Expression Regulation of of Na-K-ATPase Alpha 1 Subunit Subtypes in

Zebrasfish Gill Ionocytes

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Running head: Sodium pump isoforms in zebrafish gills

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Abstract

In zebrafish (*Danio rerio*), 6 distinct Na-K-ATPase (NKA) α1 subunit genes have been identified, and 4 of them, zATP1a1a.1, zATP1a1a.2, zATP1a1a.4, and zATP1a1a.5, are expressed in embryonic skin, where different types of ionocytes appear. The present study attempted to test a hypothesis of whether these NKA α1 paralogues are specifically expressed and function in respective ionocytes. Double fluorescence *in situ* hybridization analysis demonstrated the specific expression of zATP1a1a.1, zATP1a1a.2, and zATP1a1a.5 in NKA-rich (NaR) cells, Na⁺-Cl⁻ cotransporter (NCC)-expressing cells, and H⁺-ATPase-rich (HR) cells, respectively, based on the colocalization of the 3 NKA α1 genes with marker genes of the respective ionocytes (epithelial Ca²⁺ channel in NaR cells; NCC in NCC cells; and H⁺-ATPase and Na⁺/H⁺ exchanger 3bin HR cells). The mRNA expression (by real-time PCR) of zATP1a1a.1, zATP1a1a.2, and zATP1a1a.5 were respectively upregulated by low-Ca²⁺, low-Cl⁻, and low-Na⁺ fresh water, which had previously been reported to stimulate uptake functions of Ca²⁺, Cl⁻, and Na⁺. However, zATP1a1a.4 was not colocalized with any of the 3 types of ionocytes, nor did its mRNA respond to the ambient ions examined. Taken together, zATP1a1a.1, zATP1a1a.2, and zATP1a1a.5 may provide driving force for Na⁺ coupled cotransporter activity specifically in NaR, NCC, and HR cells, respectively.
Keywords: mitochondrion-rich cells, ion regulation, osmoregulation, fish, sodium pump
Introduction

In the post genomic era, functional studies of proteins in specific organs or cell types have become more complicated due to the discovery of additional isoforms of a target protein from bioinformatic inquiries. The zebrafish may be an alternative and suitable in vivo tool to deal with this issue because of the plentiful genetic information available and its applicability to various molecular physiological approaches (8, 21).

Na⁺-K⁺-ATPase (NKA) is essential for maintaining body fluid and electrolyte homeostasis by providing a driving force in a variety of osmoregulatory epithelia (32). The enzyme is characterized by a complex molecular heterogeneity that results from the expression and differential association of multiple isoforms of both its α- and β-subunits. Four α-, three β-, and eight γ-subunit isoforms have been identified in mammals (32, 41). The physiological role of the 4 α-isoforms of NKA (α1, α2, α3, and α4) have been extensively examined in several mammalian tissues; different α-isoforms determine the kinetic properties of NKA, and the tissue distributions of these isoforms were suggested to match the tissue-specific physiological functions (32). This notion has also been proposed in fish gills, an important osmoregulatory organ. In fish gills, mitochondrion-rich (MR) cells, the major ionocytes, express a high amount of NKA for driving different transporters related to ion uptake and ion secretion pathways in fresh water (FW) and seawater (SW) environment, respectively.
(13, 23). It was tested by co-localizing and examining the differential expression of the α1 and α3 isoforms in gill ionocytes with heterologous antibodies in tilapia (Oreochromis mossambicus) exposed to FW and SW (26). Subsequent studies cloned the cDNAs of several α-isoforms and analyzed the differential expression of those isoforms in FW and SW tilapia and salmonoid fishes by Western and Northern blotting, RT-PCR, and/or real-time PCR (3, 14, 22, 37). However, gills are composed of ionocytes and other cell types (pavement cells, mucous cells, blood cells, nervous cells, muscle cells, etc). RT-PCR (or Western blotting) analysis of a NKA isoform in gill tissues could not reflect the specific expression or function of the isoform in gill ionocytes if the target NKA isoform has not been identified to be the one specifically expressed in the gill ionocytes.

Recent studies on zebrafish (Danio rerio) skin/gills have identified 3 types of ionocytes, H^+-ATPase-rich (HR) cells, NKA-rich (NaR) cells, and Na^+-Cl^-cotransporter-expressing (NCC) cells, which express different sets of ion transporters and/or enzymes (epithelial Ca^{2+} channel in NaR cells; NCC in NCC cells; and H^+-ATPase, Na^+\textsubscript{t}/H^+ exchanger 3b, carbonic anhydrase 2-like a, and carbonic anhydrase 15a in HR cells), and subsequent molecular physiological analyses have proposed the roles of HR cells, NaR cells, and NCC cells in the respective mechanisms of acid secretion/Na^+ uptake, Ca^{2+} uptake, and Cl^- uptake (21, 29, 30, 34,
According to the model in zebrafish skin/gill ionocytes (21, 23), NKA was proposed to provide some chemical and electric gradients to drive the transport mechanisms in each type of ionocyte; however, molecular evidence is lacking so far. On the other hand, 9 distinct NKA α-subunit genes (7 α1, 1 α2 and 1 α3) have been identified in zebrafish by analyzing the expressed sequence tag (EST) and genomic databases; among them, four (α1a.1, α1a.2, α1a.4, and α1a.5) of 7 α1 genes were found to be expressed in embryonic skin mucous cells (1, 4), which were subsequently suggested to be associated with skin ionocytes (29). In addition to α-subunits, zebrafish NKA β-subunit genes were also identified; however, only one β-subunit isoform, ATP1b1b, showed ionocyte-like expression pattern in embryonic stages (4). Taken together, it is possible that various α1 paralogues may specifically exist and function in distinct types of ionocytes in zebrafish, and this would provide new insights into the physiological significances of multiple paralogues of NKA α1 genes.

The present study attempted to test the hypothesis that NKA α1 paralogues (zATP1a1a.1, zATP1a1a.2, zATP1a1a.4, and zATP1a1a.5) are specifically expressed in distinct subtypes of ionocytes to drive the respective relevant ion transport mechanisms. Experiments were designed to answer the following questions: (a) Are the NKA α1 (α1a.1, α1a.2, α1a.4, and α1a.5) paralogues co-expressed in the same
cells? (b) Is each α1 paralogue co-expressed with the relevant transporter in distinct ionocytes? and (c) Do changes in environmental ionic compositions, which can induce the transport capacities of various ions, differentially stimulate the expression of each respective α1 paralogue?
Materials and Methods

Experimental animals

Zebrafish (AB strain) brood stocks in the Institute of Cellular and Organismic Biology, Academia Sinica were kept in fresh water (local tap water) at 28.5 °C under a photoperiod of 14-h light:10-h dark before the acclimation experiments. Fish were anesthetized with 100-200 mg/l of buffered MS222 (3-aminobenzoic acid ethyl ester; Sigma, St. Louis, MO) before sampling following guidelines of the Academia Sinica Institutional Animal Care and Utilization Committee (approval no.: RFiZOOHP2007086).

Acclimation experiments

Five kinds of artificial fresh water, high (H)-Na-low (L)-Cl (10 mM Na⁺ and 0.04 mM Cl⁻), L-Na-L-Cl (0.04 mM NaCl), H-Na-H-Cl (10 mM NaCl), H-Ca (2 mM Ca), and L-Ca (0.02 mM Ca) were prepared by adding appropriate amounts of NaCl, Na₂SO₄, MgSO₄, K₂HPO₄, KH₂PO₄, and CaSO₄ to double-deionized water (Milli-RO60; Millipore, Billerica, MA). The ionic compositions (Table 1) of the 5 media were confirmed by measuring the Na⁺, K⁺, Ca²⁺, and Mg²⁺ concentrations with an atomic absorption spectrophotometer (Hitachi Z-8000, Tokyo, Japan) and by examining Cl⁻ levels with a spectrophotometer (Hitachi U-2000). The pH of the media was kept
between 6.5 and 6.9, and the water temperature was 28.5 °C. Twenty-five to thirty zebrafish for each test were acclimated to 30-l of the different artificial FW media for 2 wk and then were sampled for quantitative real-time RT-PCR (5 individuals for each test). Fish were not fed during the acclimation period. To maintain the water quality, the acclimation media were aerated with a filtered air pump, and newly prepared and changed every 3 d. The changes in the water ion compositions, pH (Table 1) and ammonia (less than 0.1 mg/l) were monitored during each water renewing.

**Preparation of total RNA and cDNA synthesis**

Isolated gill tissues were mixed and homogenized in Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was purified following the manufacturer’s protocol. The total amount of RNA was determined by spectrophotometry (ND-1000, NanoDrop Technol, Wilmington, DE), and the RNA quality was checked by running electrophoresis in RNA-denatured gels. All RNA pellets were stored at -20 °C. For cDNA synthesis, 5 μg of total RNA was reverse-transcribed in a final volume of 20 μl containing 0.5 mM dNTPs, 2.5 μM oligo (dT)$_{18}$, 5 mM dithiothreitol, and 200 units of superscript reverse transcriptase III (Invitrogen) for 1.5 h at 42 °C, followed by a 15-min incubation at 70 °C.

**RNA probe synthesis**
Fragments of the target genes (Table 2) obtained by PCR were inserted into pGEM-T Easy vectors. The inserted fragments were amplified with the T7 and SP6 primers by PCR. Digoxigenin- (Dig; Roche, Penzberg, Germany) or biotin-labeled (Roche) RNA probes were synthesized by in vitro transcription with T7 and SP6 RNA polymerase (Takara, Shiga, Japan). The qualities of the probes were examined using RNA gels, and the concentrations were determined by a dot-blot assay with standard DIG-labeled RNA (100 ng/μl) (Roche).

*Fluorescence double in situ hybridization*

Excised gills were fixed with 4% paraformaldehyde overnight at 4 °C and then washed several times with phosphate-buffered saline (PBS). After being dehydrated with methanol, whole gills were washed several times with PBS containing 0.1% Tween-20 (PBST). Samples were incubated with hybridization buffer (HyB) containing 50% formamide, 5× SSC, and 0.1% Tween-20 for 5 min at 65 °C.

Prehybridization was performed for 2 h at 65 °C with HyB+, which is the hybridization buffer supplemented with 500 μg/ml yeast transfer (t)RNA and 50 μg/mL heparin. For hybridization, samples were incubated in 100 ng of the RNA probe in 200 μl HyB+ at 65 °C overnight. Then, samples were washed at 70 °C for 30 min twice in 50% HyB and 50% 2× SSC, 30 min twice in 2× SSC, and 30 min twice in 0.2× SSC. Further washes were performed at room temperature for 5 min in 75%
0.2× SSC and 25% PBST, 5 min in 50% 0.2× SSC and 50% PBST, 5 min in 25% 0.2× SSC and 75% PBST, and 5 min in PBST. Fluorescence staining was conducted with a commercial kit of TSA Plus Fluorescence Systems (Perkin-Elmer, Waltham, MA). The hybridization signals detected by the Dig-labeled RNA probes were amplified through fluorescein-tyramide signal amplification (TSA), while cyanine 3-TSA was used for the biotin-labeled probes. Images of gill surfaces were acquired with a confocal laser scanning microscope (TCS-SP5, Leica Lasertechnik, Heidelberg, Germany).

Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted and reverse-transcribed from 5 zebrafish gill tissues (as a pooled sample) as described above. A total of 25 individuals for each test were sampled (n=5). mRNA expression of target genes were measured by qRT-PCR with an ABI Prism 7000 sequence analysis system (Applied Biosystems, Foster City, CA). Primers for all genes were designed (Table 3) using Primer Express software (vers. 2.0.0, Applied Biosystems). PCRs contained 3.2 ng of cDNA, 100 nM of each primer, and Universal SYBR green master mix (Applied Biosystems) in a final volume of 20 μl. All qRT-PCR reactions were performed as follows: 1 cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min (the set annealing temperature of all primers). PCR products were subjected to melting-curve
analysis, and representative samples were electrophoresed to verify that only a single product was present. Control reactions were conducted with sterile water to determine the levels of the background and non-specific primer dimer noise. The standard curve of each gene was confirmed to be in a linear range with β-actin as an internal control. The amplicons were sequenced to confirm the PCR products.

*Phylogenetic analysis*

Protein sequences of the NKA α1 genes were retrieved from the Ensembl or NCBI databases, and the accession numbers were listed in Supplementary Table 1. Amino acid sequences of NKA α1 from organisms representing different taxa were aligned by ClustalX vers. 1.81, and the phylogenetic tree was then calculated and drawn with MEGA vers. 4.1. A rooted phylogenetic tree was built using a Neighbor-joining (NJ) method with the pairwise deletion gaps calculating option. The bootstrap values showed beside the branches are percentage from bootstrap analysis with 1000 cycles.

*Statistical analysis*

Values are presented as the mean ± standard deviation (SD) and compared using Student’s *t*-test or one-way analysis of variance (ANOVA) (Tukey’s pairwise comparison).
Results

*Phylogenetic analysis of the ATP1α gene family*

As shown in Fig. 1A, there are 9 isoforms published in the zebrafish NKA alpha (ATP1α) gene family, and comparatively fewer isoforms were discovered in other teleost species. In order to understand the evolutionary relationship of the differences in isoform numbers, an integrated comparison of all published isoforms and those predicted from the entire genome data is essential. So far, the Ensembl database (vers. 50) has provided the genome data of 5 teleost species (*Danio rerio*, *Oryzias latipes*, *Tetraodon nigroviridis*, *Takifugu rubripes*, and *Gasterosteus aculeatus*). Twenty isoforms were retrieved from Ensembl by navigation of the gene family (Ensembl Family ID: ENSF00000000449), in which data of 9 genes of zebrafish were annotated sequences, and 2 medaka isoforms (olATP1α1a.1 and olATP1α1a.4) were cloned in our unpublished experiments (Lai et al., data not shown). Another 11 ATP1α isoforms from other teleostean species were downloaded from NCBI's GenBank (Supplementary Table 1).

According to the phylogenetic analysis (Fig. 1A), 3 major clades, ATP1α1, ATP1α2, and ATP1α3, were clustered with the teleostean orthologues and corresponding mammalian isoforms. Within the ATP1α1 group, teleostean isoforms were separated into 2 major clusters, ATP1α1a.1-like and ATP1α1a.4-like clades. In
zebrafish, zATP1a1a.2, zATP1a1a.3, and zATP1a1a.5 were closest to each other in the clade, and their genomic loci also indicated a high possibility of tandem duplication (Fig. 1B). The multiple-species genome comparison from the available genome data showed that all teleostean species have ATP1a1a.1 and ATP1a1a.4, while zATP1a1a.2, zATP1a1a.3 and zATP1a1a.5 are specifically found in zebrafish (Fig. 1B).

Expression of 4 NKA α1 paralogues in 4 types of gill cells

According to the in situ hybridization screening of zebrafish embryos (1, 4), 4 (atp1a1a.1, atp1a1a.2, atp1a1a.4, and atp1a1a.5) of the 6 α1-like genes are expressed in skin mucous cells, which showed a salt-and-pepper pattern as did the skin ionocytes (20, 29). Therefore, subsequent studies focused on these 4 genes. Because NKA α1 genes are highly conserved with 78%-83% identities of the coding region nucleotide sequences among the 4 genes, the 3’ regions including most 3’ untranslated regions (UTRs) (Table 2) were used to avoid latent cross-reactions during in situ hybridization. Fluorescence in situ hybridization results (Figs. 2A-F) showed that each of the 4 NKA α1 genes were expressed in a specific group of gill cells. Results of double-fluorescence in situ hybridization showed no colocalized signal was detected in any combination of 2 isoforms (Figs. 2A-F), indicating that the 4 α1 genes were respectively expressed in different types of cells (or ionocytes). Images of in situ
hybridization were acquired by scanning the surface of the whole-mount gills, which have complicated 3-dimensional structures in the gill filaments. Various cell sizes were observed in some images (Figs. 2-4), and this is probably because only a part of a cell, but not a whole cell, was acquired during scanning. Five individuals were used for each in situ hybridization, and the same results were confirmed in all samples.

Negative controls in situ-hybridized with respective sense riboprobes were conducted to confirm that no signals were found (data not shown).

**NKA α1 paralogues co-expressed with other ionocyte markers**

A marker of NaR cells, ECaC (trpv6), which is known to be the apical entry of transepithelial Ca\(^{2+}\) uptake (27, 34), was colocalized with only *atp1a1a.1* (Fig. 3A). A cation-chloride cotransporter, NCC-like2 (*slc12a10.2*), which was proposed to be responsible for Cl\(^{-}\) uptake in NCC cells (42), was specifically detected only in *atp1a1a.2*-positive cells (Fig. 3B). Furthermore, all signals of HR cell markers examined, including *atp6v1a* (V-ATPase subunit A) and *slc9a3b* (NHE3b, sodium/proton exchanger 3b), were detected only in *atp1a1a.5*-positive cells (Figs. 3C, D). For any other permutation combinations of the 3 NKA α1 genes (*atp1a1a.1, atp1a1a.2, and atp1a1a.5*) with the other ionocyte markers used above, non-colocalization signals were detected (Figs. 4A-F) by double fluorescence in situ
hybridization. However, none of colocalization signals were found between \textit{atp1a1a.4} and all the ionocyte marker genes (Figs. 4G-I). Five individuals were used for each \textit{in situ} hybridization, and the same results were confirmed in all samples.

5 \textit{Environmental effects on NKA} α1 \textit{parologue mRNA expression}

Adult zebrafish were acclimated to artificial fresh waters with different ion concentrations, which could stimulate the uptake capacities of various ions. Comparisons of the effects of ion compositions on the gene regulations of transcription levels of NKA α1 paralogues by qRT-PCR were conducted to examine the relationships among NKA α1 paralogues and the uptake functions of various ions.

Of the 4 NKA α1 paralogues examined in freshwater zebrafish gills, \textit{atp1a1a.2} was expressed at the highest level (around 0.2-fold relative to β-actin in arbitrary numbers, \(n=5\)), followed by \textit{atp1a1a.4}, \textit{atp1a1a.1}, and \textit{atp1a1a.5} (Figs. 5, 6). After acclimation to different media (H-Na-L-Cl, H-Na-H-Cl, L-Na-L-Cl, H-Ca or L-Ca), NKA α1 paralogues showed different expression patterns (Figs. 5A-D, 6A-D), while \textit{atp1a1a.4} was found not to respond to environmental factors (Figs. 5C, 6C). The expression of \textit{atp1a1a.1} significantly decreased in the low-Na\(^+\) condition (by comparing H-Na-L-Cl and L-Na-L-Cl media), while there was no significant difference found between the altered Cl\(^-\) factors (by comparing H-Na-L-Cl and H-Na-H-Cl media) (Fig. 5A).
atp1a1a.2 mRNA, however, was upregulated in the low-Cl\textsuperscript{−} condition (by comparing H-Na-L-Cl and H-Na-H-Cl media) and showed no difference between H-Na-L-Cl and L-Na-L-Cl (Fig. 5B). Furthermore, atp1a1a.5 mRNA showed the highest expression in L-Na-L-Cl fresh water in response to a low-Na\textsuperscript{+} environment (by comparing H-Na-L-Cl and L-Na-L-Cl media), but not to the low-Cl\textsuperscript{−} condition (by comparing H-Na-L-Cl and H-Na-H-Cl media) (Fig. 5D).

After acclimation to H-Ca and L-Ca media, the mRNA expression of the 3 NKA α\textsubscript{1} paralogues, atp1a1a.2, atp1a1a.4, and atp1a1a.5, showed no significant differences, indicating no responses to ambient Ca\textsuperscript{2+} concentration changes (Figs. 6B, 6C), while only atp1a1a.1 showed an approximately 2.3-fold higher mRNA expression level in low-Ca\textsuperscript{2+} fresh water (Fig. 6A). No fish died or showed abnormal phenotypes during the 2-wk acclimation experiments.
Discussion

The present study for the first time demonstrates the differential mRNA expression and localizations of the 3 NKA α subunit paralogues, *atp1a1a.1, atp1a1a.2,* and *atp1a1a.5,* in respective types of ionocytes of gills of zebrafish (*D. rerio*). The results suggest that these 3 paralogues of the NKA α subunit may play different roles in Ca$$^{2+},$$ Cl$$^{-},$$ and Na$$^{+}$$ uptake mechanisms in zebrafish gills.

Based on the phylogenetic analysis, 6 of the zebrafish α subunit genes are orthologues to the mammalian α1 subunit gene, and except for ATP1a1b, the other 5 α1 orthologues are near each other in chromosome 1, implying that they may have been derived from tandem duplication events in addition to genome-wide duplication (36). By comparing multiple genomic loci from 5 teleosts (Fig. 1B), ATP1a1a.1 and ATP1a1a.4 gene clusters were found in all species, and were also present at reasonable positions in the phylogenetic tree (Fig. 1A). Three α1 orthologues (ATP1a1a, ATP1a1b, and ATP1a1c) have been discovered in rainbow trout (37), and 5 α1 orthologues (ATP1a1a, ATP1a1b/i, ATP1a1b/ii, ATP1a1c/i, and ATP1a1c/ii), which include 2 pairs of putative homeologs, were also published for Atlantic salmon (16). Although ATP1a1b and ATP1a1c of both rainbow trout and Atlantic salmon are neighbors in a single linkage group (11, 16), the genomic data of these 2 tetraploid-derivative salmonid fish are not sufficiently clear to determine the
evolutionary relationships with other teleosts. Therefore, the original nomenclatures of salmonid NKA were retained in the present study. In zebrafish, retention of a high proportion of NKA α subunit gene duplicates suggests that these genes may possess specialized, rather than redundant, functional properties (15, 31). The present study provides some clues to support this notion. Four of the 6 NKA α1 paralogues of zebrafish were found to specifically be expressed and function in distinct types of gill ionocytes.

According to the whole-mount in situ hybridization analysis by Canfield et al. (4) and Blasiole et al. (1), no 2 α subunit genes were found to exhibit the same spatial distribution pattern, and 4 of the 6 NKA α1 genes were expressed in skin mucous cells, which showed a salt-and-pepper expression pattern. Mucous cells have been closely identified with large ovoid cells that are composed mostly of large apical mucous secretory granules (43). No evidence so far is available to demonstrate the presence of NKA in mucous cells. On the other hand, MR cells, a type of ionocyte, have been identified either by their characteristic morphology or by immunological staining for NKA, which has long been a marker of MR cells (13, 21, 23). Moreover, recent studies have used molecular physiological approaches to identify and functionally analyze different types of ionocytes (12, 29, 30, 34, 42, 44), which also show a salt-and-pepper expression pattern like mucous cells do (1, 4). In the present study,
the NKA α1 isoforms of atp1a1a.1, atp1a1a.2, and atp1a1a.5 were demonstrated to specifically exist in NaR, NCC, and HR cells, respectively, by the co-localizations of the α1 paralogues and the relevant ion transporters. These results do not support findings of previous studies (1, 4) that the 4 NKA α1 paralogues are expressed in zebrafish skin mucous cells.

It has been proposed that differential expression of various NKA isoforms may lead to different Na⁺ or K⁺ affinities and fulfill some of the requirements for the subtly altered enzyme behaviors of gills in fish acclimated to different salinities (14, 23). To test this, Hwang and colleagues identified the co-localization of α1 and α3 in tilapia gill MR cells and analyzed their salinity-dependent differential expression with heterologous antibodies (26), and this was supported by subsequent cloning of the 2 α-subunit genes and a Northern blot/RT-PCR investigation of their mRNA expression in gills (14). Recently, Richards et al. (37) and their subsequent study (3) identified 2 NKA α isoforms, α1a and α1b, and found the differential mRNA expression of the 2 isoforms by real-time PCR in gills of rainbow trout (Oncorhynchus mykiss), Atlantic salmon (Salmo salar), and Arctic char (Salvelinus alpinus) during salinity acclimation. Since gills are composed of various cell types, detection of mRNA with Northern blot, RT-PCR, and/or real-time PCR could not certify the specific expression or functional roles of these NKA α-subunit isoforms in fish gill ionocytes in previous studies. The
present study for the first time provides convincing molecular evidence of the distinct existence of ATP1a1a.1, ATP1a1a.2, and ATP1a1a.5 in different ionocytes in zebrafish gills based on specific co-localizations of the mRNAs of these α-subunits and the relevant transporters of ionocytes. Isoform-specific heterologous antibodies have been previously used to examine NKA α1, -2 and -3 isoforms in fish gills (26); however, no antibody is available so far to specifically detect the respective products of the NKA α1 paralogues. This is a challenging and important issue to be solved in the future.

A central unresolved issue concerning NKA is whether the multiple isoforms of the α-subunits have unique or overlapping functions, and the approaches used so far in studies of mammalian NKA isoforms have limitations for exploring this issue, as addressed by Cheng et al. (8). The mRNA expression of ATP1a1a.1, ATP1a1a.2, and ATP1a1a.5 were differentially stimulated by environmental ion compositions, and were parallel to those of the relevant transporters in each type of respective ionocyte (Table 4). HR, NaR, and NCC cells in zebrafish skin/gill have been proposed to be responsible for Na⁺ uptake/acid secretion, Ca²⁺ uptake, and Cl⁻ uptake, respectively (21). As summarized in Table 4, low-Na⁺, low-Ca²⁺, and low-Cl⁻ artificial FW media, which were reported to stimulate the uptake of distinct ions (Na⁺, Ca²⁺, and Cl⁻) by fish gills (5, 6, 7), were also found to respectively stimulate the mRNA expression of NHE3b/CA15a/ATP1a1a.5, ECaC/ATP1a1a.1, and NCC/ATP1a1a.2. This provides
molecular evidence to support the possible roles of ATP1a1a.5, ATP1a1a.1, and ATP1a1a.2 in zebrafish ionocyte functions, which are to respectively drive the uptake of Na\(^+\), Ca\(^{2+}\) and Cl\(^-\) and also suggested the distinctive but not redundant functions of these NKA \(\alpha\)-subunit paralogues in zebrafish gills \textit{in vivo}. On the other hand, ATP1a1a.4 was not co-localized in any type of zebrafish gill ionocyte (HR, NaR, or NCC cells), nor was its mRNA expression stimulated by low-ion (Na\(^+\), Ca\(^{2+}\), and Cl\(^-\)) media, indicating that ATP1a1a.4-expressing cells may be an unidentified type of ionocyte. These unidentified ionocytes were expressed in both gill filaments and lamella, and were not associated with the uptake of Na\(^+\), Ca\(^{2+}\), or Cl\(^-\). Their functions and relevant transporters are another interesting and challenging issue for future study.

The present results of the specific mRNA expression and regulation of ATP1a1a.5, ATP1a1a.1, and ATP1a1a.2 in zebrafish ionocytes allow us to make a more-comprehensive explanation for the proposed model, in which these NKA \(\alpha\)-subunits may play roles in providing some driving force for ion uptake in the respective ionocytes (Fig. 7). The Na\(^+\) uptake mechanism in zebrafish HR cells has been proposed to be similar to that in mammal kidney proximal tubular cells (30, 44). In apical HR cells, ambient HCO\(_3^-\) and H\(^+\) secreted by apical NHE3 (or H\(^+\)-ATPase) actively form H\(_2\)CO\(_3\), and H\(_2\)CO\(_3\) is dehydrated by apical CA15a, thereby enabling the passive diffusion of CO\(_2\) into cells. Then the cytosolic CA2-like a hydrates CO\(_2\) to
provide the substrates with $H^+$ and $HCO_3^-$, for the apical NHE3b and basolateral $Na^+/HCO_3^-$ cotransporter (NBC) 1, respectively. ATP1a1a.5/ATP1b1b may pump cytosolic $Na^+$ across the basolateral membrane and also provide an intracellular negative potential to drive the electrogenic $Na^+$ transport of basolateral NBC1, which has been identified in gills of Osorezan dace (*Tribolodon hakonensis*) (17) and rainbow trout (*Oncorhynchus mykiss*) (35). Similar to the mammalian renal $Ca^{2+}$ reabsorption mechanism (18), $Ca^{2+}$ passively enters zebrafish NaR cells via the apical ECaC, and then is extruded via the basolateral PMCA2 and NCX1b (27). ATP1a1a.1/ATP1b1b may creates a $Na^+$ gradient to drive the exchange by NCX1b. $Cl^-$ uptake mechanisms in zebrafish ionocytes are still in debate. A previous pharmacological study with $H^+$-ATPase and CA inhibitors proposed a proton pump- and CA-dependent $Cl^-$ uptake model in zebrafish gills (2). However, this notion does not appear to be supported by a recent loss-of-function study on $H^+$-ATPase, in which translational knockdown of $H^+$-ATPase subunit A impaired the acid secretion but without a significant effect on the $Cl^-$ content in zebrafish morphants (19). More recently, a novel NCC-like 2 isoform, zebrafish *slc12a10.2*, was found to specifically be expressed by a group of ionocytes (NCC cells) in zebrafish, and translational knockdown of NCC-like 2 caused significant decreases in both $Cl^-$ influx and $Cl^-$ content, suggesting a role of NCC in zebrafish skin/gill $Cl^-$ uptake pathways (42).
Assuming the zebrafish NCC-like 2 as an electroneutral cortansporter like the mammalian orthologue, ambient Na\(^+\)/Cl\(^-\) levels (local tap water, 0.4–0.7 mM) are unlikely to favor the operation of NCC in apical membranes of NCC cells in zebrafish, since the reported intracellular Na\(^+\)/Cl\(^-\) levels in fish gill cells are 55-62 mM (or predicted as 6-17 mM) (35) and 40-90 mM (40), respectively. The issue of the NCC’s driving force remains to be an open question (21, 42). Nevertheless, ATP1a1a.2/ATP1b1b may establish an electrochemical gradient to drive the basolateral transport pathways in NCC cells.

In mammals, NKA \(\alpha\)-subunit isoforms have been demonstrated to show differences in the affinities for ouabain and substrates (Na\(^+\) and K\(^+\)) as well as interactions with the \(\beta\) subunit \textit{in vitro} (10, 24). Interestingly, some studies demonstrated different substrate affinities for gill NKAs between SW- and FW-acclimated tilapia (28) and rainbow trout (\textit{Oncorhynchus mykiss}) (33), and both tilapia and rainbow trout were found to express NKAs with different combinations of \(\alpha\)-subunits depending on environmental salinities (14, 23, 37). On the other hand, the different responses to endocrine or environmental factors among NKA \(\alpha\) isoforms (or \(\alpha1\) paralogues) should also be considered when discussing the physiological significances of the existence of multi-isoforms of NKA \(\alpha\)-subunits. In tilapia, plasma levels of ouabain, cortisol and osmolality showed positive correlations during salinity...
acclimation, suggesting the involvement of ouabain with cortisol in the maintenance of hydromineral balance (25). In zebrafish, different ionocyte types express respective NKA α1 paralogues, which may subtly differ in ouabain affinity, implying different signaling potentials in these ionocytes. In mammalian kidneys, combinations of α1-3 and β1-2 subunits were found to be differentially expressed in various segments by RT-PCR analysis on microdissected samples (9). In overexpression experiments of mouse collecting duct mpkCCD cells, the human α1 but not the α2 isoform was stimulated by aldosterone, a steroid controlling Na⁺ reabsorption in distal nephrons (38, 39). In the present study, the ATP1a1a.1, ATP1a1a.2, and ATP1a1a.5 paralogues in zebrafish gill ionocytes showed distinctive responses to the environmental ion levels. This finding is of physiological significance and interest, and implies that regulatory pathways controlling the function and expression of each α1 paralogue in respective ionocytes may subtly differ to allow zebrafish to adapt to fluctuating environments.

**Perspectives and Significance**

In mammals, it has been for a long time an important issue to study the expressions of different combinations of NKA α and β subunits and their roles in the functions of the
various tissues and/or cell types. This phenomenon is evolutionally conserved in mammalian kidneys and zebrafish skin/gills. Compared to mammals, zebrafish is a more powerful model to provide *in vivo* molecular physiological evidence to answer if these NKA isoforms conduct respective or overlapping functions in the same organ or tissue. In zebrafish, ion transport functions in respective types of ionocytes have been explored, and the methodologies to assay these functions have also been established recently. The question about the functional redundancy of NKA isoforms in zebrafish ionocytes may be answered by examining if the functional defects in ionocytes caused by knockdown of one NKA α1 paralogue could be rescued by overexpression of another paralogue(s).
Acknowledgments

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17. Hirata T, Kaneko T, Ono T, Nakazato T, Furukawa N, Hasegawa S,
Wakabayashi S, Shigekawa M, Chang MH, Romero MF, and Hirose S.


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35. **Parks SK, Tresguerres M, and Goss GG.** Interactions between Na⁺ channels and Na⁺-HCO₃⁻ cotransporters in the freshwater fish gill MR cell: a model for


41. **Tipsmark CK.** Identification of FXYD protein genes in a teleost: tissue-specific expression and response to salinity change. *Am J Physiol Regul Integr Comp*


Table 1. Ionic compositions (mM) and pH in artificial fresh water

<table>
<thead>
<tr>
<th>Medium</th>
<th>[Na⁺]</th>
<th>[Cl⁻]</th>
<th>[Ca²⁺]</th>
<th>[K⁺]</th>
<th>[Mg²⁺]</th>
<th>pH</th>
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<tbody>
<tr>
<td>L-Na-L-Cl</td>
<td>0.04-0.05</td>
<td>0.03-0.06</td>
<td>0.18-0.19</td>
<td>0.16-0.17</td>
<td>0.18-0.19</td>
<td>6.6-6.8</td>
</tr>
<tr>
<td>H-Na-L-Cl</td>
<td>10.0-10.5</td>
<td>0.04-0.07</td>
<td>0.17-0.18</td>
<td>0.16-0.18</td>
<td>0.20-0.21</td>
<td>6.7-6.8</td>
</tr>
<tr>
<td>H-Na-H-Cl</td>
<td>9.5-10.1</td>
<td>9.8-10.2</td>
<td>0.19-0.20</td>
<td>0.15-0.17</td>
<td>0.19-0.21</td>
<td>6.7-6.9</td>
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<tr>
<td>L-Ca</td>
<td>0.46-0.47</td>
<td>0.46-0.48</td>
<td>0.01-0.02</td>
<td>0.22-023</td>
<td>0.22-0.23</td>
<td>6.5-6.8</td>
</tr>
<tr>
<td>H-Ca</td>
<td>0.45-0.48</td>
<td>0.45-0.49</td>
<td>1.91-2.20</td>
<td>0.21-023</td>
<td>0.22-0.24</td>
<td>6.7-6.9</td>
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Table 2. Probes for \textit{in situ} hybridization

<table>
<thead>
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<th>Gene name</th>
<th>GenBank accession no.</th>
<th>Region</th>
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<td>AF286372</td>
<td>nt 2596–3420</td>
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<td>\textit{atp1a1a.2}</td>
<td>AF286374</td>
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<td>\textit{atp1a1a.4}</td>
<td>AY008376</td>
<td>nt 2305–3120</td>
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<tr>
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<td>NM_178099</td>
<td>nt 2939–3761</td>
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<td>\textit{trpv6}</td>
<td>NM_001001849</td>
<td>nt 144–2268</td>
</tr>
<tr>
<td>\textit{slc12a10.2}</td>
<td>NM_001045001</td>
<td>nt 639–2162</td>
</tr>
<tr>
<td>\textit{atp6v1a}</td>
<td>NM_201135</td>
<td>nt 407–1143</td>
</tr>
<tr>
<td>\textit{slc9a3b}</td>
<td>EF591980</td>
<td>nt 1907–2745</td>
</tr>
</tbody>
</table>

\text{nt, nucleotide sequence number.}
Table 3. Primer sets for the qualitative RT-PCR analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>atp1a1a.1</td>
<td>F 5' GCCCTGAGCAATTAGACGATG 3'</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>R 5' TACGGCTACAATGGCACCCT 3'</td>
<td></td>
</tr>
<tr>
<td>atp1a1a.2</td>
<td>F 5' TCTACCTTTGGGCACCGTCAC 3'</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>R 5' TGCTTGGATCATCCCGATTT 3'</td>
<td></td>
</tr>
<tr>
<td>atp1a1a.4</td>
<td>F 5' TTCTGCCACTTCTGCTTCC 3'</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>R 5' CACCTTGATCCAGCACTCC 3'</td>
<td></td>
</tr>
<tr>
<td>atp1a1a.5</td>
<td>F 5' CGGGTTCTTTACCTAATTTGTGA 3'</td>
<td>78</td>
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<tr>
<td></td>
<td>R 5' CAGCCAATCCGTAATCCAC 3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F 5' CACCTTCCAGCAGATGTGGA 3'</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>R 5' AAAAGCCATGCAATGTTGTC 3'</td>
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</table>

F, forward primer; R, reverse primer.
Table 4. Comparisons of expression and functions of the relevant transporters/enzymes in zebrafish ionocytes

<table>
<thead>
<tr>
<th>Ionocytes</th>
<th>H⁺-ATPase-rich cells</th>
<th>Na⁺-K⁺-ATPase-rich cells</th>
<th>Na⁺-Cl⁻ cotransporter-expressing cells</th>
<th>Unidentified ionocytes</th>
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<tr>
<td>Proposed function</td>
<td>Na⁺ uptake</td>
<td>Ca⁺² uptake</td>
<td>Cl⁻ uptake</td>
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<tr>
<td>acid</td>
<td>secretion</td>
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<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Specifically-expressed transporters/enzymesᵃ</th>
<th>H⁺-ATPase</th>
<th>ECaC</th>
<th>NCC</th>
<th>Unknown</th>
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<tbody>
<tr>
<td>NHE3b</td>
<td>PMCA2</td>
<td></td>
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<tr>
<td>CA15a</td>
<td>NCX1b</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CA2-like a</td>
<td></td>
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<table>
<thead>
<tr>
<th>Na⁺-K⁺-ATPase isoform</th>
<th>ATP1a1a.5</th>
<th>ATP1a1a.1</th>
<th>ATP1a1a.2</th>
<th>ATP1a1a.4</th>
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<tbody>
<tr>
<td>ATP1b1b</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Artificial medium to stimulate functions of ionocytes</th>
<th>Low-Na⁺</th>
<th>Low-Ca⁺²</th>
<th>Low-Cl⁻</th>
<th>None</th>
</tr>
</thead>
</table>

³ Includes NHE3b, PMCA2, CA15a, NCX1b, CA2-like a.
<table>
<thead>
<tr>
<th>Transporters/enzymes</th>
<th>NHE3b</th>
<th>ECaC</th>
<th>NCC</th>
<th>None</th>
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</thead>
<tbody>
<tr>
<td>up-regulated by artificial medium</td>
<td>CA15a</td>
<td>ATP1a1a.1</td>
<td>ATP1a1a.1</td>
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<tr>
<td>medium</td>
<td>ATP1a1a.5</td>
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^a, ^b Data are from Lin et al. (29, 30), Yan et al. (44), Pan et al. (34), Liao et al. (27), and Wang et al. (42).

CA, carbonic anhydrase; ECaC, epithelial Ca\(^{2+}\) channel; HA, H\(^+\)-ATPase; NCC, Na\(^+\)-Cl\(^-\) cotransporter; NCX, Na\(^+\)/Ca\(^{2+}\) exchanger; NHE, Na\(^+\)/H\(^+\) exchanger, NKA, Na\(^+\)-K\(^+\)-ATPase; PMCA; plasma membrane Ca\(^{2+}\)-ATPase.
**Figure legends**

Fig. 1. Phylogenetic analysis of ATP1a isoform amino acid sequences (A) and a schematic picture of the genomic loci of teleostean ATP1a1 genes (B). (A) Percentages of bootstrapping branch corrections are shown beside the branches, and the orthologous relationships of clades are labeled on the right side to indicate different sub-tree regions. An asterisk (*) indicates a hypothetical protein predicted from other studies. (B) The multi-genomic alignments were retrieved from Ensembl paralogue prediction and multiple alignment of contigs. The chromosome numbers or contig names are shown on the left, and the locations of the genes in each chromosome are indicated by sequence numbers. The orthologues of ATP1a1a.1 and ATP1a1a.4 were linked with vertical filled colored boxes.

Fig. 2. Representative images of 4 distinct expression patterns of ATP1a isoforms in zebrafish gills. Whole-mount double fluorescence in situ hybridizations were used to identify expression patterns of the 6 different combinations of 2 ATP1a isoforms: (A) atp1ala.2 + atp1ala.1, (B) atp1ala.4 + atp1ala.1, (C) atp1ala.5 + atp1ala.1, (D) atp1ala.4 + atp1ala.2, (E) atp1ala.5 + atp1ala.2, and (F) atp1ala.5 + atp1ala.4. The arrow and arrowhead indicate the positive signals of the 2 NKA riboprobes in different cells. Scale bar = 20 μm.

Fig. 3. Whole-mount double fluorescence in situ hybridizations of atp1ala.1, atp1ala.2 or atp1ala.5 with ionocyte marker genes in zebrafish gills. The representative images show the colocalization for (A) atp1ala.1 + trpv6 (ECaC), (B) atp1ala.2 + slc12a10.2 (NCC-like2), (C) atp1ala.5 + atp6v1a (V-ATPase subunit A), (D) atp1ala.5 + slc9a3b (NHE3b). An asterisk (*) indicates colocalization signals. Scale bar = 20 μm.
Fig. 4. Whole-mount double fluorescence in situ hybridizations of atp1a1a.1 (A and B), atp1a1a.2 (C and D), atp1a1a.5 (E and F) or atp1a1a.4 (G-I) with ionocyte marker genes in zebrafish gills. No colocalizations between of NKA α1 genes (green fluorescence) and ionocyte markers (red fluorescence) were found: (A) atp1a1a.1 + slc12a.10.2 (NCC-like2), (B) atp1a1a.1 + atp6v1a (V-ATPase subunit A), (C) atp1a1a.2 + trpv6 (ECaC), (D) atp1a1a.2 + atp6v1a, (E) atp1a1a.5 + trpv6, (F) atp1a1a.5 + slc12a.10.2, (G) atp1a1a.4 + trpv6, (H) atp1a1a.4 + slc12a.10.2, and (I) atp1a1a.4 + atp6v1a. Scale bar = 20 μm.

Fig. 5. Effects of environmental Na\(^+\) or Cl\(^-\) concentration on gill transcriptions by quantitative RT-PCR of atp1a1a.1 (A), atp1a1a.2 (B), atp1a1a.4 (C), and atp1a1a.5 (D) in zebrafish. The mean and SD (n=5) are shown. One-way ANOVA pair-wise comparisons were conducted among treatments.

Fig. 6. Effects of environmental Ca\(^{2+}\) concentration on gill transcriptions (by quantitative RT-PCR) of atp1a1a.1 (A), atp1a1a.2 (B), atp1a1a.4 (C), and atp1a1a.5 (D) in zebrafish. An asterisk (*) indicates a significant difference between the 2 groups (Student's t-test, \(p<0.05\)).

Fig. 7. Model of the ion regulation mechanisms in zebrafish gill/skin ionocytes. Most of the proposed mechanisms are derived from Hwang and Lee (23) and Hwang (21) with some updates. A question mark, “?”, indicates an unidentified ionocyte, transporter, or pathway. CA2, carbonic anhydrase 2-like a; CA15, carbonic anhydrate 15a; ClC, Cl\(^-\) channel; ECaC, epithelial Ca\(^{2+}\) channel; HA, H\(^{+}\)-ATPase; NBC, Na\(^+\)/HCO\(3^-\)cotransporter; NCC, Na\(^+\)-Cl\(^-\) cotransporter; NCX, Na\(^+\)/Ca\(^{2+}\) exchanger 1b; NHE, Na\(^+\)/H\(^+\) exchanger 3b; NKA, Na\(^+\)-K\(^-\)-ATPase; PMCA2; plasma membrane Ca\(^{2+}\)-ATPase.
Fig. 1

A. Phylogenetic tree showing the relationships among various ATP1a isoforms from different species. The tree is divided into multiple clades, including ATP1a.1-like clade, ATP1a.1 clade, ATP1a.4-like clade, ATP1a.4 clade, ATP1a2 clade, and ATP1a3 clade.

B. Chromosomal localization of ATP1a isoforms across different species. The map shows the locations of ATP1a isoforms on various chromosomes, with distances and sizes indicated.
Fig. 2

A: atp1a1.2 + atp1a1.1

B: atp1a1.4 + atp1a1.1

C: atp1a1.5 + atp1a1.1

D: atp1a1.4 + atp1a1.2

E: atp1a1.5 + atp1a1.2

F: atp1a1.5 + atp1a1.4
Fig. 3

(A) atp1a1a.1 + trpv6

(B) atp1a1a.2 + slc12a10.2

(C) atp1a1a.5 + atp6v1a

(D) atp1a1a.5 + slc9a3b
Fig. 4

A. atp1a1a.1 + slc12a10.2
B. atp1a1a.1 + atp6v1a
C. atp1a1a.2 + trpv6
D. atp1a1a.2 + atp6v1a
E. atp1a1a.5 + trpv6
F. atp1a1a.5 + slc12a10.2
G. atp1a1a.4 + trpv6
H. atp1a1a.4 + slc12a10.2
I. atp1a1a.4 + atp6v1a
Fig. 5

(A) atp1a1a.1 mRNA
(B) atp1a1a.2 mRNA
(C) atp1a1a.4 mRNA
(D) atp1a1a.5 mRNA
Fig. 7

Apical

NCC cell

ECaC

NaR cell

PMCA

NCX

HR cell

CA15

HCO₃⁻ + H⁺

HA

CO₂

NHE

Na⁺ Na⁺ Na⁺ Na⁺ Na⁺

H⁺ H⁺ H⁺ H⁺ H⁺

HCO₃⁻ + H⁺

CA2

CO₂

NBC

ATP1a1a.4 ATP1b1b

ATP1a1a.2 ATP1b1b

ATP1a1a.1 ATP1b1b

ATP1a1a.5 ATP1b1b

ATP1a1a.4 ATP1b1b

ATP1a1a.2 ATP1b1b

ATP1a1a.1 ATP1b1b

ATP1a1a.5 ATP1b1b