with the severity and duration of hypoxia (Le et al., under review), suggesting that *ndrg1a* is implicated in hypoxia adaptation. However, others have shown that NDRG1 plays a key role in kidney cell differentiation in *Xenopus* (Kyuno, Fukui, Michiue, & Asashima, 2003; Zhang, Guo, & Chen, 2013), which, if conserved, would preclude directly testing the function of this NDRG family member in cell physiology. We therefore began by investigating whether differentiation of PD cells and ionocytes is impaired in Ndrg1a-deficient zebrafish embryos.

Ndrg1a was targeted using a translation-blocking morpholino (MO) and CRISPR/Cas 9 mutagenesis to produce a null allele, *ndrg1a*^{mbc1} (Supplemental Fig. 1). *ndrg1a*^{mbc1} mutants (referred to henceforth as *ndrg1a*$^{-/-}$) are homozygous viable and morphologically normal (Supplemental Fig. 1E, F). Whole-mount *in situ* hybridization (WISH) using several markers (*slc20a1a*: anterior PD, *slc12a3*: posterior PD, *atp1a1a*, the catalytic subunit of NKA: entire length of the PD and all ionocytes and *kcnj1a.1*: middle part of the PD and ionocytes), revealed that the distribution of these transcripts is normal in *ndrg1a* loss-of-function (LOF) embryos at 24 hpf, when PDs are fully formed (Kimmel, Ballard, Kimmel,

Ullmann, & Schilling, 1995) (Fig. 1A). Furthermore, the expression of *slc20a1a* and *atp1a1a* is properly maintained in 48 hpf *ndrg1a* LOF embryos (Supplemental Fig. 2A), when the kidney becomes fully functional (Drummond et al., 1998). Immunofluorescence using anti-ATP1A1A and anti-GFP in the transgenic line Tg[*enpep:GFP*] (entire length of the PD) further confirmed proper protein expression in the PD (Fig. 1B).

Kidney physiology is tightly linked to cell polarity, which enables the directional flow of ions. Analysis of the subcellular distribution of polarity markers F-actin and Zonula-Occludens-1 (ZO-1) showed that *ndrg1a* is not required for apico-basal polarization of PD cells (Supplemental Fig. 2B).

We next tested the function of the embryonic kidney using a kidney clearance assay (KCA) (Christou-Savina et al., 2015). Rhodamine dextran was injected into the pericardial cavity of 48 hpf embryos and the ability of the kidney to clear the rhodamine dextran was tested by measuring fluorescence intensity after 3 and 24 hours post-injection. This analysis revealed that kidney filtration is normal in *ndrg1a*$^{-/-}$ mutants (Supplemental Fig. 2C, D).

These findings indicate that *ndrg1a* is neither required for the differentiation of the PD and ionocytes nor for the function of the PD under normoxic conditions, enabling us to directly test the function of *ndrg1a* in physiological adaptation to hypoxia.

Ndrg1a promotes organismal and cell survival under prolonged hypoxia

To test whether *ndrg1a* promotes hypoxia survival, we exposed 24 hpf embryos to increasing durations of anoxia (6, 12, 18 and 24 h) and scored viability immediately post-treatment. This analysis showed that WT embryos survived prolonged anoxia exposure somewhat better than *ndrg1a* $^{-/-}$ mutants (18 h: WT 78.5% vs. *ndrg1a* $^{-/-}$ 52.6%; 24 h: WT 47.8% vs. *ndrg1a* $^{-/-}$ 23.2%) (Fig. 2A). However, when surviving embryos were returned to normal oxygen for 2 days, we noticed a dramatic reduction in viability of *ndrg1a* $^{-/-}$ mutants relative to WT embryos (18h: WT 79.0% vs. *ndrg1a* $^{-/-}$ 35.5%; 24h: WT 58.8% vs. *ndrg1a* $^{-/-}$ 7.1%) (Fig. 2B). Furthermore, a large percentage of the *ndrg1a* $^{-/-}$ mutants that survived immediately post-anoxia developed mild or severe edema (Fig. 2C) (6h: WT 97.3% no edema, 2.7% mild edema, 0% severe edema vs. *ndrg1a* $^{-/-}$ 69.5% no edema, 30.5% mild edema, 0% severe edema; 12h: WT 94.5% no edema, 5.5% mild edema, 0% severe edema vs. *ndrg1a* $^{-/-}$ 65.2% no edema, 28.2% mild edema, 6.6% severe edema; 18h: WT 93.1% no edema, 6.9% mild edema, 0% severe edema vs. *ndrg1a* $^{-/-}$ 50% no edema, 8.8% mild edema, 41.2% severe edema). Edema results from water retention and has multiple proximal causes, including malfunction of the PD (Elmonem et al., 2018) and ionocytes (Hwang & Chou, 2013). We therefore tested whether renal damage occurred post-anoxia using the kidney clearance assay. 24 hpf embryos were subjected to 12 h of anoxia and 2

days of re-oxygenation, following which rhodamine dextran was injected into their pericardial cavity and fluorescence measurements were made 3 and 24 hours post-injection. This analysis revealed that *ndrg1a* $^{-/-}$ mutants retained more rhodamine dextran than WT embryos post-anoxia (WT: 44.3% fluorescence vs. *ndrg1a* $^{-/-}$ mutants: 60.3% fluorescence)(Fig. 2D, E), indicative of reduced kidney filtration capability.

Together, these data suggest that *ndrg1a* protects the kidney (and possibly ionocytes) from hypoxia-induced cellular damage, which reduces edema formation and enhances survival.

NKA is downregulated following prolonged anoxia

The expression of *ndrg1a* in the PD and ionocytes and the edema observed in mutants exposed to severe and prolonged hypoxia suggest that Ndrg1a plays a regulatory role, perhaps by controlling ion homeostasis. Of the multiple ion channels and pumps expressed in these cells (e.g. Fig. 1), the catalytic subunit of the NKA pump, ATP1A1A, was selected for further analysis. This enzyme is estimated to consume 30% of the cell's energy in maintaining Na^+ and K^+ gradients (Pirahanchi, Jessu, & Aeddula, 2021). Given this high-energy demand, an evolutionarily conserved response of cells exposed to low oxygen is to

downregulate NKA activity or to degrade the pump itself (Bogdanova et al., 2016; Buck & Hochachka, 1993; Gusarova et al., 2011).

To address whether Ndrg1a and ATP1A1A interact in the zebrafish embryo, we began by examining their distribution. There are five genes encoding the alpha 1 subunit in the zebrafish genome (*atp1a1a.1-atp1a1a.5*), each with a distinct expression profile (Blasiole et al., 2002; Canfield et al., 2002). The distribution of *atp1a1a.2* is very similar to that of *ndrg1a*, both of which are expressed in the PD and ionocytes (Fig. 3a1,a2). Double immunolabeling using anti-NDRG1 and anti-ATP1A1A (against the chick alpha 1 subunit) confirmed that Ndrg1a and ATP1A1A are co-expressed in the kidney and ionocytes (Fig. 3a3), although a small percentage of ionocytes are positive for only Ndrg1a or ATP1A1A (magnified image Fig 3a3, colored boxes in Fig. 4a1).

We next tested whether NKA is hypoxia-responsive in zebrafish by examining ATP1A1A levels in embryos that were exposed to different durations of anoxia. Under normoxic conditions (0 h time point), ATP1A1A was distributed along the entire length of the PD but its levels were noticeably higher in the posterior PD (Fig. 3b1). Following 6 h of anoxia, the intensity of the signal was reduced throughout the PD, but most noticeably in the anterior region (open arrowheads in Fig. 3b2; quantified in Fig. 3C). By 12 h of anoxia, ATP1A1A levels were further decreased along the full anterior-posterior extent of the PD

(open arrowheads in Fig. 3b3; quantified in Fig. 3C). Additional timepoints of anoxia (18 and 24 h) resulted in further NKA downregulation (WT anterior PD: 48% reduction of NKA level between 0h and 24h anoxia; WT posterior PD: 49% reduction of NKA level between 0h and 24h anoxia) (Fig. 3C), but not complete depletion, consistent with prior observations in human and rat lung cells (Wodopia et al., 2000). Transverse sections of WT embryos subjected to anoxia and double-labeled with anti-ATP1A1A and the cell surface marker phalloidin (cortical F-actin) revealed that NKA levels were gradually reduced in the baso-lateral plasma membrane compartment of anterior (Fig. 3d1-d3', top panels) and posterior (Fig. 3d4-d6', bottom panels) PD cells.

ATP1A1A was also downregulated under anoxia in ionocytes that co-expressed Ndrg1a (33% reduction of NKA level between 0h and 24h anoxia), (yellow boxes in Fig. 4a1-b3, a1'-b3', quantified in Fig. 4B). In contrast, ionocytes that were Ndrg1a-negative retained high levels of ATP1A1A following 6 and 12 h of anoxia (green boxes in Fig 4a1-b3, quantified in Fig. 4B). These observations reveal a negative correlation between the presence of Ndrg1a and ATP1A1A levels.

We also observed that the downregulation of ATP1A1A is reversible. Indeed, following 18 h of anoxia, ATP1A1A levels in both the PD and ionocytes

increased steadily when embryos were re-introduced to normoxia, plateauing at 6 h post-re-oxygenation (anterior PD) (Supplemental Fig. 3).

Overall, these data indicate that downregulation of NKA is a gradual, long-term response to anoxia that is reversible when oxygen levels return to normal.

Degradation of NKA is Ndrg1a-dependent

The spatial overlap between Ndrg1a and ATP1A1A and the negative correlation between the levels of these proteins in ionocytes, suggested that Ndrg1a may control NKA abundance under hypoxia. To test this, *ndrg1a* $^{-/-}$ mutants were exposed to different durations of anoxia and ATP1A1A levels were quantified. Unexpectedly, analysis of normoxic controls (0 h anoxia time point) revealed that the PD (Fig. 3b1 vs. b4, C) and ionocytes (Fig. 4b1 vs. c1, B) of mutants expressed higher levels of ATP1A1A than cells in WT embryos (58% and 20% increase in anterior and posterior PD, respectively, of mutant vs. WT), suggesting that Ndrg1a regulates ATP1A1A levels, even under normal oxygen. However, despite these elevated normoxic levels, the relative amount of ATP1A1A in the PD of mutants was only marginally reduced following prolonged anoxia compared to WT embryos in which ATP1A1A levels decreased proportionately to time of anoxia exposure (24 h anoxia, anterior PD: 8.3% reduction for *ndrg1a*$^{-/-}$ vs. 48% reduction for WT; 24 h anoxia, posterior PD: 25% reduction for *ndrg1a*$^{-/-}$

vs. 49% reduction for WT) (Fig. 3b2,b3 vs. b5, b6, C) and even slightly increased in ionocytes of mutants (7% increase) (Fig. 4B).

Furthermore, analysis of cross sections double-labeled with phalloidin showed that ATP1A1A mostly remained localized in the baso-lateral membrane of PD cells (Fig. 3D) in *ndrg1a* $^{-/-}$ mutants, suggesting that the pump is not endocytosed.

These data suggest that Ndrg1a negatively regulates NKA abundance in the plasma membrane under normal oxygen and that this activity is somehow enhanced or accelerated in response to prolonged anoxia.

Ndrg1a and NKA interact under severe hypoxia

A previous study identified ATP1A1A as a potential binding partner of NDRG1 in human prostate cancer cells (Tu, Yan, Hood, & Lin, 2007), raising the question of whether Ndrg1a physically interacts with ATP1A1A to bring about its downregulation. ATP1A1A is a transmembrane protein, in contrast to NDRG1 whose peptide sequence does not include an obvious membrane localization signal (Shi, Larkin, Chen, & Sadovsky, 2013). Furthermore, NDRG1 intracellular distribution appears to vary between cell types and in response to environmental signals, including hypoxia (Caruso et al., 2004; Kim et al., 2004; Kurdistani et al., 1998; Lachat et al., 2002; Shi et al., 2013; Sibold et al., 2007; Song et al., 2010).

Given that ATP1A1A downregulation is triggered downstream of hypoxia, in an Ndrg1a-dependent manner, we reasoned that these proteins may co-localize near the cell cortex. This was confirmed in embryos that were double-labeled with anti-NDRG1 and anti-ATP1A1A. Under normoxic conditions, we observed that Ndrg1a was distributed throughout the cytosol and overlapped extensively with ATP1A1A at the cell cortex of ionocytes (Fig 4a1') and PD cells (Fig. 5a1, a4). The overlap was reduced following 6 and 12 h of anoxia in both the ionocytes (insets in Fig. 4a2, a3**)** and the anterior PD, coinciding with significant downregulation of ATP1A1A (Fig. 5a2, a3). In contrast to the anterior PD, the posterior kidney showed extensive overlap of Ndrg1a and ATP1A1A at the cell cortex, even after 12 h of anoxia (Fig 5a5, a6), which is consistent with retention of NKA in the distal kidney tubule (Fig. 3b3, d6) and indicative of distinct physiological properties of these cells.

To test whether Ndrg1a binds to ATP1A1A, we adapted the proximity ligation assay (PLA) for use on whole-mount zebrafish embryos. PLA, uses antibodies to two proteins of interest associated with DNA primers; a productive PCR product is produced and detected by fluorescent labeling only when the two antibodies are within less than 40 nm, suggesting that the two target proteins are in close physical proximity and may interact (Weibrecht et al., 2010). PLA performed on anoxia-treated embryos using anti-NDRG1 and anti-ATP1A1A

antibodies revealed a low signal in the PD for the 0 h anoxia time point (Fig. 5b1, c1, c4), which noticeably increased by 6 and 12 h of anoxia (Fig. 5b2, b3, c2, c3, c5, c6). Cross sections further showed that the PLA signal in the anterior PD was scattered, localizing to subcortical and possibly intracellular storage compartments (Fig. 5c2, c3), whereas it appeared mostly associated with the cell cortex in the posterior PD (Fig. 5c5, c6). No PLA signal was detected in *ndrg1a*$^{-/-}$ mutants, confirming that the assay is specific for Ndrg1a-ATP1A1A interaction (Fig. 5b4-b6).

The ability to detect strong PLA signal after 12 h of anoxia, a time point that correlates with significantly reduced ATP1A1A levels in the PD, is consistent with the high level of sensitivity of this assay and indicates that interaction between Ndrg1a and ATP1A1A is enhanced by hypoxia.

NKA is downregulated via lysosomal and proteasomal degradation pathways

While ATP1A1A protein levels are not fully depleted following prolonged anoxia, they are greatly reduced. Degradation of transmembrane proteins usually occurs through the lysosome, although these proteins, including potentially NKA, can also be directed to the proteosome (Helenius, Dada, & Sznajder, 2010; Hirsch & Ploegh, 2000). Hence, blocking lysosomal or/and proteasomal degradation is expected to enhance ATP1A1A levels in embryos exposed to anoxia. To

determine the ATP1A1A degradation route, WT embryos were exposed to anoxia (12 h) and simultaneously incubated in either MG-132 (26S proteasome inhibitor) or Chloroquine (autophagy inhibitor) (Mathai, Meijer, & Simonsen, 2017) or a combination thereof, and immunolabeled using anti-ATP1A1A. Measurements of fluorescence intensity showed that either drug slightly enhanced ATP1A1A levels in the PD relative to control embryos (12h anoxia, no drug) (Fig. 5d2-d4, E) (anterior PD: 7% increase under 12 h anoxia + MG-132 vs. 12 h anoxia, no drug; 59% increase under 12 h anoxia + Chloroquine vs. 12 h anoxia, no drug; posterior PD: 24% increase under 12 h anoxia + MG-132 vs. 12 h anoxia, no drug; 36% increase under 12 h anoxia + Chloroquine vs. 12 h anoxia, no drug), but the combined use of both drugs had the strongest effect relative to control embryos (12h anoxia, no drug) (Fig. 5d5, E) (anterior PD: 87% increase under 12 h anoxia + MG-132 and Chloroquine vs. 12 h anoxia, no drug; posterior PD: 45% increase under 12 h anoxia + MG-132 and Chloroquine vs. 12 h anoxia, no drug). These results suggest that ATP1A1A is partially degraded following prolonged anoxia via both lysosomal and proteasomal pathways. It is possible that Ndrg1a mediates both degradation pathways given that previous studies have identified three subunits of the 26S proteasome (Tu et al., 2007) and lysosomal-associated membrane protein 1 (LAMP1) (Liu et al., 2018) as putative NDRG1 binding partners

Ndrg1a preserves ATP levels under severe hypoxia

Hypoxia-induced downregulation of NKA is thought to be a key aspect of metabolic suppression, the ultimate goal of which is to preserve cellular energy (Bogdanova et al., 2016; Buck & Hochachka, 1993; Gusarova et al., 2011). To assess whether the activity of Ndrg1a contributes to the establishment of hypometabolism, the total level of ATP (from whole embryo lysates) was quantified using a luminescent ATP assay (Fig. 5F). We found that ATP levels were similar between WT and *ndrg1a*$^{-/-}$ mutants under normoxia (0 h anoxia time point). Surprisingly, ATP concentration transiently increased in embryos of both genotypes (6 h anoxia: WT: 47% increase; *ndrg1a*$^{-/-}$: 50% increase) before declining after 12 h of anoxia. Remarkably, despite the fact that Ndrg1a-positive cells represent a small fraction of total cell number in the embryo, we observed that the reduction in ATP levels was more pronounced in the mutants than in WT embryos for this duration in anoxia (WT: 21.6% decrease; *ndrg1a*$^{-/-}$: 33.2% decrease). Given that WT and *ndrg1a*$^{-/-}$ embryos have similar survival rates following 12 h of anoxia exposure (Fig. 2A), the reduced ATP levels in mutants cannot readily be explained by lethality.

These results therefore suggest that Ndrg1a promotes energy conservation following prolonged exposure to anoxia, which coincides with a period of significant NKA downregulation. The transient rise in ATP is likely due to

sustained oxidative phosphorylation between 0 and 6 h of anoxia, topped by an increase in glycolysis.

Lactate as a candidate proximal signal for metabolic suppression

Thus far, our data suggest that activation of Ndrg1a accelerates NKA degradation following prolonged anoxia. However, the proximal signal that activates Ndrg1a downstream of anoxia is unknown. We reasoned that this signal could be a small molecule whose levels accumulate following hypoxia exposure. To gain insight into the identity of this putative signal, we carried out metabolite profiling using pre-gastrula zebrafish embryos. Early embryos have a relatively homogenous cell population, facilitating the identification of candidate small molecules. Furthermore, they arrest rapidly following anoxia exposure (Padilla & Roth, 2001), which provides a convenient read out for metabolic suppression. We therefore carried out metabolite profiling using dome-stage WT embryos subjected to 1 h of anoxia and age- and developmental stage-matched controls raised under normoxic conditions. Following extraction of metabolites, mass spectrometry was performed to identify small molecules whose levels are either up or down regulated (Fig. 6A-C). This analysis revealed three candidate ions, sulfite, sulfate and lactate, which lied outside of the 99th percentile confidence interval for outliers (a-c in Fig. 6A). We chose to further pursue lactate, given that

this metabolite, the end product of glycolysis, was previously shown to bind to NDRG3 in hypoxic cancer cells, resulting in NDRG3 stabilization and activation of Ras-Erk signaling (Lee et al., 2015).

Lactate accumulation in anoxic samples was verified by measuring HILIC LC-MS extracted ion counts (Fig. 6B) and quantified using integrated ion count areas (Fig. 6C). To test whether an increase in L-lactate also occurs in older 24 hpf embryos and determine the temporal profile of lactate accumulation, we performed a lactate fluorometric assay (Fig. 6D). This analysis revealed that lactate concentration increased linearly with time in anoxia, consistent with previous observations (Podrabsky, Lopez, Fan, Higashi, & Somero, 2007; Van Heel, 2001; Vianen & Steffens, 2001; Virani & Rees, 2000). This conserved response reflects a shift from oxidative phosphorylation to anaerobic respiration.

Inhibition of oxidative-phosphorylation increases lactate and induces ATP1A1A degradation

A protein alignment of members of the human, mouse and zebrafish NDRG family revealed that the NDRG3 amino acid residues previously implicated in lactate binding (Lee et al., 2015) are conserved across members of this family (Fig. 6E), suggesting that lactate may also bind to and possibly activate NDRG1.

We next asked whether lactate may be sufficient to promote ATP1A1A degradation under normoxic conditions. Sodium azide, an electron transport chain inhibitor, was used to increase endogenous lactate levels as previously shown (Oginuma et al., 2017) and was confirmed to be effective (Fig. 7A). We then tested whether exposure of WT embryos to sodium azide (6, 12, 18, 24 h duration) under normal oxygen caused a decrease in ATP1A1A levels (Fig. 7B). This analysis revealed that NKA levels were indeed reduced in response to sodium azide treatment (WT anterior PD: 73% reduction in ATP1A1A between 0 h and 24 h anoxia; WT posterior PD: 63% reduction in ATP1A1A between 0 h and 24 h anoxia) (Fig. 7C). If Ndrg1a is activated downstream of sodium azide, ATP1A1A levels should not decrease in *ndrg1a*$^{-/-}$ mutants exposed to this drug. Analysis of sodium azide-treated *ndrg1a*$^{-/-}$ mutants immunolabeled with anti-ATP1A1A revealed that NKA levels were comparably higher in mutants than in WT embryos for the same treatment groups (*ndrg1a*$^{-/-}$ anterior PD: 20% reduction in ATP1A1A between 0 h and 24 h anoxia; *ndrg1a*$^{-/-}$ posterior PD: 7.5% reduction in ATP1A1A between 0 h and 24 h anoxia) (Fig. 7C), corroborating with this prediction.

Consistent with the ability of sodium azide to downregulate NKA under normoxic conditions, we also observed that drug-treated embryos exhibited enhanced Ndrg1a-ATP1A1A interaction, as PLA labeling increased

proportionately with the duration of the treatment (Fig 7D, d1-4; E). In contrast, *ndrg1a*$^{-/-}$ mutants subjected to the same treatment had no PLA signal, confirming the specificity of this assay (Fig 7d5). These data are consistent with lactate being a proximal signal that contributes to NKA downregulation by promoting Ndrg1a-ATP1A1A interaction.

DISCUSSION

Increasing evidence indicates that members of the NDRG family of α−β hydrolases are implicated in the hypoxia response (Melotte et al., 2010). However, the mechanisms by which they function in the physiological adaptation of normal cells to low oxygen are, for the most part, unknown. We uncover here a novel and essential role for Ndrg1a in promoting long-term adaptation to hypoxia via metabolic suppression. Overall, our findings support a model whereby prolonged and severe hypoxia activates or enhances Ndrg1a signaling in the zebrafish embryo, possibly via lactate binding. Activated Ndrg1a accelerates the rate of endocytosis and degradation of the energy-demanding NKA pump in the PD and ionocytes, thereby preserving ATP. The high level of homology between NDRG1 in vertebrates along with evidence pointing towards conserved molecular interactions, suggest that the proposed model for Ndrg1a signaling may be broadly applicable.

Ndrg1a as a molecular switch for long-term adaptation to hypoxia

Given that the molecular cascade leading to ATP1A1A internalization and degradation is triggered by prolonged anoxia and dependent on *ndrg1a*, we propose that Ndrg1a functions as a molecular switch for long-term adaptation to hypoxia. The mechanisms underlying Ndrg1a activation have not been fully elucidated, however two non-mutually exclusive models can be envisaged, which

we have coined quantitative vs. qualitative. In the quantitative model, Ndrg1a normoxic activity is enhanced in response to hypoxia, possibly via post-translational modifications (PTM) or/and transcriptional up-regulation of *ndrg1a* (Le et al., under revision). Consistent with this model, we have shown that *ndrg1a* is required to maintain normoxic levels of NKA in the plasma membrane and to accelerate NKA degradation under hypoxia. Others have reported on similar NDRG1-dependent regulation of LDLR and E-Cadherin levels under normal oxygen (Kachhap et al., 2007; Pietiainen et al., 2013), however it is unknown whether such activities are enhanced under hypoxia.

In the qualitative model, Ndrg1a acquires new properties under hypoxia due to a PTM or binding to a small molecule, which brings about a change in protein conformation or/and binding partner affinity. In support of this model, a previous study showed that binding of lactate to NDRG3 in hypoxic cancer cells is sufficient to stabilize NDRG3 and activate Raf-ERK signaling (Lee et al., 2015). We have shown that sodium azide treatment, which blocks oxidative-phosphorylation and increases the intracellular concentration of lactate, is sufficient to induce ATP1A1A degradation under normoxic conditions in an Ndrg1a-dependent manner. While several metabolites could function downstream of sodium azide as effectors of this response, our data point to lactate as a prime candidate.

There is currently no consensus on the molecular role of activated NDRG1 (Melotte et al., 2010), however we favor the idea that Ndrg1a functions as an adapter protein to link protein-binding partners together. Indeed, there is a growing list of NDRG1 binding partners that localize to several cellular compartments (Tu et al., 2007). Our findings suggest that one of the protein complexes that NDRG1 could bring together under hypoxic conditions includes ATP1A1A, endosomal components or/and protein degradation machinery.

Hypoxia-induced NKA downregulation as a mechanism to preserve ATP

The NKA isoforms that are expressed in the kidney are essential for the active translocation of Na^+ and K^+ ions across the membrane and in the secondary active transport of other solutes, including glucose (Katz, 1982). The cost of operating NKA is considerable, and hence an evolutionarily conserved response to hypoxia is to downregulate this pump to conserve ATP. Mechanisms of downregulation include reducing pump activity (inactivation via several redox-sensitive modifications) or/and triggering its endocytosis and degradation (Bogdanova et al., 2016; Buck & Hochachka, 1993; Gusarova et al., 2011). In the zebrafish, downregulation of NKA is a slow response to prolonged anoxia, taking place over the course of several hours. This response correlates with ATP preservation and is most likely delayed because it is a last resort for the cell.

In order for NKA downregulation to be considered a hypoxia adaptation, it would have to be reversible upon return to normoxia, which we have confirmed. The cellular source of the post-anoxia protein is unknown, but may involve intracellular stores of endocytosed NKA or/and protein synthesized *de novo*. The former could provide an immediate source of NKA for rapid restoration of pump activity, while the latter, a more delayed response, could fully replenish the cell's NKA supply.

Context-specific function of NDRG1

Our data support a physiological role for *ndrg1a* in hypoxia-induced NKA degradation. The NKA pump plays an important role in freshwater teleosts such as zebrafish. These fish are faced with the challenge of potential over-hydration and salt depletion, since their body fluids are hyperosmotic with respect to the environment. To compensate for the tendency towards water influx, zebrafish excrete large volumes of water and limit NaCl loss via reabsorption of Na^+ and Cl^- in the kidney distal (collecting) duct and bladder, and also through ionocyte-dependent absorption of these ions from the environment (Takvam, Wood, Kryvi, & Nilsen, 2021). Consistent with these findings, zebrafish NKA is expressed more highly in the posterior PD, where its activity may power the bulk of Na^+ reabsorption. PLA further revealed regional differences in the manner in which

NKA is regulated in the anterior and posterior PD, as ATP1A1A and Ndrg1a appear to interact in posterior cells even under normoxic conditions. It is possible that, owing to the high level of activity of NKA in the posterior tubules, there is a build up of lactate in these cells. If correct, constitutive Ndrg1a-ATP1A1A interaction in posterior cells may prime them for a more effective response to hypoxia.

Studies in amphibians show that NDRG1 controls cell differentiation of endodermal organs (Kyuno et al., 2003; Zhang et al., 2013), with no indication of a physiological role for this protein. It is unclear what accounts for the difference in NDRG1 function between teleosts and amphibians, but one possibility is the increased number of *Ndrg* paralogs in zebrafish (6 NDRGs in zebrafish versus 4 in *Xenopus laevis*). This increase might have enabled functional diversification of some members of the NDRG family and for *ndrg1a* in particular to evolve a role in hypoxia adaptation. However, there is ample evidence in mouse and human cells that NDRG1 is hypoxia-responsive (Melotte et al., 2010), making this an unlikely explanation. Alternatively, *ndrg1a* may be required for PD and ionocyte differentiation but its function is masked by other members of the NDRG family that compensate for *ndrg1a* LOF.

NDRG1 and ion homeostasis

While NKA degradation preserves ATP, the loss of this pump can cause a rise in intracellular Na^+ concentration and subsequent water influx, culminating in cellular edema (Bogdanova, Grenacher, Nikinmaa, & Gassmann, 2005; Leaf, 1956; Mudge, 1951). However, hypoxia-tolerant organisms are thought to counter NKA depletion with a general reduction in membrane permeability via blockage of leak and water channels (Boutilier, 2001; Doll, Hochachka, & Reiner, 1991; LaMacchia & Roth, 2015; Lutz & Nilsson, 1997). The underlying mechanisms for this phenomenon, known as channel arrest, have remained controversial, especially with regards to the identity of the proximal signal that triggers channel arrest (Buck & Hochachka, 1993; Hochachka & Lutz, 2001).

PD cells and ionocytes in zebrafish embryos exposed to prolonged anoxia do not show overt signs of cellular edema (Fig. 3D and 4A), suggesting that channel arrest may apply to this organism. We further speculate that NDRG1 may negatively regulate ion transporters in zebrafish embryos, in addition to mediating NKA degradation. Indeed, *ndrg1a*$^{-/-}$ mutants exposed to prolonged anoxia develop severe edema. Moreover, NDRG1 is known to interact with ion transporters CLNS1A and SLC25A6 and ion transport regulator FXYD (Arystarkhova, Bouley, Liu, & Sweadner, 2017; Yang, Kang, Hsu, Lin, & Lee, 2016) and the level of several ion transporters in cichlids exposed to high salinity was shown to be inversely proportional to that of NDRG1 (Kultz, Li, Gardell, & Sacchi, 2013).

If correct, the implication of this prediction is that NDRG1 could function as a master switch that coordinates both metabolic suppression/energy preservation (NKA downregulation) and ion homeostasis (reduction in membrane permeability).

Given that human NDRG1 is expressed in the kidney as well as other metabolically-demanding tissues (Melotte et al., 2010) and interacts with NKA and several ion transporters, it is intriguing to consider whether NDRG1-lactate signaling could be modulated to mitigate the cellular damage caused by hypoxic injury.

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COMPETING INTERESTS

The authors confirm that there are no financial or non-financial competing interests.

FIGURE LEGENDS

Figure 1. Ndrg1a is not required for the differentiation of the pronephric duct and ionocytes.
(A, B) Lateral views of 24 hpf WT, standard MO-injected, *ndrg1a* MO-injected and *ndrg1a*$^{-/-}$ mutant embryos labeled using (A) wholemount *in situ* hybridization to detect the mRNA distribution of *slc20a1a, slc12a3, atp1a1a and kcnj1a.1* and (B) immunolabeling with anti-ATP1A1A or anti-GFP (Tg[*enpep:GFP* transgenic line). n = 3 embryos imaged for each probe. Scale bar 300 μm for *slc20a1a*, *kcnj1a.1*, and ATP1a1a. Annotations: arrows point to signal in the pronephric duct; arrowheads: ionocytes.

Figure 2. *ndrg1a* confers protection from hypoxic injury
(A) % Survival of WT embryos and *ndrg1a*$^{-/-}$ mutants immediately following 6, 12, 18 and 24 h of anoxia exposure. Embryos were 24 hpf at the time of exposure. A significant difference between WT and mutants was observed for 12 and 18 h of anoxia (Two-way ANOVA analysis was performed; 0 h WT vs. *ndrg1a*$^{-/-}$: p value > 0.9999, ns; 6 h WT vs. *ndrg1a*$^{-/-}$: p value = 0.1275, ns; 12 h WT vs. *ndrg1a*$^{-/-}$: p value = 0.9940, ns; 18 h WT vs. *ndrg1a*$^{-/-}$: p value <0.0001, ****; 24 h WT vs. *ndrg1a*$^{-/-}$: p value < 0.0001, ****). n = average of 30 embryos per experimental group; n = 5 experiments. (B) % Survival of WT embryos and *ndrg1a*$^{-/-}$mutants following 6, 12, 18 and 24 h of anoxia exposure and 2 days of re-oxygenation (re-ox). Embryos were 24 hpf at the time of initial anoxia exposure. A significant difference between WT and mutants was observed for 18 and 24 h of anoxia + 2 re-ox (Two-way ANOVA analysis was performed; 0h + re-ox WT vs. *ndrg1a*$^{-/-}$: p value >0.9999, ns; 6h + re-ox WT vs. *ndrg1a*$^{-/-}$: p value >0.9999, ns; 12h + re-ox WT vs. *ndrg1a*$^{-/-}$: p value >0.9999, ns; 18h + re-ox WT vs. *ndrg1a*$^{-/-}$: p value < 0.0001, ****; 24 h + re-ox WT vs. *ndrg1a*$^{-/-}$: p value <0.0001, ****). n = average of 9-26 embryos per experimental group; n=2-5 experiments. (C, left) % Edema observed in WT embryos and *ndrg1a*$^{-/-}$mutants following 0, 6, 12, 18 and 24 h of anoxia exposure and 2 days re-ox. Edema phenotypes were divided into 3 categories: no edema, mild edema and severe edema. A significant difference between WT and mutants was observed for 6, 12, and 18 h of anoxia + 2 re-ox for the presence of either mild or severe edema (Two-way ANOVA analysis was performed; 0h + re-ox WT vs. *ndrg1a*$^{-/-}$: p value

> 0.9999, ns; 6h + re-ox WT vs. *ndrg1a*$^{-/-}$: p value = 0.0224, *; 12 h + re-ox WT vs. *ndrg1a*$^{-/-}$: p value = 0.0118, *; 18h + re-ox WT vs. *ndrg1a*$^{-/-}$: p value = 0.0001, ***). (C, right) Representative images of embryos in each phenotypic category. n = average of 9-26 embryos per experimental group; n = 2-3 experiments. Scale bar 200 μm. (D) Quantification of kidney clearance assay using 48 hpf WT embryos and *ndrg1a*$^{-/-}$mutants exposed to 12 h of anoxia, followed by 2 days re-ox and injection with rhodamine dextran. A significant difference between WT and mutants was observed 24 hpi (unpaired t test; p value < 0.0001, ****. n = average of 9 embryos per experimental group; n = 2 experiments. (E) Lateral views of injected embryos were taken 3 and 24 h post-injection (hpi). White boxes indicate where fluorescence measurements were taken. Scale bar 200 μm.

Figure 3. NKA downregulatation in the pronephric duct in response to anoxia is *ndrg1a*-dependent

(A) Lateral views of 24 hpf WT embryos revealing (a.1,2) *ndrg1a* and *atp1a1a* transcript distribution using whole-mount *in situ* hybridization. (a.3) Ndrg1a and ATP1A1A protein distribution using double immunolabeling. Scale bars: a1, a2: 300 μm, a3: 100 μm for both wholemount and inset. (B) Lateral views of WT embryos and *ndrg1a*$^{-/-}$ mutants exposed at 24 hpf to increasing duration of anoxia: (b1, b4) 0 h; (b2, b5) 6 h; (b3, b6) 12 h and immunolabeled to reveal ATP1A1A. n = average of 5 embryos per experimental group; n=2 experiments. Scale bar 100 μm. Abbreviations: ant = anterior, post = posterior. Annotations: yellow boxes show where anterior and posterior pronephric duct measurements were taken. Arrowheads indicate decreased signal. (C) Normalized fluorescence intensity in the (c1) anterior and (c2) posterior pronephric duct of WT embryos and *ndrg1a*$^{-/-}$ mutants. Embryos were exposed at 24 hpf to increasing duration of anoxia (0, 6, 12, 18 and 24 h) and immunolabeled to reveal ATP1A1A levels. n = average of 5 pronephric duct per experimental group; n=2 experiments. (D) Cross sections views of the anterior (d1-d3', d7-d9') and posterior (d4-d6', d10-d12') pronephric ducts in WT embryos (d1-d3', d4-d6') and *ndrg1a*$^{-/-}$ mutants (d7-d9', d10-d12'), labeled with anti-ATP1A1A and phalloidin (F-actin). Scale bar 20 μm. Annotations: asterisks indicate the lumen (apical surface of PD cells). n = average of 6 pronephric ducts imaged per treatment group or 3 embryos; n = 2 experiments.

Figure 4. **NKA is co-expressed with Ndrg1a in a subset of ionocytes and downregulated under anoxia in an *ndrg1a*-dependent manner**
(A, left) Lateral views of WT embryos (a1-b3) and *ndrg1a*$^{-/-}$ mutants (c1-c3) exposed to anoxia for 0 h (a1-c1), 6 h (a2-c2), 12 h (a3-c3) and immunolabeled with anti-ATP1A1A (green) and anti-Ndrg1a (red); (a1-a3) show merged channels, while (b1-c3) show the green (ATP1A1A) channel only. Annotations: yellow and green boxes identify ionocytes that are positive for Ndrg1a and ATP1A1A (yellow) or ATP1A1A-only (green). Scale bar: a1: 100μm; a1': 2μm. (A, right) Higher magnification images of yellow boxed in areas in a1-b3, showing merged channels (a1-a3') and green channel only (b1-b3'). (B) Normalized fluorescence intensity of ATP1A1A in ionocytes of WT embryos and *ndrg1a*$^{-/-}$ mutants exposed to anoxia for 0, 6, 12, 18, and 24h.

Figure 5. NKA interacts with Ndrg1a under anoxia and is degraded via lysosomal and proteasomal pathways to preserve ATP.
(A) Cross sections of WT embryos exposed to anoxia for 0 h (a1, a4), 6 h (a2, a5) and 12 h (a3, a6) and immunolabeled with anti-ATP1A1A (green) and anti-Ndrg1a (red). (a1-a3) Anterior pronephric duct. (a4-a6) Posterior pronephric duct. Scale bar =10 μm. n = average of 3 embryos per experimental group; n = 2 experiments. (B) Lateral views of WT (b1-b3) and *ndrg1a*$^{-/-}$ (b4-b6) embryos labeled using whole-mount proximity ligation assay (PLA) to reveal Ndrg1a and ATP1A1A interaction. Embryos were exposed to 0 h (b1, b4), 6 h (b2, b5), and 12 h (b3, b6) of anoxia. Scale bar = 300 μm. n = average of 3 embryos per experimental group; n = 2 experiments. (C) Cross sections through the anterior (c1-c3) and posterior (c4-c6) pronephric duct of WT embryos treated as in (b1-b4). Scale bar = 10 μm. (D) Lateral views of WT embryos exposed to 0 h (d1) or 12 h of anoxia (d2-d5) in presence or absence of MG-132 (100uM, proteasome inhibitor), or Chloroquine (10 mM, autophagy inhibitor), or both and immunolabeled using anti-ATP1A1A. n = average of 4 embryos per experimental group; n = 2 experiments. Scale bar 200 μm. Annotation: yellow boxes show where anterior and posterior pronephric duct measurements were taken. (E) Quantification of ATP1A1A fluorescence intensity in the pronephric duct of embryos subjected to the same anoxia and drug treatments as shown in (D). Anterior PD: 7% increase in Anoxia 12h, mg132 vs Anoxia 12h, no drug $p>0.9999$ ns; 59% increase in Anoxia 12h, chloroquine vs Anoxia 12h, no drug

p=0.0298 *; 87% increase in Anoxia 12h mg132+chloroquine vs Anoxia 12h, no drug p=0.0013 **. Posterior PD: 24% increase in Anoxia 12h, mg132 vs Anoxia 12h, no drug p=0.0640 ns, 36% increase in Anoxia 12h, chloroquine vs Anoxia 12h, no drug p=0.0002 ***, 45% increase in Anoxia 12h mg132+chloroquine vs Anoxia 12h, no drug p<0.0001 ****. (F) Quantification of total ATP concentration per WT embryo or *ndrg1a*$^{-/-}$ mutant exposed to 0, 6, 12 h of anoxia. A significant difference between WT and mutants was observed for 12 h of anoxia (Two-way ANOVA analysis was performed; 0 h WT vs. *ndrg1a*$^{-/-}$: p value = 0.8355, ns; 6 h WT vs. *ndrg1a*$^{-/-}$: p value = 0.7236, ns; 12 h WT vs. *ndrg1a*$^{-/-}$: p value = 0.0353, *). n = average of 15-20 embryos per experimental group; n = 3 experiments.

Figure 6. Lactate concentration increases under anoxia

(A, top) Metabolites enriched in anoxia-treated embryos relative to normoxic controls. Y axis: metabolite levels (integrated ion counts) in extracts from sphere stage (4 hpf) embryos exposed to 1 hour of anoxia; X axis: metabolite levels (integrated ion counts) in extracts from age-matched (5 hpf) normoxic controls. The red lines show the predicted correlation line (99% confidence). Blue lines demarcate outliers (99% confidence). Three potential outliers (a, b and c) were identified. n = 10 embryos per sample and 2 technical repeats. (A, bottom) Identity of the three outliers (a,-c) that are significantly enriched in the anoxia-treated sample. (B) Extracted ion counts for different elution timepoints are shown for two extracts from sphere stage (4 hpf) embryos exposed to 1 h of anoxia (orange and red lines) and two control samples from 5 hpf normoxic embryos (yellow and green lines). The peak corresponds to lactate. (C) Comparison of lactate levels (highest peak intensity) in extracts from sphere stage (4 hpf) embryo exposed to 1 h of anoxia and 5 hpf normoxic controls reveals an 18 fold increase (unpaired t test; $p < 0.01$). (D) Fluorometric lactate assay showing lactate concentration (nmol lactate/embryo) in whole embryo extracts from 24 hpf WT embryos exposed to increasing durations of anoxia (0-7 hours). n = average of 20 embryos per experimental group; n = 3 experiments. (E) Alignment of members of the NDRG family revealing lactate-docking residues, identified in NDRG3 by Lee et al., 2015. Green and red residues represent amino acids in NDRG3 that are 5 Angstrom from lactate. Red residues represent amino acids that may be critical for the interaction between NDRG3 and lactate.

Figure 7. Lactate concentration increases following sodium azide treatment under normoxia and correlates with ATP1A1A downregulation

(A) Fluorometric lactate assay showing pmol of lactate in whole embryo extracts from WT embryos and *ndrg1a*$^{-/-}$ mutants raised under normoxic conditions and exposed to sodium azide for 0, 6, 12 hours. Embryos were 24 hpf at the time of treatment and untreated embryos were stage-matched to drug-treated embryos. n = average of 15 embryos per experimental group; n = 2 experiments. (B) Lateral views of WT embryos and *ndrg1a*$^{-/-}$ mutants exposed at 24 hpf to increasing duration of azide under normoxia: (b1-5) 0 h; (b1, b4) 12 h; (b3, 5) 24 h and immunolabeled to reveal ATP1A1A. n = average of 3 embryos per experimental group. Scale bar 300 μm. Arrowheads indicate decreased signal. (C) Normalized NKA fluorescence intensity in the anterior (circle) and posterior (triangle) pronephric duct of WT (black) embryos and *ndrg1a*$^{-/-}$ (red) mutants. Embryos were exposed at 24 hpf to increasing duration of azide under normoxia (0, 6, 12, 18 and 24 h) and immunolabeled to reveal ATP1A1A levels. n = average of 3 pronephric duct per experimental group. (D) Lateral views of WT embryos (d1-4) and an *ndrg1a*$^{-/-}$ mutant (d5) labeled using whole-mount proximity ligation assay (PLA) to reveal Ndrg1a and ATP1A1A interaction. Embryos were raised under normoxic conditions in the presence (6, 12, 18, 24 h exposure) or absence of sodium azide. n = average of 3 embryos per experimental group; n = 2 experiments. Annotations: arrowheads point to PLA signal. (E) Normalized PLA intensity in the anterior (circle) and posterior (triangle) pronephric duct of WT embryo.

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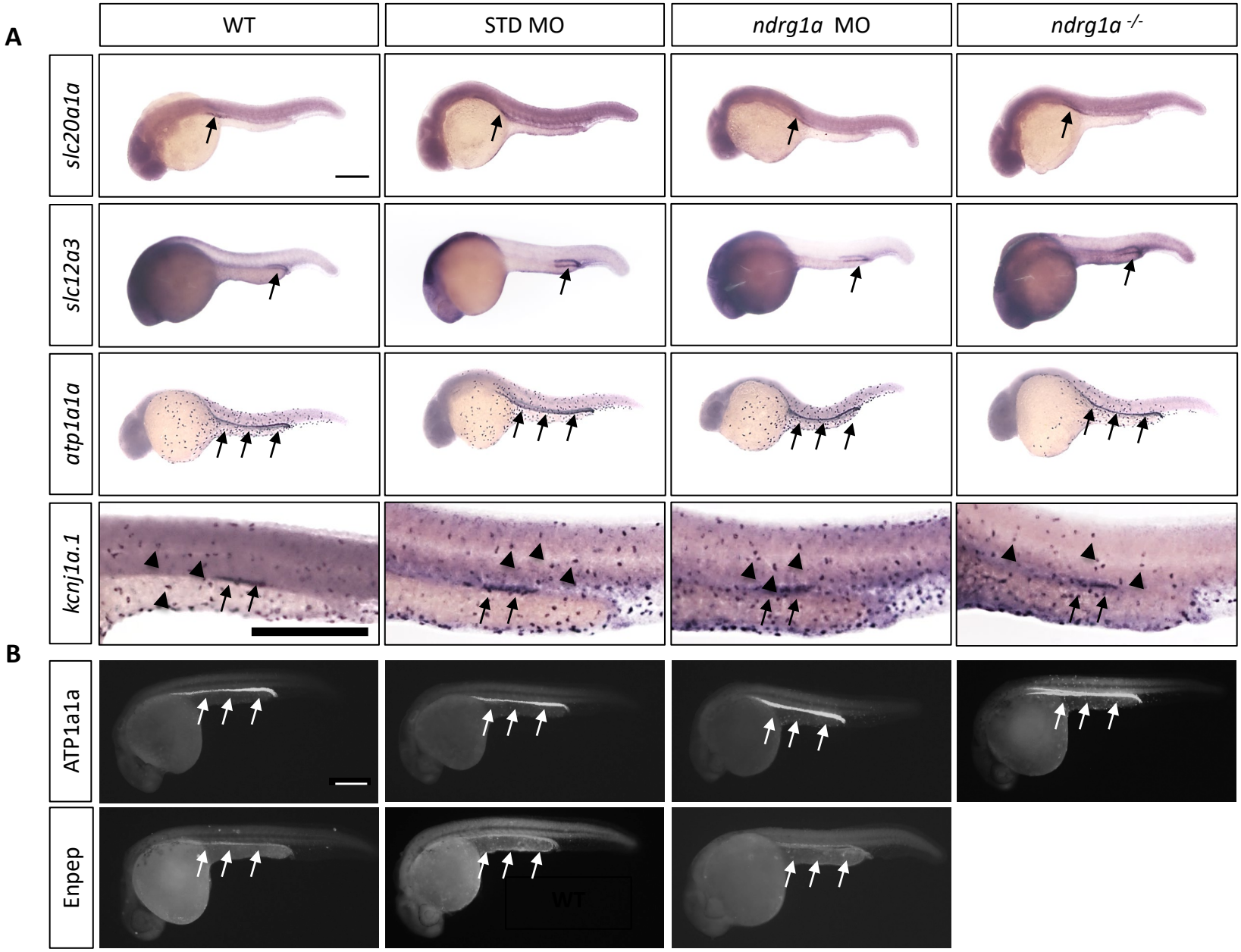
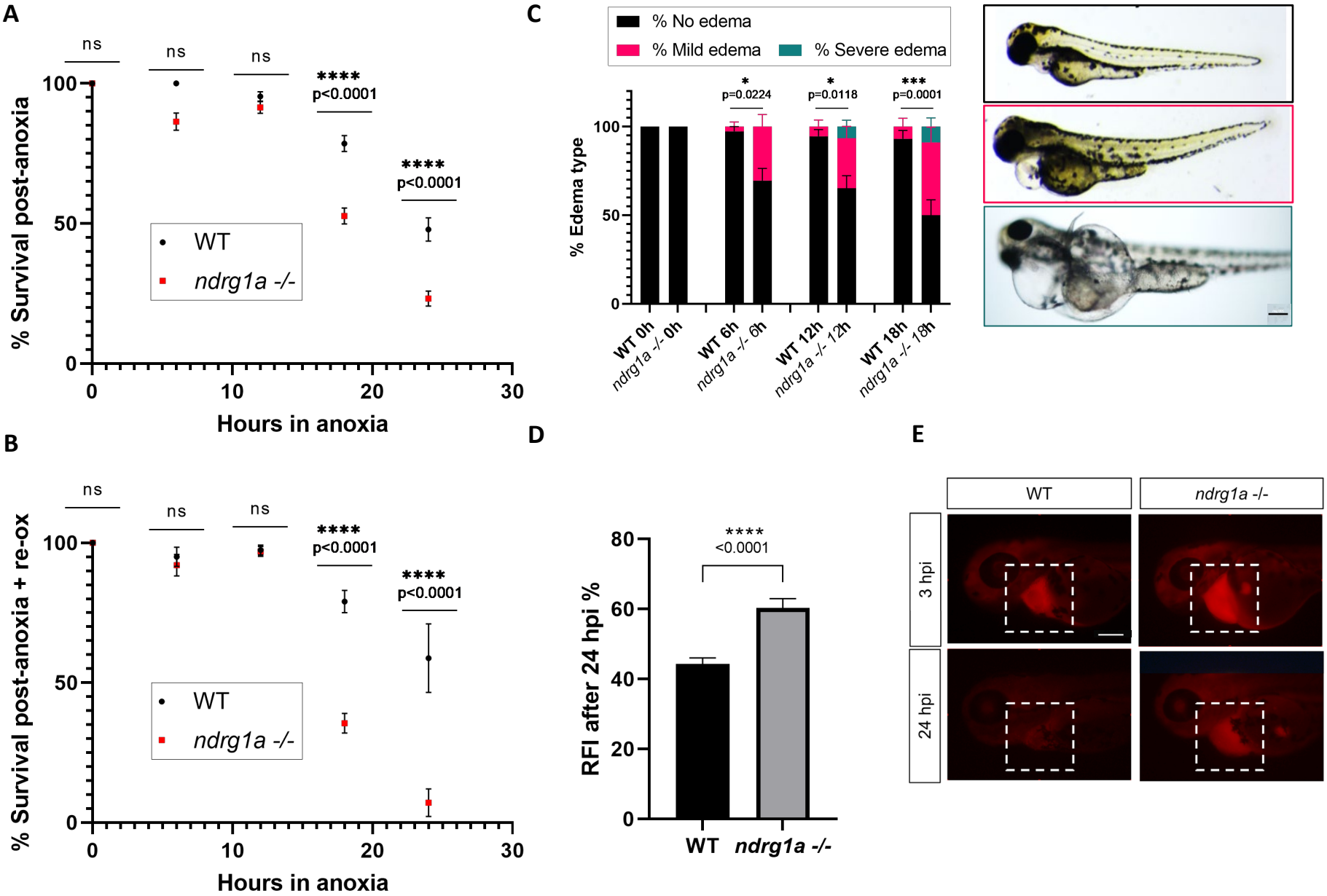
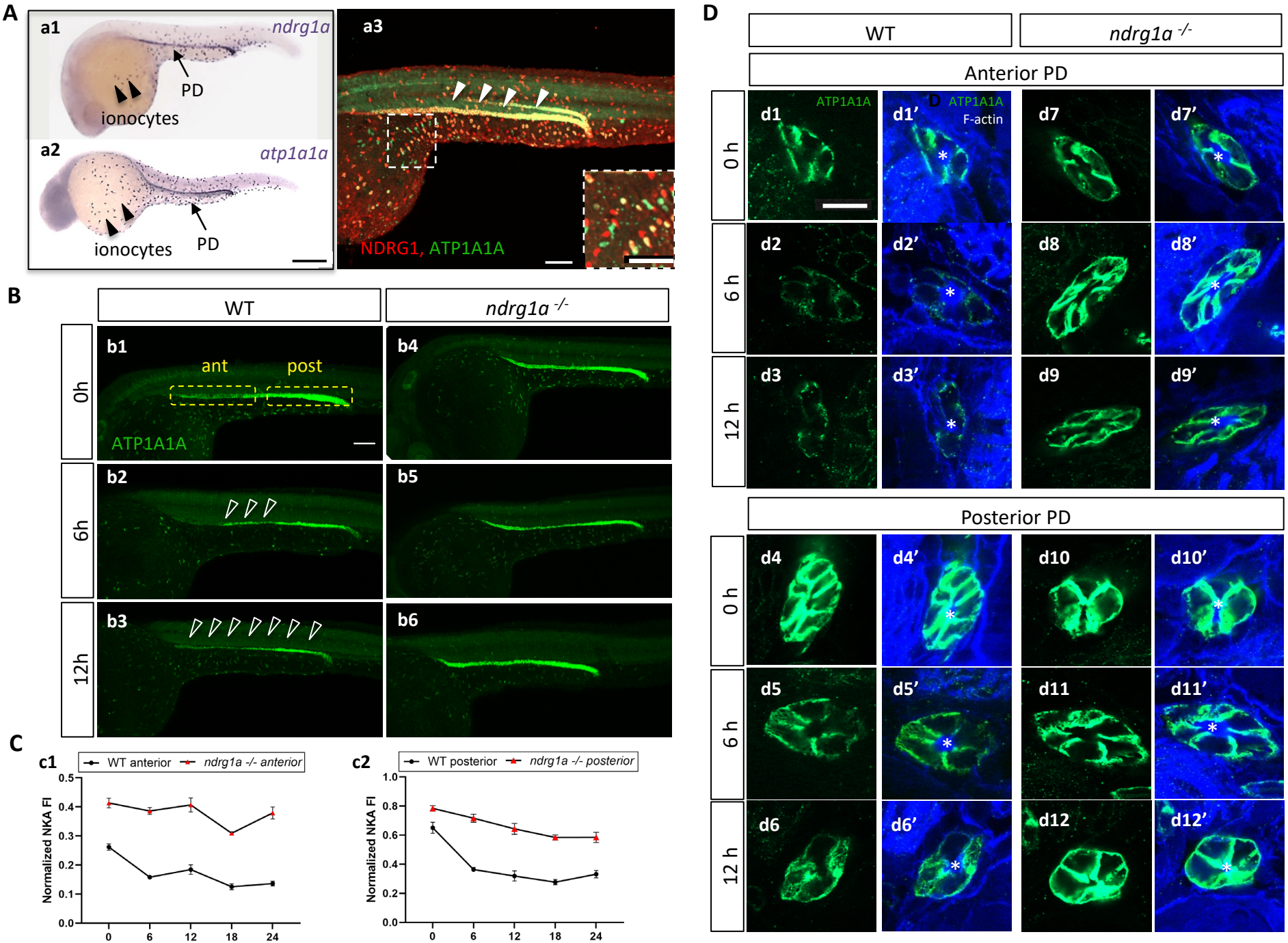
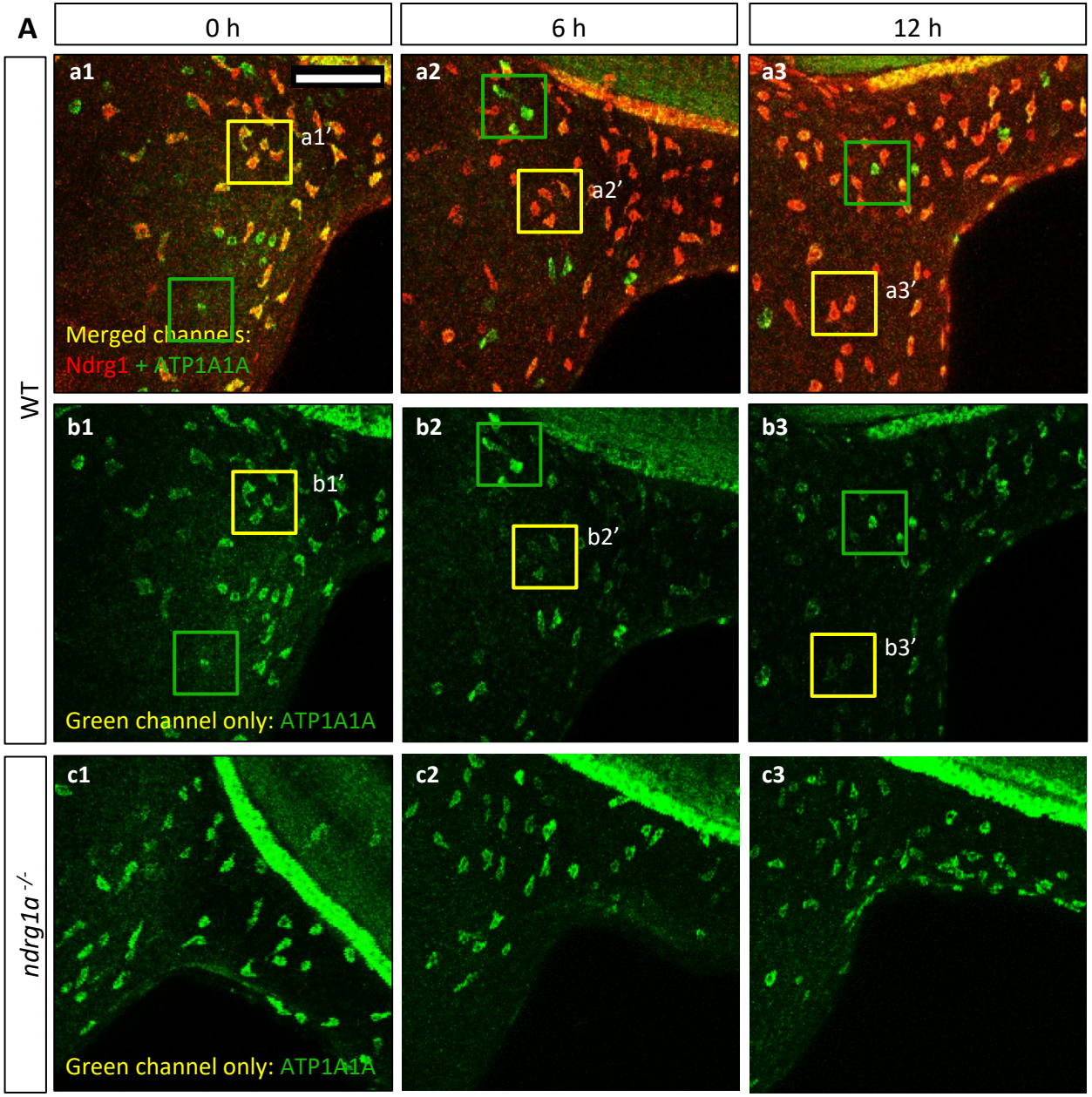
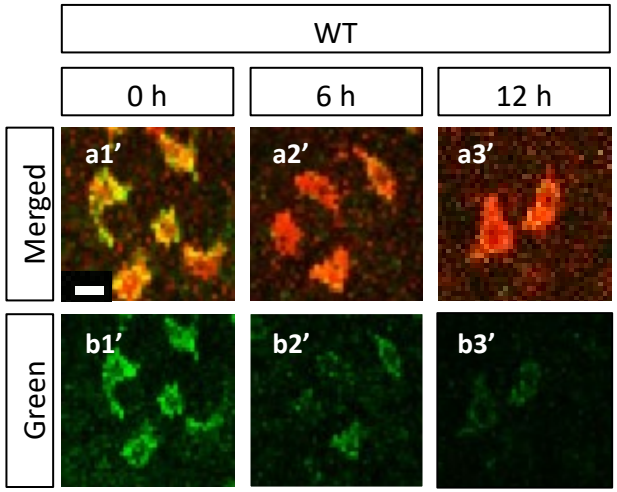
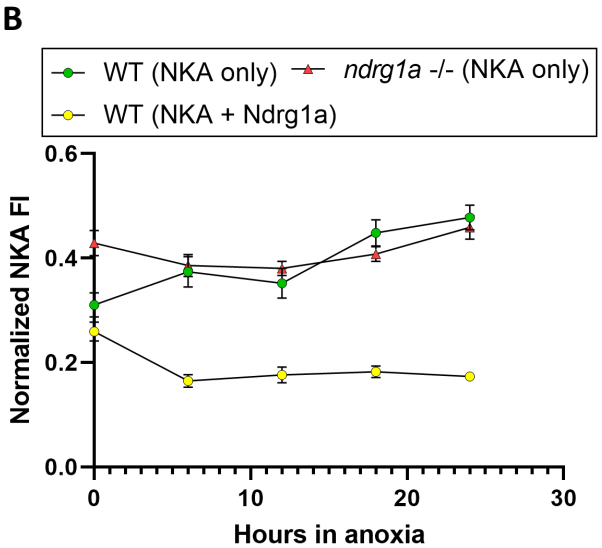

A
WT
STD MO
ndrg1a MO
ndrg1a -/-
slc20a1a
slc12a3
atp1a1a
kcnj1a.1
B
ATP1a1a
Enpep
WT

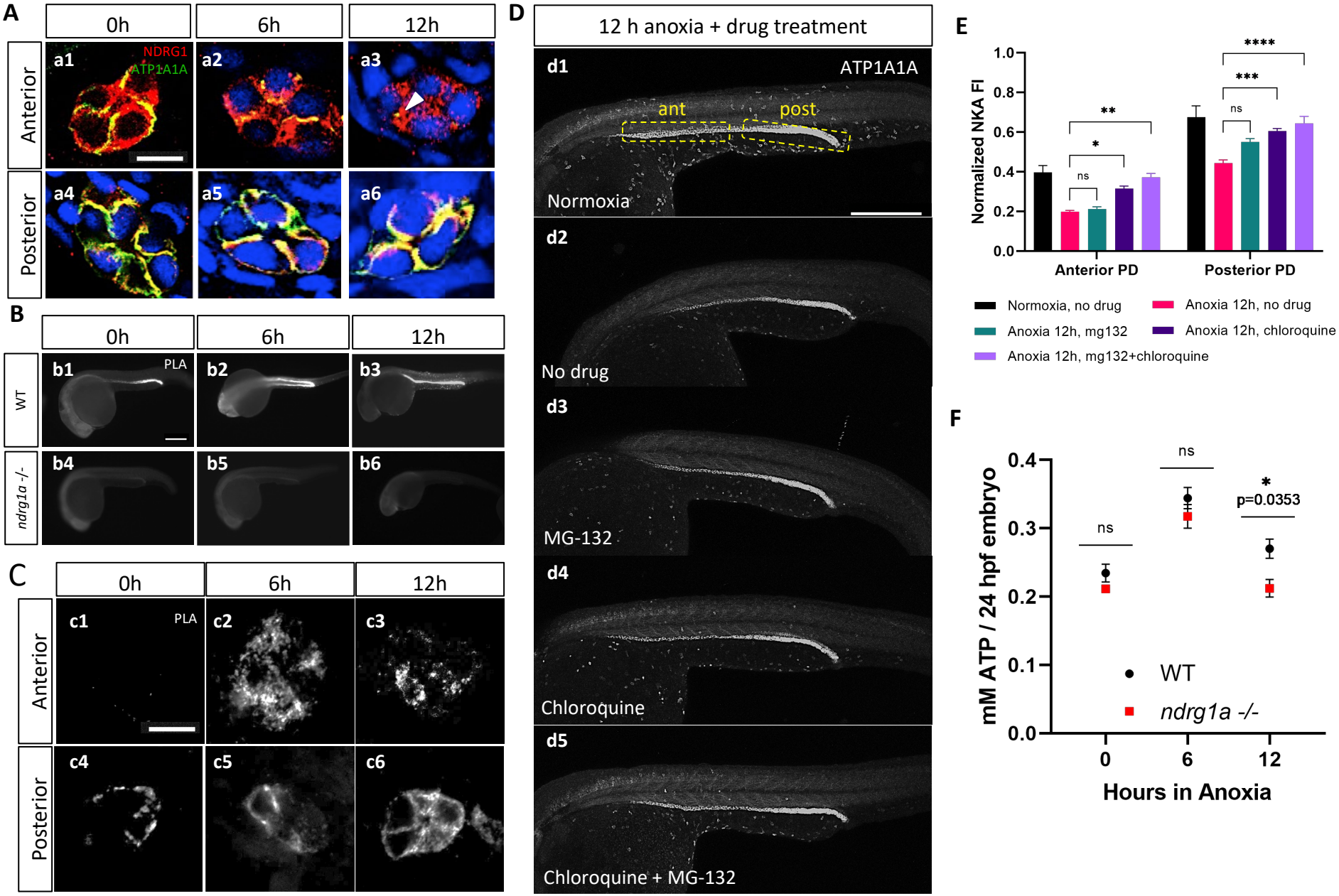

A
ns
ns
ns
****
p<0.0001
****
p<0.0001
% Survival post-anoxia
100
50
0
WT
ndrg1a -/-
0
10
20
30
Hours in anoxia
B
ns
ns
ns
****
p<0.0001
****
p<0.0001
% Survival post-anoxia + re-ox
100
50
0
WT
ndrg1a -/-
0
10
20
30
Hours in anoxia
C
% No edema
% Mild edema
% Severe edema
*
p=0.0224
*
p=0.0118
***
p=0.0001
% Edema type
100
50
0
WT 0h
ndrg1a -/- 0h
WT 6h
ndrg1a -/- 6h
WT 12h
ndrg1a -/- 12h
WT 18h
ndrg1a -/- 18h
D
****
<0.0001
RFI after 24 hpi %
80
60
40
20
0
WT
ndrg1a -/-
E
WT
ndrg1a -/-
3 hpi
24 hpi

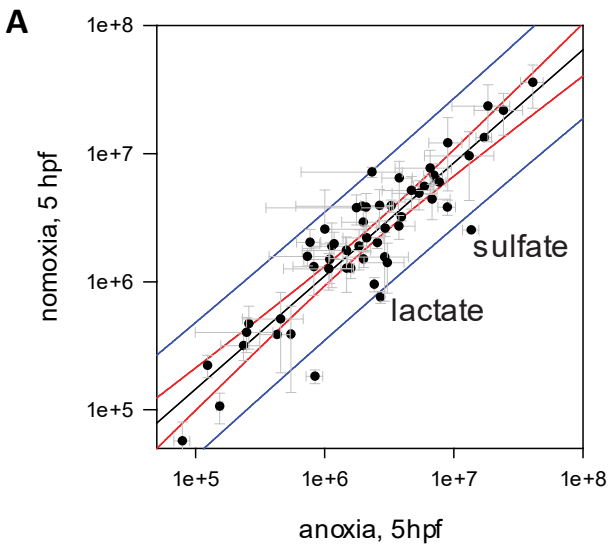

A
a1
ndrg1a
PD
ionocytes
a2
atp1a1a
ionocytes
PD
a3
NDRG1, ATP1A1A
B
WT
ndrg1a -/-
0h
6h
12h
b1
ant
post
ATP1A1A
b2
b3
b4
b5
b6
C
c1
WT anterior
ndrg1a -/- anterior
Normalized NKA FI
Hours in anoxia
c2
WT posterior
ndrg1a -/- posterior
Normalized NKA FI
Hours in anoxia
D
WT
ndrg1a -/-
Anterior PD
Posterior PD
0 h
6 h
12 h
ATP1A1A
F-actin
d1
d1'
d2
d2'
d3
d3'
d4
d4'
d5
d5'
d6
d6'
d7
d7'
d8
d8'
d9
d9'
d10
d10'
d11
d11'
d12
d12'


A
0 h
6 h
12 h
WT
a1
a1'
a2
a2'
a3
a3'
Merged channels:
Ndrg1 + ATP1A1A
b1
b1'
b2
b2'
b3
b3'
Green channel only: ATP1A1A
ndrg1a -/-
c1
c2
c3
Green channel only: ATP1A1A

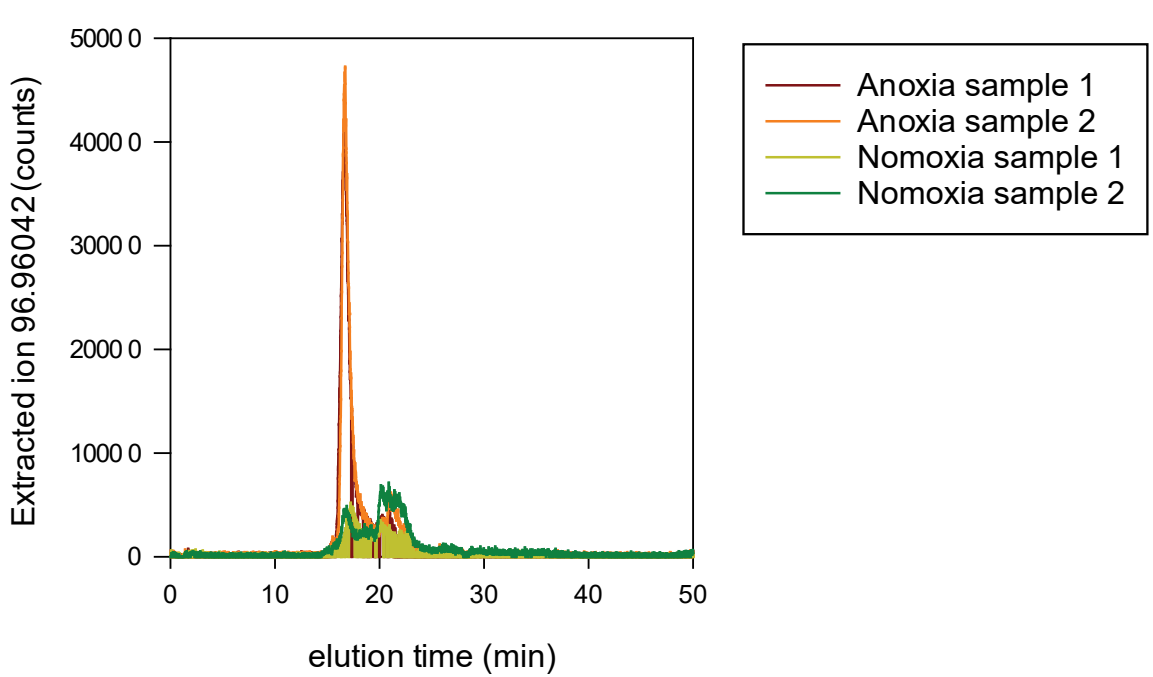

WT
0 h
6 h
12 h
Merged
Green
a1'
a2'
a3'
b1'
b2'
b3'

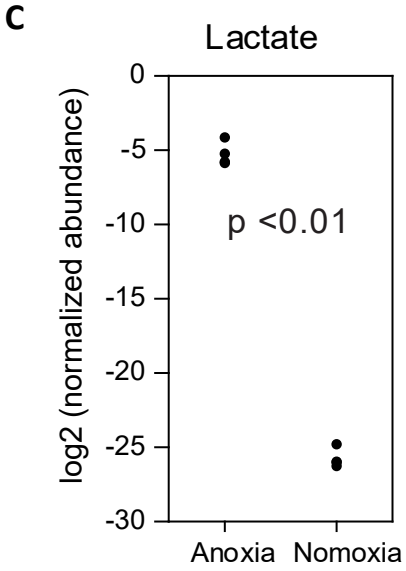

B
WT (NKA only)
ndrg1a -/- (NKA only)
WT (NKA + Ndrg1a)
Normalized NKA FI
0.0
0.2
0.4
0.6
0
10
20
30
Hours in anoxia

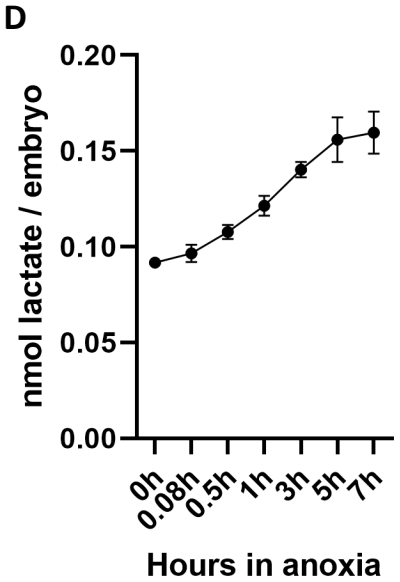

A
0h
6h
12h
Anterior
Posterior
a1
NDRG1
ATP1A1A
a2
a3
a4
a5
a6
B
0h
6h
12h
WT
ndrg1a -/-
b1
PLA
b2
b3
b4
b5
b6
C
0h
6h
12h
Anterior
Posterior
c1
PLA
c2
c3
c4
c5
c6
D
12 h anoxia + drug treatment
d1
ATP1A1A
ant
post
Normoxia
d2
No drug
d3
MG-132
d4
Chloroquine
d5
Chloroquine + MG-132
E
Normalized NKA FI
1.0
0.8
0.6
0.4
0.2
0.0
****
***
**
*
ns
ns
Anterior PD
Posterior PD
Normoxia, no drug
Anoxia 12h, no drug
Anoxia 12h, mg132
Anoxia 12h, chloroquine
Anoxia 12h, mg132+chloroquine
F
mM ATP / 24 hpf embryo
0.4
0.3
0.2
0.1
0.0
ns
ns
*
p=0.0353
WT
ndrg1a -/-
0
6
12
Hours in Anoxia

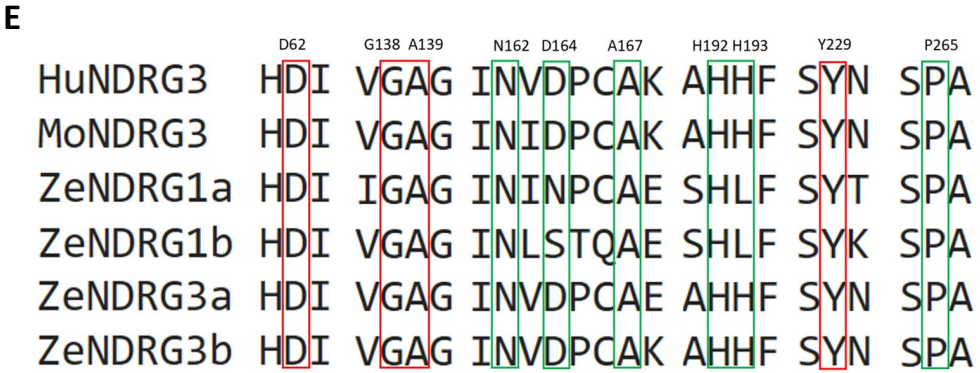


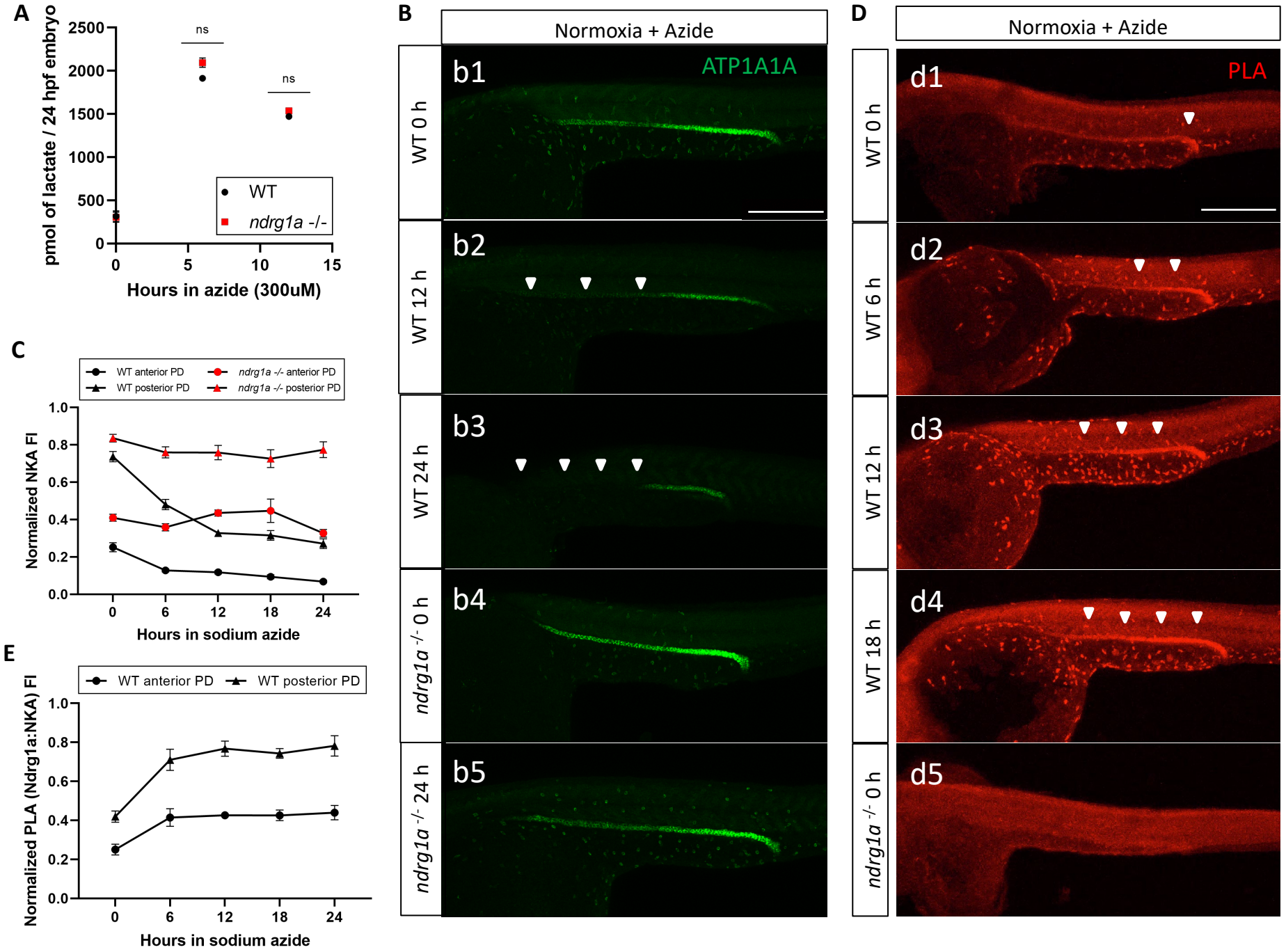
	m/z	identity
a	79.9571	SO_3^-
b	89.0241	lactate
c	96.9600	$H_2SO_4^-$










A
pmol of lactate / 24 hpf embryo
2500
2000
1500
1000
500
0
ns
ns
WT
ndrg1a -/-
0
5
10
15
Hours in azide (300uM)
B
Normoxia + Azide
ATP1A1A
b1
b2
b3
b4
b5
WT 0 h
WT 12 h
WT 24 h
ndrg1a -/- 0 h
ndrg1a -/- 24 h
C
WT anterior PD
WT posterior PD
ndrg1a -/- anterior PD
ndrg1a -/- posterior PD
Normalized NKA FI
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
18
24
Hours in sodium azide
D
Normoxia + Azide
PLA
d1
d2
d3
d4
d5
WT 0 h
WT 6 h
WT 12 h
WT 18 h
ndrg1a -/- 0 h
E
WT anterior PD
WT posterior PD
Normalized PLA (Ndrg1a:NKA) FI
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
18
24
Hours in sodium azide