Phylogenetic and developmental analyses indicate complex functions of calcium-activated potassium channels in zebrafish embryonic development

Martin R. Silic | Maya M Black | GuangJun Zhang

1Department of Comparative Pathobiology, Purdue University, West Lafayette, Indiana
2Purdue University Center for Cancer Research, West Lafayette, Indiana
3Purdue Institute for Inflammation, Immunology and Infectious Diseases (PI4D), West Lafayette, Indiana
4Purdue Institute for Integrative Neuroscience; Purdue University, West Lafayette, Indiana

Correspondence
GuangJun Zhang, Department of Comparative Pathobiology, Purdue University, West Lafayette, Indiana, USA. Email: gjzhang@purdue.edu

Funding information
National Institute of General Medical Sciences of the National Institutes of Health, Grant/Award Number: R35 GM-124913

Abstract
Background: Calcium-activated potassium channels (KCa) are a specific type of potassium channel activated by intracellular calcium concentration changes. This group of potassium channels plays fundamental roles ranging from regulating neuronal excitability to immune cell activation. Many human diseases such as schizophrenia, hypertension, epilepsy, and cancers have been linked to mutations in this group of potassium channels. Although the KCa channels have been extensively studied electrophysiologically and pharmacologically, their spatiotemporal gene expression during embryogenesis remains mostly unknown.

Results: Using zebrafish as a model, we identified and renamed 14 KCa genes. We further performed phylogenetic and syntenic analyses on vertebrate KCa genes. Our data revealed that the number of KCa genes in zebrafish was increased, most likely due to teleost-specific whole-genome duplication. Moreover, we examined zebrafish KCa gene expression during early embryogenesis. The duplicated ohnologous genes show distinct and overlapped gene expression. Furthermore, we found that zebrafish KCa genes are expressed in various tissues and organs (somites, fins, olfactory regions, eye, kidney, and so on) and neuronal tissues, suggesting that they may play important roles during zebrafish embryogenesis.

Conclusions: Our phylogenetic and developmental analyses shed light on the potential functions of the KCa genes during embryogenesis related to congenital diseases and human channelopathies.

KEYWORDS
calcium-activated potassium ion channels, in situ hybridization, KCa channels, KCNMA, KCNMB, KCNN, KCNT, phylogeny, whole genome duplication (WGD), zebrafish

1 | INTRODUCTION

Potassium ion channels play critical roles in regulating fundamental physiological processes, from membrane potential maintenance, neuronal action potential firing, to hormone secretion, cellular volume control, cell migration, cell cycle, cell death, and cancers.1,2 There are 78 potassium channel coding genes in the human...
KCNN4 genes, and IK 
KCNN3 subunits are encoded by the 
KCNN1 (humans, the BK 

cell activation. They are also known to cause various 
ranging from regulating neuronal excitability to immune 
group of potassium channels plays fundamental roles 
mutation, and epilepsy. Based on single-channel conduc-
tion, and examined their gene expression during early 
embryogenesis. We found that the 

Calcium-activated potassium ion channels (KCa) are a 
specific type of potassium channel activated by intracel-
lar calcium as a ligand to hyperpolarize cell membrane 
potential by changing potassium permeability. This 
group of potassium channels plays fundamental roles 
ranging from regulating neuronal excitability to immune 
cell activation. They are also known to cause various 
diseases when mutated, such as schizophrenia, hyperten-
sion, and epilepsy. Based on single-channel conduct-
tance, KCa channels are generally further classified into 
three subgroups: large (BK, 300-200pS), intermediate (IK, 
39-32pS), and small conductance (SK, 14-4pS) channels. 
All the KCa channels exist as tetramers of 
α-subunits in the plasma membrane. In humans, SK α 
subunits are encoded by the KCNNA1, KCNN2, and 
KCNN3 genes, and IK α subunits are encoded by the 
KCN4 gene. The SK and IK channels share similar 
protein domains and a calmodulin binding-domain 
(S0) at their C-terminus. In contrast, the BK α subunit 
has one additional transmembrane domain at its N-
terminal and a calcium bowl (RCK1-RCK2) at its C-
terminus that directly detects changes to calcium ion 
levels.

BK channel function can be modulated by auxiliary 
proteins referred to as beta and gamma units. These pro-
teins do not actually form ion pores in the cell membrane 
for potassium ions but can be either inhibitory or excit-
atory regarding channel conductance regulation. In 
humans, the BK α subunits are encoded by the KCNMA1 
(SLO1, KCa1.1) gene, β and γ subunits are products of 
KCMNB1-KCNNB4 genes and LRRC genes, respectively. 
In addition to the BK channel, there are three structu-
ral potassium channels: KCNT1 (SLO2.2/ SLACK), 
KCNT2 (SLO2.1/SLICK), and KCNU1 (SLO3) in 
the human genome. However, the first two can also be 
activated by sodium and chloride, and the last one by 
protons. This group of genes plays a diverse and 
important role in physiology and human diseases.

Although the KCa channels have been extensively 
studied for electrophysiological and pharmacological 
properties, their gene expression during embryogenesis 
remains largely unknown. Here, we systematically cloned 
KCa genes from zebrafish, performed phylogenetic analy-
sis, and examined their gene expression during early 
embryonic development. We found that the KCa gene 
family was expanded through teleost whole-genome 
duplication (WGD) events. The KCa genes are expressed 
in both neuronal and somatic tissues in early fish 
embryos, suggesting they play important roles in verte-
brate embryogenesis.

2 | RESULTS

2.1 | The kcnn1 gene was duplicated in zebrafish and teleost

To better understand KCa gene functions during embry-
genesis, we chose zebrafish due to its prominent advan-
tages such as tractable genetics, rapid, and transparent 
external embryonic development. To identify zebrafish 
KCa genes, we first searched for all the zebrafish small 
and intermediate conductance KCa channels by BLAST 
in the current zebrafish reference genome, GRCz11, in 
Ensembl, using human KCa genes as baits. Consistent 
with the ZFIN collection, we found there is only one 
kcnn2, kcnn3, and kcnn4 genes in the zebrafish genome 
(Table 1). However, there are two kcnn1 genes (kcnn1a 
and kcnn1b). To clarify the zebrafish gene identifies, we 
built a phylogeny with human, mouse, and zebrafish 
gene protein sequences (Figure 1A). Both zebrafish 
KCNN1A and KCNN1B genes were grouped with the human 
and mouse KCNN1 genes (Figure 1A). It is well known 
that teleost genomes underwent a WGD event. Thus, 
these two kcnn1 genes likely resulted from the tel-
ost WGD event. To test this, we performed a phylo-
getic analysis with a few more teleost species and 
tetrapod representatives. Indeed, teleost Kcnn1 forms 
two clades (Kcnn1a and Kcnn1b, Figure 1B), suggesting 
that the two genes likely originated from the WGD 
event. The spotted gar separated from other teleosts 
before the WGD and it only has one kcnn1 gene. Interest-
ingly, it was grouped with Kcnn1b genes with a low 
supporting value, suggesting that the teleost kcnn1b 
sequences might evolve slower from ancestor sequences 
than kcnn1a genes.

2.2 | Zebrafish small and intermediate 
KCa channel genes are expressed distinctly 
during early embryogenesis

As gene spatial expression may provide insights into 
where the genes function, we examined gene expression 
patterns using whole-mount in situ hybridization. At 
12hpf (hours post fertilization), kcnn1a is expressed in 
the whole body, with higher expression in the posterior 
area. It is also expressed at the posterior lateral plate
mesoderm (Figure 2A, E). At 24hpf, the posterior expression recesses, and 
kcnn1a is expressed mainly in the brain and anterior neural tube (Figure 2B, F). Cross-section revealed that it is expressed in the brain telencephalon and the neural tube's lateral side (Figure 2B, F). Once the fish embryo reaches 48hpf and 72hpf, the 
kcnn1a expression is limited to the neurons in various parts of the brain (Figure 2C, D, G, H, C'.0, C'.00, G'.0, G'.00).

The 
kcnn1b gene has some overlap and distinct expression with the 
kcnn1a gene. At 12hpf, it has a similar expression pattern with 
kcnn1a, but is not found expressed in the lateral plate (Figure 2I, M). At 24hpf, it is expressed in the central nervous system and a new domain, presomitic mesoderm (Figure 2J), which later differentiates into paraxial mesoderm. It is also expressed at the neural tube (Figure 2N), but with a broader domain than 
kcnn1a (Figure 2J, N). The difference of 
kcnn1a and 
kcnn1b is more evident at the 48hpf stage, at which 
kcnn1b is in the intermediate hypothalamus, posterior lateral line ganglia, and pectoral fin buds (Figure 2K, K'.0, K'.00). At 72hpf, 
kcnn1b expression becomes evident (Figure 2AA, EE). In the neural tube, it is expressed at the upper lateral sides. Its somite expression retreats (Figure 2AA, AA', EE), but can still be detected at the posterior trunk dermomyotome (Figure 2AA, EE', EE'). In contrast, this somite expression is entirely not detectable by 72hpf. At this stage, it is limited to the brain area (Figure 2BB, FF).

In contrast to other 
kcnn genes, the intermediate conductance channel gene, 
kcnn4, is only detected in the distal early tubes of the nephric duct at the 24hpf and 48hpf stage, but not 12hpf (Figure 3A–E, G). This expression recesses by 72hpf (Figure 3F, H), indicating 
kcnn4 has a temporal function in kidney development.

### 2.3 Zebrafish has two BK channel genes

In the current zebrafish genome and NCBI database, we identified two 
kcna1 genes: One is listed as 
kcna1a
ENSDARG00000079840, and the other as KCNMA1 in Ensembl. The later one has an identical mRNA, XM_021480452.1, in NCBI (Table 1). We refer to this gene as kcnma1b. To verify these two genes’ homology with the tetrapod KCNMA1 gene, we performed phylogenetic analysis (Figure 4A). Our result suggested that these two duplicated paralogous genes resulted from teleost WGD,17 as they formed distinct clads with other kcnma1 genes from teleost species. This result is consistent with a previous report on the teleost kcnma1 gene evolutionary analysis.18 To further demonstrate these two genes are ohnologs, we conducted syntenic analysis. If they originated from the teleost WGD, they likely share neighbor genes. Indeed, zebrafish kcnma1a shares a conserved syntenic (dlg5-kcnma1-irmd-znrf503-comtd1) with the human, mouse, and spotted gar (Figure 4B). While the kcnma1b gene shares two neighbor genes (dlg5 and anxa11) with kcnma1a (Figure 4B), suggesting that this gene might have evolved faster.

Next, we examined the expression of the BK genes in zebrafish embryos. The kcnma1a gene is expressed in the somites and hypothalamus at the 12hpf stage. It is also weakly detected at the midbrain and hindbrain areas (Figure 5A,E). By 24hpf, its somite expression remains, and telencephalon and olfactory organ expression become evident (Figure 5B, F,insets). This somite expression recesses at 48hpf. At this
stage, the \textit{kcnn1a} gene is mainly found in the brain. It is also detected at the pectoral fin buds (Figure 5C,G). This expression pattern extends to 72hpf (Figure 5D,H). In contrast, the \textit{kcnn1b} gene expression is located at the anterior somite, hypothalamus, and hindbrain at 12hpf (Figure 5I,M). At 24hpf, it is expressed in all somites and hypothalamus similar to \textit{kcnn1a} (Figure 5J,N). However, it is also expressed at the floor plate of the neural tube, midbrain, and hindbrain (Figure 5J). By 48hpf and 72hpf, the somite expression retreats, while the neural expression is restricted to the brain region (Figure 5K,O). Both genes are expressed in the otic vesicle at 72hpf.

These two genes' expression domains are consistent with a previous report on their function in the zebrafish sensory organs.\textsuperscript{19,20}

\subsection*{2.4 Zebrafish beta units of BK channel genes were duplicated}

BK channels are known to function as a complex with other proteins. The beta unit is one of the channel modifiers. To better understand the BK gene function in early embryogenesis, we examined the beta unit coding genes.
By BLAST search using human genes as bait, we identified two *kcnmb2* genes (*kcnmb2a, kcnmb2b/si:ch211-247n2.1*) and one *kcnmb3* (*si:ch211-38m6.7; Table 1). Interestingly, we did not identify any *kcnmb1* and *kcnmb4* genes in zebrafish and any other teleosts from current genome databases, suggesting the two genes were lost after the separation of teleost from the rest of the vertebrate lineage after WGDs. To resolve homologous gene relationships, we chose *kcnmb* genes from some representative species and conducted a phylogenetic analysis using corresponding protein sequences. All candidate zebrafish *kcnmb* genes formed four distinct clades with other species (Figure 6A). The two *kcnmb2* genes formed sub-groups within the *kcnmb2* clade, suggesting that they are gene duplicates. All analyzed teleost genes were consistent with zebrafish, indicating the two duplicated resulted from the teleost WGD event. To further demonstrate the two *kcnmb2* genes are ohnologs, we performed synteny analysis in zebrafish, human, mouse, and spotted gar. A stable synteny (*kcnmb2-zmat3-pik3ca-kcnmb3-mfn1-gnb4-actl6a*) was identified among spotted gar, human, and mouse (Figure 6B). The zebrafish *kcnmb2a* is linked with tbl1x1b and kcnmb3. In contrast, *kcnmb2b* is linked with tbl1x1a. These results suggest that there was a gene positional reshuffling after the teleost WGD.

We then examined these three *kcnmb* genes’ expression in zebrafish embryos. The *kcnmb2a* gene is in the brain and anterior neural tube at 12hpf (Figure 7A,E). By 24hpf, it is mainly expressed in the anterior somites (Figure 7B,F). Within each somite, it is expressed mediolaterally to the dermomyotome (Figure 7B',F'). This somite expression remains at 48hpf. At this stage, it is also expressed in the midbrain, tegmentum (Figure 7C, G). However, it is barely detected once the embryo reaches 72hpf (Figure 7D,H). In contrast to *kcnmb2a*, the *kcnmb2b* gene is more ubiquitously expressed in 12hpf fish embryos (Figure 7I,M). This ubiquitous expression remains at 24hpf (Figure 7J,J',N,N'). After that, it retracts from posterior to anterior, and is expressed in the whole brain and the eye. It is also found at the pectoral fin buds (Figure 7K,O). At 72hpf, its brain expression recesses, and it is mainly detected in the gill arches and pectoral fins (Figure 7L,P). Although the *kcnmb3* gene is physically linked with *kcnmb2a* on zebrafish chromosome 2 (Figure 6B), it has a similar expression pattern to *kcnmb2b* instead of *kcnmb2a* (Figure 6Q-X). From 12hpf to 24hpf, it is ubiquitously expressed in zebrafish embryos with relatively low level in the notochord (Figure 6Q-V,R,V'). At 48hpf, it is mainly expressed in the brain region in addition to pectoral fin buds (Figure 6S,W). While, at 72hpf, it is expressed in the gill...
**2.5 Zebrafish have duplicated kcnt genes**

The kcnt gene has a similar structure to the kcnma1 gene, but lack the S0 transmembrane domain. These channels are more sodium activated instead of calcium-activated. Here, we also searched for kcnt genes in the zebrafish genome. We identified 4 kcnt genes (kcnt1a, kcnt1b, kcnt2a, and kcnt2b) by BLAST (Table 1). Phylogenetic analysis placed these four genes in kcnt1 and kcnt2 clades, respectively. The teleost species has two copies of kcnt1 and kcnt2 (Figure 8), indicating they originated from the teleost-specific WGD event.

To better understand their functions during early development, we examined their expression in zebrafish embryos. The kcnt1a gene is not detectable in 12hpf fish embryos (Figure 9A,E). By 24hpf, it is found at the...
olfactory organ (Figure 9B,B₁,B₁₀,F). This pattern is maintained at 48hpf and 72hpf (Figure 9C-D,G-H). It is also expressed at the tegmentum and midbrain at 72hpf (Figure 9D,H). The kcnt1b gene is found at the Kupffer’s vesicle (Figure 9I,M) at 12hpf. It starts to be expressed at the hypothalamus, midbrain, hindbrain, and Rohan beard neurons of the neural tube at 24hpf (Figure 9J,J₁₀,N,N₁₀,J₁₀,N₁₀). Whereas its expression is limited to the olfactory organs, subpallium, otic vesicle, and some neural nuclei at 48hpf and 72hpf (Figure 9,K-K₁₀,Z-Z₁₀,K₁₀,Z₁₀). The kcnt2b gene is expressed at the subpallium, posterior tuberculum, and thalamus at 48hpf (Figure 9AA,EE). This expression pattern remains at 72hpf, but it is also found the hindbrain (Figure 9BB,FF).

**DISCUSSION**

We have identified 14 KCa channel genes in the zebrafish genome and found that some of them were duplicated. Some mammalian orthologues were lost after the split of the teleosts. Moreover, we examined their gene expression during zebrafish early embryogenesis. Most of them showed variable expression patterns through each embryonic stage. Some of them are expressed in non-neural tissues, suggesting that they might be needed for normal embryonic developmental processes, such as differentiation and patterning.

**3.1 Evolution of vertebrate KCa channel genes**

The KCa channel genes have been extensively studied in model organisms such as fly and human cells. Their functions are generally evolutionarily conserved. As reported in the literature, there is usually one Kcnn and kcnt gene
FIGURE 6  Legend on next page.
in invertebrates such as fruit fly. However, there are four KCNN genes (KCNN1-KCNN4) in most mammalian species. This is a general phenomenon found with many other gene families such as BMP (BMP2, BMP4, BMP16), Hedgehog (SHH, IHH, DHH), and KANK (KANK1-KANK4) genes.\textsuperscript{23-25} The best representatives are the HOX gene clusters.\textsuperscript{26,27} There is only one hox gene cluster in the fruit fly and other invertebrates, but there are 4 HOX gene clusters in tetrapods (HOXA, HOXB, HOXC, and HOXD). These gene number expansions most likely resulted from the two rounds of WGD around the vertebrates’ dawn.\textsuperscript{15,28,29} The two rounds of WGDs are critical for the vertebrate morphological novelties since they led to the expansion of the genetic component for the developmental tool kit.\textsuperscript{30,31} In addition to the two rounds of vertebrate WGD (2R), there was a third round of WGD (third R) in teleost lineages before the split of spotted gar that happened about 300 million years ago.\textsuperscript{13,15,32,33} This teleost specific WGD further increases the gene numbers for teleost. For example, there are seven hox gene clusters in zebrafish. It is worth noting that not all the duplicated genes remain, some duplicated ones were lost or became pseudogenes after the WGD event.\textsuperscript{34,35} Thus, there could be an odd number of genes in certain gene families.

Based on our genomic and phylogenetic analyses, we propose a model of KCa gene evolution in vertebrates (Figure 10). All the vertebrate KCa genes resulted from the vertebrate WGDs (1R and 2R). After the 2R, two of the slo1 ohnologs and two of the kcnt ohnologs (kcnt3 and kcnt4) were lost, only kcnma1 and kcnu1, kcnt1 and kcnt2 genes were retained in the early vertebrate lineages (Figure 10). All the duplicated kcn and kcnmb ohnologous genes remains (Figure 10). The teleost-specific WGD (3R) further reshaped the gene numbers of this group of genes through gene duplication and gene loss. A few genes (kcnma1a & kcnma1b, kcnt2a & kcnt2b, kcnmb2a & kcnmb2b, and kcnna1 & kcnnb1) were duplicated in zebrafish and other teleost species. All the teleost lost the kcnu1 (KCa5.1, Kcnma3, and Slo3) and kcnmb1. Zebrafish also lost kcnmb4, but this gene is present in other teleost species, indicating the KCa gene number could be further modified through speciation after WGDs.

### 3.2 Overlapping but distinct gene expression of the duplicated zebrafish genes

For these retained duplicated genes, they became redundant, and they are expressed in the same type of cell or tissue immediately after WGD. As the protein coding region and regulatory region (enhancers and silencers) evolve at different rates due to the pleiotropic functional constraints on the protein-coding regions, two scenarios emerge as proposed by the DDC model.\textsuperscript{36} (a) Sub-functionalization: The duplicates split the functions of their ancestor gene. (b) Neofunctionalization: one of the copies is expressed at a new cell or tissue type once its regulatory region changed dramatically. Consistent with this theory, our results of the zebrafish kcnna1a and kcnna1b showed similar expression domains during development. For example, both genes are expressed at the brain and neural tubes, but kcnna1a is uniquely expressed at lateral plate mesoderm, and kcnna1b has a distinct pretotal mesoderm expression at 24hpf, and pectoral fin bud expression at 48hpf. Similarly, both kcnna1a and kcnna1b are expressed at somite and dermomyotome at 12hpf and 24hpf, respectively. However, only kcnna1a is found at the fin bud at 48hpf and 72hpf. The two pairs of kcnt1 (kcnt1a and kcnt1b) and kcnt2 (kcnt2a and kcnt2b) are mainly expressed in the neural nucleus, but they do not share many expression domains. Interestingly, kcnt1b and kcnt2a are expressed in the Rohon beard neurons suggesting their ancestor gene might play important roles in this type of neuron. It would be interesting to compare these gene expression patterns from basal vertebrates such as hagfish, lamprey, and sharks.

### 3.3 KCa channel expression in non-neuronal tissues during early embryogenesis

While ion channel function in neuronal tissues is widely accepted, other cells and tissues are relatively less appreciated. Here, we show that most of the KCa channel genes are expressed in the developing zebrafish brain and neural tubes/spine. They are also found in various non-
excitatory cells, such as somites, eyes, fins, kidneys, olfactory organs, and other embryonic tissues. Our findings on KCa genes during embryogenesis is consistent with previous reports that some voltage-gated ion channels are also expressed at specific stages during Xenopus and mammalian embryogenesis. Understanding the distinct expression patterns of these channels during embryonic development could be essential to reveal new mechanisms for human channelopathies and congenital diseases. Accumulating evidence is revealing that ion channels are key players for embryogenesis through bioelectricity, which is endogenous electrical signaling mediated by the dynamic distribution of charged molecules via channels, pumps, and gap junctions. Mutations of these genes were reported to cause abnormal structural development in multiple species. The kcnk5 and slc12a7a mutant zebrafish develop elongated fins in zebrafish. The kcnj2 gene was found to regulate wing disc and human craniofacial development. The CaV1.2 can regulate chondrogenesis during limb development. We recently demonstrated that the ectopic expression of the kcnj13 gene in zebrafish somite could lead to elongated fins in zebrafish. This suggests that the ion channel genes in the non-excitatory cells could be important for tissue and organ patterning and differentiation during embryogenesis. Along this direction, future functional studies may be pursued to target expression domains. For example, kcn3 and kcnma1a are also expressed explicitly in the dermomyotome around 24hpf. It will be interesting to know whether they are needed for zebrafish fin patterning as the kcnj13 gene. To address this, we could employ the CRISPR approach to make

![Figure 7](image-url)
conventional large knockout\textsuperscript{45} or tissue-specific knock-out\textsuperscript{46,47} to inactivate the two genes and examine fin developmental abnormalities or defects in the future.

4 | EXPERIMENTAL PROCEDURES

4.1 | Zebrafish strains and husbandry

Zebrafish were raised and maintained following AAALAC approved standards at the Purdue animal housing facility. Experiments were performed according to approved protocols by the Purdue Animal Care and Use Committee (PACUC). All the zebrafish experiments were carried out in wildtype TAB background. Zebrafish were maintained according to the zebrafish book.\textsuperscript{48} Zebrafish embryo were staged based on Kimmel staging guide.\textsuperscript{49}

4.2 | Bioinformatics and phylogenetic analysis

Zebrafish KCa channel protein sequences were first identified through searches of the NCBI database using human gene symbols. The un-annotated protein sequences were identified through BLASTp in Ensembl and NCBI using according human orthologous protein sequences as a query. Representative protein sequences from the major metazoan taxa were selected based on their taxonomic positions. Whenever there were multiple isoforms, the longest sequence was chosen for analysis. The final protein accession numbers and species are listed in the Table S1.

Multiple protein sequences were aligned using MUSLE program.\textsuperscript{50} To identify the best evolutionary model for phylogenetic analysis, we carried out a best model test using maximum likelihood (ML) and default
The models with lowest Bayesian Information Criterion (BIC) scores were considered to describe the substitution pattern the best, and JTT + G was chosen. Then, we constructed phylogenetic trees using PhyML3.1. ML phylogenetic analysis was performed using JTT + G with 1000 bootstrap replicates. The final phylogenetic trees were viewed and generated with FigTree V1.4.2 (http://tree.bio.ed.ac.uk/software/figtree). The syntenic analysis was first carried out in Genomics browser (version 100.01). Individual gene position was then verified in Ensembl, UCSC genome browser, Synteny Database.
4.3 | Zebrafish gene cloning

A whole open reading frame was amplified with RT-PCR using gene-specific primers for each identified zebrafish KCa channel gene. Primers were designed in ApeE program using default settings. CACC Kozak sequences were added at the 5' end of forward primer to prepare direct Topo cloning. Total RNAs were exacted from pooled fish embryos composed of about a hundred 1-3 dpf fish embryos using TRIzol reagent (Thermo Fisher) according to manufactory guidance. After quality checking and quantification by Nanodrop, Reverse transcription was carried out with the SuperScript III First-Strand Synthesis System (Thermo Fisher) following manual instructions. Phusion High-Fidelity DNA Polymerase master mix (New England Biolabs) or PrimeSTAR GXL DNA Polymerase (Takara Bio) were chosen for PCR amplification. PCR primers utilized are listed in Table S2. PCR products were then purified with NucleoSpin Gel and PCR Clean-up Kit (Takara Bio). Purified PCR products of each gene were cloned into the pENTR-D-TOPO or pDONR221 vector and transformed into Top10 Escherichia coli cells using the pENTR Directional TOPO Cloning Kit (Thermo Scientific) according to its manual instruction. Correct clones with the right gene orientation and sequences were verified with Sanger sequencing. All the zebrafish KCa gene clones are available through Addgene (Plasmid #164951-164964).

4.4 | Whole-mount in situ hybridization, cryosection, and imaging

Riboprobe DNA templates were prepared either by PCR (size <2 kbps) or plasmid linearization (size>2 kbps) with an endonuclease at the 5' end of the protein-coding region in the pENTR vector. DNA templates were purified with the NucleoSpin Gel and PCR Clean-up Kit (Takara Bio). Anti-sense riboprobes were synthesized with T7 RNA polymerase (Thermo Scientific) and DIG RNA Labeling Mix (Roche, 11277073910) by in vitro transcription. Sense riboprobes were synthesized with Sp6 RNA polymerase (Thermo Scientific) and DNA templates that were generated by PCRs (Sp6 promoter was added to the 5 end of gene-specific forward primers). Synthesized riboprobes were purified with Sigma Spin post-reaction clean-up columns (Sigma, S5059). About 1-2 μL of these probes were added per reaction tube for in situ hybridization.

Whole-mount in situ hybridizations were performed according to the previous published method with some modifications. Briefly, zebrafish embryos were collected, maintained, and staged according to the zebrafish development staging guide. Chorions were removed using pronase (Sigma) treatment before fixation. Zebrafish embryos were then fixed with 4% PFA overnight at 4°C and then dehydrated using serial methanol (25%, 50%, 75%, and 100%) with PBST (Phosphate-
buffered embryos were stored at −20°C until used for experiments. To perform the WISH, fish embryos were rehydrated using the reverse gradient methanol in PBST. Embryos (12-24hpf) were then bleached with 6% H2O2 in PBT until all tissues were clear. 48 to 72hpf embryos were bleached with 3% H2O2 and 0.5% KOH to remove pigmentation. Fish embryos were permeabilized with proteinase K (10 μg/mL in PBT): 8 to 18hpf, 3 minutes; 24hpf, 5 minutes; 48 hpf, 20 to 30 minutes; 72hpf, 45 to 60 minutes at room temperature. After permeabilization, embryos were washed with PBT and fixed in 4% PFA with 0.2% glutaraldehyde for 3 hours at room temperature to inactivate proteinase K. Next, fish embryos were washed with PBT (2 × 10 minutes) to remove fixatives. Then, fish embryos were incubated in pre-hybridization solution [50% formamide, 5XSSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0), 2% Roche blocking powder, 0.1% Triton-X, 50 mg/mL heparin, 1 mg/mL Torula yeast RNA, 1 mM EDTA, 0.1% CHAPS, DEPC-treated ddH2O] overnight at 65°C in hybridization oven with gentle shaking (40 rpm). Riboprobes were added on the second day. Then, embryos were further hybridized for another 48 to 72 hours before washing out unbound riboprobes using 2× SSC and 0.2× SSC (3 × 30 minutes for each solution). Next, fish embryos were washed with KBT2 2× for 10 minutes each (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 1.0% Tween-20) before antigen blocking using 20% sheep serum in KBT2 for 3 hours. Antidigoxigenin antibody conjugated to alkaline phosphatase (Roche) was added into the blocking solution in a ratio of 1:5000, and embryos were incubated overnight at 4°C with gentle shaking. After 6× 1-hour washing with KBT2 and overnight KBT2 incubation at 4°C with gentle shaking, the following day, color reaction was carried out in NTMT solution (100 mM Tris-HCl, pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.5% Tween-20) with 75 mg/mL NBT (Roche), 50 mg/mL BCIP (Roche), and 10% DMF (N,N-dimethylformamide). Color development was carried out in the dark with gentle rocking. We monitor the color density of each reaction closely. Once the embryos developed suitable color density, the reaction was stopped with NTMT washing. The finished samples were imaged immediately or stored in 4% paraformaldehyde for later imaging.

For histological analysis, posthybridization embryos were equilibrated in 15% sucrose overnight, then 30% sucrose in 20% gelatin, after which they were embedded in 20% gelatin for cryosection (25 μm) on a cryotome. Images were acquired using Axio CamMRc camera on Zeiss Stereo Discovery.V12 and Axio Imager 2 compound microscope. Whole-mount embryos were imaged in 3% methylcellulose.

ACKNOWLEDGMENTS
The research was supported by the National Institute of General Medical Sciences of the National Institutes of Health (R35 GM-124913) to GuangJun Zhang. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agents.

CONFLICT OF INTEREST
The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS
Martin Silic: Formal analysis, Investigation, Methodology, Writing-review and editing. Maya Black: Investigation, Writing-review and editing. GuangJun Zhang: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing-original draft, Writing-review and editing.

ORCID
Martin R. Silic © https://orcid.org/0000-0003-3433-3922
GuangJun Zhang © https://orcid.org/0000-0002-0839-5161

REFERENCES
1. Huang X, Jan LY. Targeting potassium channels in cancer. J Cell Biol. 2014;206(2):151-162. https://doi.org/10.1083/jcb.201404136.
2. Brown BM, Shim H, Christophersen P, Wulff H. Pharmacology of small- and intermediate-conductance calcium-activated potassium channels. Annu Rev Pharmacol Toxicol. 2020;60:219-240. https://doi.org/10.1146/annurev-pharmtox-010919-023420.
3. Kaczmarek LK, Aldrich RW, Chandy KG, Grissmer S, Wei AD, Wulff H. International union of basic and clinical pharmacology. C. Nomenclature and properties of calcium-activated and sodium-activated potassium channels. Pharmacol Rev. 2017;69 (1):1-11. https://doi.org/10.1124/pr.116.012864.
4. Khatri AS, Gonzalez-Hernandez A, Physiological Roles GT. Therapeutic potential of Ca(2+) activated potassium channels in the nervous system. Front Mol Neurosci. 2018;11:258. https://doi.org/10.3389/fnmol.2018.00258.
5. Vergara C, Latorre R, Marrion NV, Adelman JP. Calcium-activated potassium channels. Curr Opin Neurobiol. 1998;8(3):321-329. https://doi.org/10.1016/s0959-4388(98)80056-1.
6. Feske S, Wulff H, Skolnik EY. Ion channels in innate and adaptive immunity. Annu Rev Immunol. 2015;33:291-353. https://doi.org/10.1146/annurev-immunol-032414-112212.
7. Kohler M, Hirschberg B, Bond CT, et al. Small-conductance, calcium-activated potassium channels from mammalian brain. Science. 1996;273(5282):1709-1714. https://doi.org/10.1126/science.273.5282.1709.
8. Joiner WJ, Wang LY, Tang MD, Kaczmarek LK. hSK4, a member of a novel subfamily of calcium-activated potassium channels. Proc Natl Acad Sci U S A. 1997;94(20):11013-11018. https://doi.org/10.1073/pnas.94.20.11013.
11. Ishii TM, Silvia C, Hirschberg B, Bond CT, Adelman JP, Maylie J. A human intermediate conductance calcium-activated potassium channel. *Proc Natl Acad Sci U S A*. 1997;94(21):11651-11656. https://doi.org/10.1073/pnas.94.21.11651.

10. Contreras GF, Castillo K, Enrique N, et al. A BK (Slo1) channel journey from molecule to physiology. *Channels (Austin)*. 2013;7(6):442-458. https://doi.org/10.4161/chann.26242.

9. Ishii TM, Silvia C, Hirschberg B, Bond CT, Adelman JP, Maylie J. A human intermediate conductance calcium-activated potassium channel. *Proc Natl Acad Sci U S A*. 1997;94(21):11651-11656. https://doi.org/10.1073/pnas.94.21.11651.

8. Salkoff L, Butler A, Ferreira G, Wei A. High-conductance potassium channels of the SLO family. *Nat Rev Neurosci*. 2006;7(12):921-931. https://doi.org/10.1038/nrn1992.

7. Bailey CS, Moldenhauer HJ, Park SM, Keros S, Meredith AL. KCNMA1-linked channelopathy. *J Gen Physiol*. 2019;151(10):1173-1189. https://doi.org/10.1085/jgp.201912457.

6. Posts JH, Woods IG, Ngo-Hazelett P, et al. Zebrafish KCNMA1-linked channelopathy. *PLoS Biol*. 2005;3(10):e314. https://doi.org/10.1371/journal.pbio.0030314.

5. Ohno S. *Evolution by Gene Duplication*. New York: Springer Science+Business Media, LLC; 1970.

4. Wagner GP, Lynch VJ. Evolutionary novelties. *Curr Biol*; 2010; 20(2):R48-R52. https://doi.org/10.1016/j.cub.2009.11.010.

3. Van de Peer Y, Maere S, Meyer A. The evolutionary significance of ancient genome duplications. *Nat Rev Genet*. 2009;10(10):725-732. https://doi.org/10.1038/nrg2600.

2. Woods IG, Kelly PD, Chu P, et al. A comparative map of the zebrafish genome. *Genome Res*. 2000;10(12):1903-1914.

1. Buss JH, Woods IG, Ngo-Hazelett P, et al. Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res*. 2000;10(12):1890-1902.

Wolfe K. Robustness—it’s not where you think it is. *Nat Genet*. 2000;25(1):3-4. https://doi.org/10.1038/75560.

Rohmann KN, Deitche D, Bass AH. Calcium-activated potassium (BK) channels are encoded by duplicate slo1 genes in teleost fishes. *Mol Biol Evol*. 2009;26(7):1509-1521. https://doi.org/10.1093/molbev/msp060.

Rohmann KN, Tripp JA, Genova RM, Bass AH. Manipulation of BK channel expression is sufficient to alter auditory hair cell thresholds in larval zebrafish. *J Exp Biol*. 2014;217(14):2531-2539. https://doi.org/10.1242/jeb.103093.

Cabo R, Zichichi R, Vina E, et al. Calcium-activated potassium channel SK1 is widely expressed in the peripheral nervous system and sensory organs of adult zebrafish. *Neurosci Lett*. 2013;555:62-67. https://doi.org/10.1016/j.neulet.2013.09.026.

McGrath CL, Gout JF, Jöhr P, Doak TG, Lynch M. Differential retention and divergent resolution of duplicate genes following whole-genome duplication. *Genome Res*. 2014;24(10):1665-1675. https://doi.org/10.1101/gr.173740.114.

Paszuner J, Cabau C, Nguyen T, et al. Gene evolution and gene expression after whole genome duplication in fish: the PhylloFish database. *BMC Genomics*. 2016;17:368. https://doi.org/10.1186/s12864-016-2709-z.

Hensley MR, Cui Z, Chua RFM, et al. Evolutionary and developmental analysis reveals KANK genes were co-opted for vertebrate vascular development. *Sci Rep*. 2016;6:27816. https://doi.org/10.1038/srep27816.

Marques CL, Fernandez I, Viegas MN, et al. Comparative analysis of zebrafish bone morphogenetic proteins 2, 4 and 16: molecular and evolutionary perspectives. *Cell Mol Life Sci*. 2016;73(4):841-857. https://doi.org/10.1007/s00018-015-2024-x.

Pereira J, Johnson WE, O’Brien SJ, et al. Evolutionary genomics and adaptive evolution of the hedgehog gene family (Shh, Ihh and Dhh) in vertebrates. *PLoS One*. 2014;9(12):e74132. https://doi.org/10.1371/journal.pone.0074132.

Wagner GP, Amemiya C, Ruddle F. Hox cluster duplications and the opportunity for evolutionary novelties. *Proc Natl Acad Sci U S A*. 2003;100(25):14603-14606. https://doi.org/10.1073/pnas.2536656100.

Amores A, Force A, Yan YL, et al. Zebrafish hox clusters and vertebrate genome evolution. *Science*. 1998;282(5394):1711-1714. https://doi.org/10.1126/science.282.5394.1711.

Deh Pal, Boore JL. Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol*. 2005;3(10):e314. https://doi.org/10.1371/journal.pbio.0030314.

Ohno S. *Evolution by Gene Duplication*. New York: Springer Science+Business Media, LLC; 1970.

Wagner GP, Lynch VJ. Evolutionary novelties. *Curr Biol*; 2010; 20(2):R48-R52. https://doi.org/10.1016/j.cub.2009.11.010.

Van de Peer Y, Maere S, Meyer A. The evolutionary significance of ancient genome duplications. *Nat Rev Genet*. 2009;10(10):725-732. https://doi.org/10.1038/nrg2600.

Woods IG, Kelly PD, Chu P, et al. A comparative map of the zebrafish genome. *Genome Res*. 2000;10(12):1903-1914.

Brunet FG, Croilüss H, Paris M, et al. Gene loss and evolutionary rates following whole-genome duplication in teleost fishes. *Mol Biol Evol*. 2006;23(9):1808-1816. https://doi.org/10.1093/molbev/ms049.

Postlethwait JH. The zebrafish genome in context: ohnologs gone missing. *J Exp Zool B Mol Dev Evol*. 2007;308(5):563-577. https://doi.org/10.1002/jexz.b21137.

Albalat R, Canestro C. Evolution by gene loss. *Nat Rev Genet*. 2016;17(7):379-391. https://doi.org/10.1038/nrg.2016.39.

Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics*. 1999;151(4):1531-1545.

Moody WJ. The development of voltage-gated ion channels and its relation to activity-dependent development events. *Curr Top Dev Biol*. 1998;39:159-185. https://doi.org/10.1016/s0070-2153(08)60455-x.

Bates E. Ion channels in development and cancer. *Annu Rev Cell Dev Biol*. 2015;31:231-247. https://doi.org/10.1146/annurev-cellbio-100814-125338.

Levin M. Molecular bioelectricity: how endogenous voltage potentials control cell behavior and instruct pattern regulation in vivo. *Mol Biol Cell*. 2014;25(24):3835-3850. https://doi.org/10.1091/mbc.E13-12-0708.

Dahal GR, Pradhan SJ, Bates EA. Inwardly rectifying potassium channels influence Drosophilawing morphogenesis by regulating Dpp release. *Development*. 2017;144(15):2771-2783. https://doi.org/10.1242/dev.146647.

Belus MT, Rogers MA, Elzubeir A, et al. Kir2.1 is important for efficient BMP signaling in mammalian face development. *Dev Biol*. 2018;444:S297-S307. https://doi.org/10.1016/j.ydbio.2018.02.012.

Adams DS, Uzel SGM, Akagi J, et al. Bioelectric signalling via potassium channels: a mechanism for craniofacial dysmorphogenesis in KCNJ2-associated Andersen-Tawil Syndrome. *Journal of Physiology-London*. 2016;594(12):3245-3270. https://doi.org/10.1113/jp271930.
limb development. *Proc Natl Acad Sci U S A.* 2019;116(43):21592-21601. https://doi.org/10.1073/pnas.1908981116.

44. Silic MR, Wu Q, Kim BH, et al. Potassium channel-associated bioelectricity of the dermomyotome determines fin patterning in zebrafish. *Genetics.* 2020;215:1084. https://doi.org/10.1534/genetics.120.303390.

45. Kim BH, Zhang G. Generating stable knockout zebrafish lines by deleting large chromosomal fragments using multiple gRNAs. *G3 (Bethesda).* 2020;10(3):1029-1037. https://doi.org/10.1534/g3.119.401035.

46. Yin L, Maddison LA, Li M, et al. Multiplex conditional mutagenesis using transgenic expression of Cas9 and sgRNAs. *Genetics.* 2015;200(2):431-441. https://doi.org/10.1534/genetics.115.176917.

47. Ablain J, Durand EM, Yang S, Zhou Y, Zon LIA. CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Dev Cell.* 2015;32(6):756-764. https://doi.org/10.1016/j.devcel.2015.01.032.

48. Westerfield M. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio Rerio).* 4th ed. Eugene: University of Oregon Press; 2000.

49. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn.* 1995;203(3):253-310. https://doi.org/10.1002/aja.1002030302.

50. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 2004;32(5):1792-1797. https://doi.org/10.1093/nar/gkh340.

51. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol.* 2013;30(12):2725-2729. https://doi.org/10.1093/molbev/mst197.

52. Felsenstein J. *Inferring Phylogenies.* Sunderland: Sinauer; 2004.

53. Zhang G, Miyamoto MM, Cohn MJ. Lamprey type II collagen and Sox9 reveal an ancient origin of the vertebrate collagenous skeleton. *Proc Natl Acad Sci U S A.* 2006;103(9):3180-3185. https://doi.org/10.1073/pnas.0508313103.

54. Zhang G, Cohn MJ. Hagfish and lancelet fibrillar collagens reveal that type II collagen-based cartilage evolved in stem vertebrates. *Proc Natl Acad Sci U S A.* 2006;103(45):16829-16833. https://doi.org/10.1073/pnas.0605630103.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Silic MR, Black MM, Zhang GJ. Phylogenetic and developmental analyses indicate complex functions of calcium-activated potassium channels in zebrafish embryonic development. *Developmental Dynamics.* 2021;1–17. https://doi.org/10.1002/dvdy.329