Characterization and Expression of the Zebrafish qki Paralogs

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Abstract

Quaking (QKI) is an RNA-binding protein involved in post-transcriptional mRNA processing. This gene is found to be associated with several human neurological disorders. Early expression of QKI proteins in the developing mouse neuroepithelium, together with neural tube defects in Qk mouse mutants, suggest the functional requirement of Qk for the establishment of the nervous system. As a knockout of Qk is embryonic lethal in mice, other model systems like the zebrafish could serve as a tool to study the developmental functions of qki. In the present study we sought to characterize the evolutionary relationship and spatiotemporal expression of qkia, qki2, and qkib; zebrafish homologs of human QKI. We found that qkia is an ancestral paralog of the single tetrapod Qk gene that was likely lost during the fin-to-limb transition. Conversely, qkib and qki2 are orthologs, emerging at the root of the vertebrate and teleost lineage, respectively. Both qki2 and qkib, but not qkia, were expressed in the progenitor domains of the central nervous system, similar to expression of the single gene in mice. Despite having partially overlapping expression domains, each gene has a unique expression pattern, suggesting that these genes have undergone sub-functionalization following duplication. Therefore, we suggest the zebrafish could be used to study the separate functions of qki genes during embryonic development.

Introduction

Quaking (QKI) is an evolutionarily conserved RNA-binding protein involved in post-transcriptional mRNA processing, ranging from facilitating spliceosomal complex formation [1], to mRNA stability [2] and localization [3]. Bioinformatic analysis of the QKI response element (QRE) identified 1,433 putative RNA targets associated with processes such as development, organogenesis and cell differentiation [4]. Similar to other members of the Signal Transduction and Activation of RNA (STAR) protein family, QKI contains a single K-Homology (KH) motif directly involved in RNA binding [5]. The KH domain is expressed within each of the multiple...
isofoms of QKI (QKI5, QKI6, QKI7, and QKI7b), which are distinguished by both the size of the mRNA molecule and the C-terminal amino acid sequence [6]. In the human prefrontal cortex, QKI isoforms present differential expression patterns over time, with high QKI5 expression in the fetal brain and an increased postnatal expression of QKI6, 7 and 7b [7]. Alterations in the expression levels of specific QKI isoforms have also been associated with schizophrenia [8–11], major depression [12], anxiety [13], ataxia [14], and glioma [15–18], highlighting the importance of a better understanding of the diverse functions of QKI, particularly during nervous system development.

The function of Qk has predominantly been investigated in rodents where RNA coding for each isoform of Qk can be detected in most tissues of the adult mouse (Kondo et al., 1999). Within the nervous system, Qk is abundantly expressed in glial cells, including oligodendrocytes and astrocytes in the central nervous system (CNS), and Schwann cells in the peripheral nervous system (PNS), while it is absent from mature neurons, with the exception of transient expression of Qk5 in motor neurons of the embryonic spinal cord [6, 19]. Similar to humans, expression of the mouse Qk6 and Qk7 isoforms peak during the active phase of myelination, while Qk5 is upregulated during embryogenesis [6, 7, 20]. Consistent with their expression patterns, loss of Qk6 and Qk7 proteins specifically from myelin-forming glia (oligodendrocytes and Schwann cells) resulted in severe dysmyelination in both the CNS and PNS, in the original Qk viable (Qk<sup>v</sup>) mutant line, which carries a spontaneous deletion in the 5' regulatory region of the gene. Consequently, Qk<sup>v/v</sup> mice develop characteristic tremors (“quaking”) of the hindquarters around the second postnatal week, followed by tonic-clonic seizures in adults [6, 21–24]. A more severe dysmyelination phenotype, accompanied by early onset seizures and severe ataxia, was observed in mice carrying another viable Qk recessive allele [25]. This phenotype was attributed to the loss of Qk6 and Qk7, similar to what has been observed in Qk<sup>v</sup>, but showing an additional reduction in the expression of the Qk5 isoform. Finally, a complete knockout of Qk results in neural tube defects and mid-embryonic lethality [26], obfuscating functional characterization of this gene at later developmental timepoints.

In an attempt to identify an alternative model to study QKI functions during early vertebrate development <i>in vivo</i>, we investigated the homologs of QKI in zebrafish (<i>Danio rerio</i>). One of the zebrafish homologs, <i>qkia</i> (located on chromosome 17), has previously been cloned [27] and has been proposed to promote slow muscle formation by attenuating Hedgehog signaling through stabilization of gli2a (GLI family zinc finger 2a) mRNA [28]. However, two additional copies of the <i>qki</i> gene that have not been previously studied are present in the zebrafish genome, <i>qki2</i> (on chromosome 13) and <i>qki2b</i> (on chromosome 12). Here we present the evolutionary relationship of the three zebrafish QKI homologs and describe their developmental expression, both spatially and temporally.

**Materials and Methods**

**Sequences retrieval and exon-intron organization**

Nucleotide and amino acid (aa) sequences of human and zebrafish Quaking were retrieved from Genbank (version 197). The accession numbers of the selected sequences are: QKI5 (NP_006766.1), QKI6 (NP_996735.1), QKI7 (NP_996736.1) and QKI7B (NP_996737.1), Qkia<sub>_tv1</sub> (NP_571299.1), Qkia<sub>_tv2</sub> (XP_005158811.1), Qkia<sub>_tv1</sub> (XP_005156410.1), Qkia<sub>_tv2</sub> (NP_957136.2), Qki2<sub>_tv1</sub> (XP_005156558.1), Qki2<sub>_tv2</sub> (XP_005156558.1), Qkib<sub>_tv1</sub> (XP_005156655.1), Qkib<sub>_tv2</sub> (XP_005156655.1) and Qkib<sub>_tv4</sub> (XP_005156655.1). Genbank sequences were compared against the Ensembl assembly Zv9 (release 70) to verify their presence in the genome. In addition, Qki2<sub>_tv3</sub> and Qkib<sub>_tv3</sub> were manually retrieved from Ensembl using BLAST with QKI5 as the query. The Qkib<sub>_tv1</sub> (XP_005156655.1) was modified by the addition of exon 8. To obtain the exon-intron structure
of qki in other species, and to identify additional putative transcript variants in zebrafish, the four human QKI aa sequences listed above were used for a BLAST query against the genomes of *Branchiostoma floridae* (amphioxus), *Danio rerio* (zebrafish), *Lepisosteus oculatus* (spotted gar), *Latimeria chalumnae* (coelacanth), and *Gallus gallus* (chicken). This analysis identified the translated exon sequences from each genome and the aligned human exons were used to manually annotate the splice sites in each species (listed in S1 Fig). Small exons not detected by BLAST were identified using six-frame translation of the relevant genomic sequence.

**Bioinformatic analysis**

Sequences were aligned using Clustal Omega 1.2.0 [29]. The resulting aa alignment and corresponding DNA sequences were loaded in PAL2NAL, v14 [30] to construct a codon alignment. Manual editing of alignments was done in JalView, v2.8.0b1 [31] and the resulting alignment was used to assess the nucleotide substitution model with jModelTest 2.1.4 [32, 33]. A phylogenetic tree was constructed with MrBayes 3.2.1 [34], using the standard nucleotide substitution model, allowing all rate to vary as predicted in the general time reversible model, and assuming a gamma distribution of rates with invariable sites. The analysis was run for 1,000,000 generations with a sample frequency of 100 and a relative burnin of 25%.

For synteny analysis between human and zebrafish chromosomes containing the QKI/qki genes, two different methods were employed. For an analysis of larger chromosome regions, the Synteny Database (http://syntenydb.uoregon.edu/synteny_db/) [35] was used, and microsynteny was investigated manually with Ensembl.

**Zebrafish**

Zebrafish AB strain embryos were raised in system water at 28.5°C and staged according to either hours post-fertilization (hpf) or morphological criteria [36] as indicated in the text. For *in situ* hybridization experiments, embryos older than 24 hpf were treated with 0.003% 1-phenyl 2-thiourea (Sigma) to inhibit pigmentaion. All animal studies were carried out with approval from the animal ethics committee (Uppsala djurförsökssetiska nämnd) in Uppsala, Sweden, permit number: C262/11 and C161/14.

**RNA extraction and real-time RT-PCR**

Total RNA was extracted from pooled embryos or larvae using Trizol (Life Technologies) according to the manufacturer’s protocol. RNA concentration and quality were determined using a NanoDrop ND-1000 spectrophotometer. From each biological replicate, 400 ng of RNA were reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) according to manufacturer’s protocol. cDNA samples were diluted with RNAse-free water to a final concentration of 5 ng/μl and stored at -20°C.

All real-time PCR experiments were performed using an ABI Prism 7500 Sequence Detector System (Applied Biosystems) with optical tubes (Applied Biosystems). Amplification was achieved using Power SYBR Green PCR Master Mix (Applied Biosystems), equal amounts of cDNA, and gene specific primers sets: *qkiα*, 5'- CCCACCTGGAGTTACAGACG, 5' - CA GTTGCCTCGGTGTAGTC; *qkiβ*, 5' - CCAGAGTCCGCCATCATGA, 5' - TTTGTTC GGGAAGGCCATAC; *qkiβ*, 5' - TGGAGTATCCATCGACTCC, 5' - TGGGAATGTGACA GGTCTGA. Following completion of each real-time PCR reaction, a dissociation step was added and melt curve analysis was performed to validate the specificity of PCR amplicons. Data were processed by ABI 7500 system SDS software version 2.0.6. Relative qki mRNA levels were quantified using a standard curve and normalized against expression of two endogenous control genes previously validated as suitable reference genes for developmental studies in
zebrafish [37]: eukaryotic translation elongation factor 1 alpha 1, like 1 (eef1a1l1): 5'- AGCA GCAGCTGAGGAGGTGAT, 5'- CCGCATTGTAGATCAGATGG, and actin, beta 1 (actb1): 5'- GATGATGAAATTGCCGCACCTG, 5'- ACCAACATGACACCCCCTGATGT.

cRNA probe synthesis and whole mount in situ hybridization

Templates for probe synthesis were PCR amplified from embryonic zebrafish cDNA using primers including SP6 or T7 RNA polymerase promoter sequences. To minimize cross-reactivity, the 5' untranslated regions of qkib and qki2 were used for primer design. Primer sets were designed as follows: qkia, 5'-CTGTAATACGACTCACTATAGGGCGTAATGAACACAGAAGAAAC, 5'-GGGATTTAGGTGACACTATAGAAACCCAGTTTAAGAGAAAG; qki2, 5'-CTGTAATACGACTCACTATAGGGCGAGAACACAGCAGACAGCCTCAAC, 5'-GGGA TTAGGTGACACTATAGAAATGTTGTTGGATTTGACG; qkib, 5'-CTGTAATACGACTCACTATAGGGCGAGAACACAGCAGACAGCCTCAAC, 5'-CTGTAATACGACTCACTATAGGGCGAGAACACAGCAGACAGCCTCAAC. All PCR products were of expected size as inspected by agarose gel electrophoresis. Purified PCR products were in vitro transcribed and labeled using digoxigenin (DIG) RNA Labeling Kit (Roche) according to manufacturer's protocol. cRNA probes were precipitated with LiCl and stored at -80°C. Whole mount in-situ hybridization was performed according to a standard protocol [38]. Between 100 and 250 ng of each probe was hybridized overnight at 67°C.

Fluorescent in situ hybridization and immunofluorescence

Embryos were fixed in 4% paraformaldehyde (PFA) in PBS at specified times post-fertilization, cryoprotected in 30% sucrose, and sectioned at 12 μm using a Leica CM3050 S cryostat. Sections were permeabilized with 2.5 μg/ml Proteinase K for 2 minutes at room temperature, and post-fixed in 4% PFA/PBS for 15 minutes. Sections were then treated with 0.1 M triethanolamine/ 0.25% acetic anhydride for 10 minutes and incubated with either 150 ng of DIG or FITC-labeled probe per slide overnight at 65°C. Probe was detected with an anti-DIG or anti-FITC antibody (1:100) conjugated to horseradish peroxidase (Roche). Signal was produced by treatment with TSA Plus Cyanine 3 / Cyanine 5 system (PerkinElmer) and washed overnight at 4°C. Immunofluorescence was completed using anti-HuC/D (6 μg/ml, Life Technologies), anti-SOX2 (3 μg/ml, Abcam), and primary antibodies were detected using an anti-rabbit antibody conjugated to Alexa-488 (Abcam) and anti-mouse antibody conjugated to Alexa-594 (Life Technologies). DAPI was added at a concentration of 1 μg/ml.

Imaging

Zebrafish embryos for whole mount in situ hybridization were embedded in 75% glycerol/PBS and imaged using a Nikon SMZ 1500 microscope equipped with a DS-Vi1 camera. Fluorescent images of transverse cryostat sections were acquired using a Leica TCS SP5 laser-scanning confocal microscope.

Results

qki phylogeny in chordates

As a first point of investigation of the qki genes in zebrafish, we examined the evolution of qki genes during chordate radiation. We identified three zebrafish genes encoding for proteins with high amino acid similarity to the single human QKI gene (> 75%). BLAST searches also revealed that the human QKI5 isoform had annotated transcript structures similar to those in the investigated species. These QKI5-related sequences were used to construct a phylogenetic
tree, using the single qki gene in amphioxus as an outgroup (Fig 1A). This analysis revealed that the zebrafish qkia gene diverged early in vertebrate evolution, forming a separate clade with one of the two qki genes present in both L. oculatus (spotted gar) and L. chalumnae (coelocanth). The other two identified zebrafish qki genes, qki2 and qkib, cluster with the second qki gene present both in spotted gar and coelocanth, along with the single qki gene identified in tetrapods, a selection of which is shown in the phylogenetic tree (Fig 1A). This topology suggests that the zebrafish qki2 and qkib are orthologs of human QKI, while the zebrafish qkia gene is a paralog of qki2 and qkib in the zebrafish and of QKI in humans.

Exon-intron structure of human and zebrafish QKI gene transcripts

To examine the conservation of gene structure between zebrafish qki genes and human QKI we compared the exon-intron structure of the different predicted transcript variants (tv) of the zebrafish against the human transcripts (Fig 1B). The human exon terminology is used for zebrafish, numbering the exons 1–6, 7a, 7b, 7c and 8. Note that the numbering is based on exon structure similarity. The exon size is generally well conserved with exceptions in exons 1, 6, and 7a (S1 Fig).

Exon-intron structure of predicted qki transcript variants presents additional evidence of the relationships between the three zebrafish qki genes and human QKI (Fig 1B). The qkia in zebrafish includes two transcript variants that differ only in the extension of exon 6, as previously shown by Lobbardi et al. 2011. The first transcript variant, qkia_tv1, includes an extended sequence of exon 6 together with exon 7c and 8. This combination of exons is not found in any other gene analyzed in this study. The second transcript, qkia_tv2, shares identical exon structure with human QKI5 (marked in yellow). Three transcript variants were predicted for qki2,
among which qki2_tv1 and qki2_tv2 have structure similar to human QKI5 and QKI6, respectively. It should be noted that the third qki2 predicted transcript, qki2_tv3, is not annotated in either the Genbank or the Vertebrate genome annotation (Vega) databases. Furthermore, the extended versions of exon 6 shares reduced sequence similarity between humans and zebrafish. Therefore, the C-terminus of qki2_tv3 transcript, if produced, is not conserved with human QKI7B. The third zebrafish qki gene, qkib, encodes four predicted transcripts, where the first three share the same structure with the three transcripts identified for qki2. The fourth predicted transcript shares a similar structure with human QKI7.

**Shared synteny between zebrafish and human QKI genes**

Given the high similarity between the qki genes, we investigated the synteny of these genes in multiple vertebrate species. Overall, a large degree of rearrangements were observed between all three zebrafish chromosome regions compared to the human region (Fig 2A). However, both qkib and qkia in the zebrafish share multiple genes, on a macrosyntenic level, with human QKI. The chromosome containing qki2 did not share any synteny with the human QKI genomic region, and was therefore not included in the figure. However, the qki2 genomic region of zebrafish shares a large degree of synteny with other teleost fish species, such as the region surrounding qki2 of the tetraodon and qkib of the spotted gar (Fig 2B). Conversely, analysis of the same region anchored to zebrafish qkib shows a relatively increased synteny with the qkib gene of spotted gar. This suggests that qki2 is a teleost-specific gene that likely originated from the whole genome duplication at the root of the teleost lineage.

**Protein alignments between zebrafish and human QKI**

The alignment of all translated and predicted zebrafish and human QKI protein isoforms demonstrates high amino acid (aa) conservation within the first five exons encoding for the functional KH domain (Fig 3). Highest similarity was found between zebrafish Qkib and human QKI, with only two aa substitutions located in exons 2 and 4. Qki2 differs in six positions with the human protein while Qkia is the most divergent zebrafish Qki isoform in this region. In exon 6, larger amounts of aa substitutions are present between the two species, with the highest conservation still present between human and Qkib. The extended version of exon 6, only present in one protein isoform encoded by each gene in both species, is highly dissimilar. The same is true for exon 7a included in the sequence of Qkib_tv4 and QKI7. Within most C-terminal exons, both isoforms of Qkia align with QKI5, while the isoforms of Qki2 and Qkib align either with QKI5 or QKI6.

**Developmental expression profiles of qki genes in zebrafish**

Developmental gene expression profiles of qkia, qki2 and qkib were determined using quantitative real-time PCR (qPCR) on RNA extracted from whole zebrafish embryos and larvae, ranging from 7 hours post-fertilization (hpf) to 21 days post-fertilization (dpf) (Fig 4). Primers used were designed to include all potential transcript variants for each gene. The qkia gene showed the highest expression during early embryogenesis (7–14 hpf) and its transcription rapidly declined at the end of somitogenesis (19 hpf) by approximately 50% (Fig 4A). Over the following 6 days of development qkia expression gradually decreased to about 25% of the initial transcript level, which remained stable until 21 dpf. The relative mRNA levels of qki2 and qkib, while distinct from qkia, followed similar developmental patterns, including up-regulation during early development (between 7–14 hpf for qki2 and 24–36 hpf for qkib). Expression of both qki2 and qkib peaked at 3 dpf, and gradually declined to about 30% of the maximum level by 7 dpf, which remained stable until 21 dpf (Fig 4B and 4C).
Fig 2. Syntenic conservation of qki genes. (A) Shared synteny between the genomic region containing QKI in human chromosome 6 and zebrafish chromosomes 13 and 17. Colored boxes represent genes. Genes in common between human chromosome 6 and zebrafish chromosome 17 are marked in yellow and genes shared between human chromosome 6 and zebrafish chromosome 13 are colored blue. QKI is framed with a thick line. Gene names are...
To compare the spatiotemporal expression of mRNAs encoding each of the three qki genes, zebrafish of various developmental stages were subjected to whole mount in situ hybridization using gene-specific cRNA probes (Fig 5). At the 4-cell stage, maternally transcribed qkia was detected, while no obvious staining was observed for either qki2 or qkib probes, even after prolonged incubation with an alkaline phosphatase substrate. During early somitogenesis (5 somites), the qkia probe homogeneously labeled a subset of the presomitic mesoderm, named adaxial cells (ad), located in two rows tightly flanking the notochord (Fig 5A). Weaker and more diffuse staining was also detected in the lateral portion of paraxial mesoderm (lpm) as well as in the mesoderm of the head region. Expression of qki2 mRNA was also found in the adaxial cells, however it was upregulated in newly formed somites (som) as compared to the presomitic paraxial mesoderm (Fig 5B). Different from qkia, qki2 expression was also evident in two discrete stripes in the hindbrain primordium (arrows in Fig 5B, 5 somite stage). Transcription of qkib was detected in two clusters likely corresponding to the midbrain (mb) and hindbrain (hb) (indicated by arrows in Fig 5C, 5 somite stage), and in the neural plate of the trunk. As somites matured, the territories of both qkia and qki2 transcription expanded laterally within the somite, with qkia expansion being more pronounced in the posterior segmental plate (Fig 5A and 5B). At the same time, qki2 showed a similar pattern to qkib within the developing neural tube, where both transcripts were present in newly formed neuromeres, including forebrain (fb), midbrain and hindbrain (Fig 5B and 5C). However, qkib mRNA was more ubiquitously expressed within the developing neural tube and extended along the anterior-posterior axis.

By 24 hpf, the somite formation was complete and differentiated muscle fibers were labeled with both qkia and qki2 probes (Fig 5A and 5B). Furthermore, qkia was diffusely expressed in the head, potentially labeling the craniofacial musculature, which became more apparent at later stages. On the other hand, qki2 was strongly expressed in the heart primordium (h). Both qkib and qki2, but not qkia, were clearly expressed in the developing brain. The qkia staining was apparent in discrete regions, including the ventral and dorsal diencephalon (di), midbrain-hindbrain boundary (mhb) and hindbrain rhombomeres. Although qkib labeling was broadly expressed along the entire length of the neural tube (indicated by arrows in Fig 5C, 72 hpf), a more intense signal was detected in the diencephalon, midbrain and hindbrain.

During the hatching period (48–72 hpf), qkia was strongly expressed in the craniofacial muscles (cm) supporting the extending jaw and eye capsule. Labeling was also prominent in pectoral fin muscles (pf). However, neither qkia nor qki2 remained transcribed in skeletal trunk muscles. Instead, qki2 continued to be expressed in the heart and brain, where previously broader expression domains became progressively restricted to the ventricular zone (vz) across the developing brain. Moreover, two columns of qki2-positive cells could be distinguished in the dorsolateral hindbrain (dhb). From 2 to 3 dpf the pattern of qkib transcription in the brain was similar to qki2 expression (Fig 5C). Additionally, at 3 dpf qki2, but not qkib, was detected along the anterior lateral line (indicated by arrows in Fig 5B, 72 hpf), suggesting qki2 is present in Schwann cells myelinating the lateral line nerve. As the myelination progressed, qki2 territory also expanded caudally along the entire length of the lateral line (not shown), consistent with the anterior-posterior maturation gradient of the trunk.

Together, the detected gene expression patterns suggest the possibility of both complementary and distinct functions of the qki genes during zebrafish development.
**qki2** and **qkib** expression in the developing nervous system

We next sought to further examine the expression of *qki2* and *qkib* in the developing nervous system using *in situ* hybridization combined with immunofluorescence on transverse sections.

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**Fig 3. Sequence alignment and conservation between zebrafish and human QKI proteins.** Amino acid sequence alignment including all isoforms found in human and zebrafish. The position of each exon is indicated with black lines above the alignment. The human exon terminology is used for both species. The KH domain is marked with a red line below the alignment. Black background indicates identical amino acids and grey indicates conservative changes.

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A. qki1a

B. qki2

C. qki3

Relative mRNA Expression vs. Developmental Time Point (post-fertilisation)
of developing zebrafish (Figs 6 and 7). At 3 dpf, both qki2 and qkib were localized to the mid-line ventricular zone of the forebrain, visualized by co-labeling with Sox2, a marker of neural progenitor cell populations (Fig 6). In the hindbrain, qki2 and qkib were expressed in cells located in the dorsolateral region overlapping with Sox2 expression (Fig 6). Within the spinal cord, signal for both qki2 and qkib was detected in the central progenitor zone at 2 dpf and 3 dpf, respectively, as shown by co-
localization with Sox2 (Figs 6 and 7). At 3 dpf qki2 expression was also detected in a ventral region, likely corresponding to the ventral floor plate of the developing spinal cord, outside of the labeled Sox2 progenitor zone (Fig 6). Notably both qki2 and qkib were absent from differentiated neurons, demonstrated by lack of co-staining with an anti-HuC/D antibody (Fig 7). In summary qki2 and qkib are predominantly expressed in neural progenitor cells but not in differentiated neurons.

Discussion

In this study, we investigated the evolution and expression of three qki genes in zebrafish: qki2 and qkib which are orthologs of human QKI, and qkia which is a paralog. Expression of qkia was primarily restricted to the mesoderm, whereas qkib mRNA was detected in the developing nervous system and qki2 had a partially overlapping expression with both genes. Within the nervous system, qki2 and qkib were both expressed in midline progenitor zones, and colocalized with Sox2, a marker of neural progenitor cells.

To investigate the evolution of qki genes, phylogenetic analysis was performed and revealed the segregation of two qki clades in vertebrates. Because only a single gene was identified in amphioxus, it is likely that two qki genes existed in the last common ancestor of tetrapods and ray-finned fish, a hypothesis supported by the presence of two distinct qki genes in both the spotted gar and coelocanth. The absence of two qki genes in the examined tetrapod genomes suggests that one of the ancestral paralogs was lost during the fin-to-limb transition. The gene qki2 is likely the result of the teleost-specific whole genome duplication. This duplication event is supported by the increased syntenic conservation of the qki2 genes in tetraodon and zebrafish, but not spotted gar. Furthermore, the human QKI locus shared neighboring genes with both qkia and qkib, but not qki2, supporting the teleost-specific origin of qki2. Similar conclusions were obtained by alignment of the human splice variants against the zebrafish genome; both qki2 and qkib included putative transcripts with similar structures to human QKI5 and QKI6, whereas of the two putative qkia transcripts, only one shared a similar transcript structure to QKI. At the amino acid level, there is a striking degree of conservation among all chordate Qki proteins throughout the RNA-binding KH-domain, suggesting a strong selective pressure on the composition of this domain. The C-terminus of QKI5 has previously been shown to be critical in the nuclear localization of this isoform [39], and sequence similarity between all three zebrafish isoforms (Qkia_tv1 and 2, Qki2_tv1 and Qkib_tv1) is highly conserved with human QKI5, suggesting that a nuclear localization of Qki is an ancestral trait.

Similarly, the C-terminus of QKI6, which is capable of binding the TGE (tra-2 and GLI) translation regulation element [40], is well conserved with both Qki2_tv2 and Qkib_tv2, suggesting that this function of the gene arose before the teleost-specific qki duplication. More divergence is apparent in the C-terminus of the QKI7 and QKI7B isoforms, suggesting that these isoforms are the result of more evolutionarily recent isoform acquisition.

Gene expression analysis of the qki genes in zebrafish demonstrated that the mRNA profiles of the three zebrafish qki genes exhibit unique, although partially overlapping expression patterns during embryonic and larval development. This is in contrast to the widespread expression of the single mammalian QKI gene in the heart, vascular system, muscles, brain and spinal cord [15, 20, 41]. The differences in expression between fish and mammals suggest that
following the loss of qkia in the tetrapod lineage, the enhancer elements for QKI became more generalized, driving expression in both mesoderm and neural ectoderm lineages. Conversely, following the duplication events in the teleost, the qki2 and qkib promoters diverged, resulting in retention of the two paralogs.

In accordance with a previous publication [27], qkia was detected in the trunk paraxial mesoderm and its derivatives, including somites and somite muscles as well as in the craniofacial and pectoral fin musculature. However, the strong qkia staining in the brain observed by Tanaka et al. could not be reproduced in our experiments.

qkib was detected in the neural plate during early somitogenesis with subsequent expression in the developing neural tube, where initially broad expression domains became progressively restricted to the CNS ventricular zone across brain and spinal cord. This spatiotemporal profile is indicative of qkib expression in neural progenitor cells, supported by both colocalization with Sox2, and a lack of qkib mRNA differentiated neurons, consistent with patterns previously reported for QKI isoforms in the mouse [6]. The function of qkib in this area is not yet known.

qki2 was detected in partially overlapping expression domains with qkia within the paraxial mesoderm and somite muscles, and qkib within the developing neural tube. Additionally, in the nervous system, qki2 exhibited unique expression patterns to qkib. The localization of qki2 in the ventral spinal cord and upregulation of qki2 transcription during the active phase of myelination (3 dpf), suggests that qki2 may play a role in zebrafish myelination [42], similar to what has been found in the mouse [43]. However, additional cellular markers will be needed to further delineate the exact cellular expression of qkib and qki2 within the nervous system.

Taken together, the distinct spatiotemporal localizations of the qki genes in zebrafish suggest that following duplication these paralogs have undergone subfunctionalization by mutations in their enhancer elements. Therefore, the zebrafish presents a unique model system with the potential to selectively dissect the role of qki in specific cell types. Future efforts will utilize directed genomic inactivation using gene editing techniques.

**Supporting Information**

S1 Fig. Genomic organization of human QKI and zebrafish qki. Known and predicted splice variants of the human QKI and the three zebrafish qki genes are detailed. The splice acceptor (sa) and splice donor (sd) sites for each exon and the exon length (in nucleotides) are compared between species.

(TIF)

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**Author Contributions**

Conceived and designed the experiments: KJR JS BF EJ LSE. Performed the experiments: KJR JS BF CP. Analyzed the data: KJR JS BF ÅTR GT CP. Contributed reagents/materials/analysis...
tools: EJ LSE. Wrote the paper: KJR JS BF ÅTR CP PK EJ LSE. Approved the last version of the manuscript: KJR JS BF ÅTR GT CP PK EJ LSE.

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