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Developmental expression and functional characterization of quaking genes in zebrafish *(Danio rerio)*

Μελέτη της αναπτυξιακής έκφρασης και λειτουργικός χαρακτηριασμός των γονιδίων quaking στο zebrafish(Danio rerio)

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ABSTRACT

Schizophrenia (SCZ) is a mental disorder characterized by a breakdown of thinking process and by absence of typical emotions. One of its etiologies is the abnormal myelination and disorganization of white matter. Studies in mice have shown that, KH domain containing, RNA binding (*QKI*) has an essential role in myelination, which might indicate contribution in SCZ. Our studies focus on understanding the role of *QKI* during early development, using zebrafish. For this reason we developed glia markers for future co-localization with *qki* in zebrafish and also qPCR to examine the developmental expression of the genes. Our results shown similar expression patters of quaking between zebrafish, human and mice. Combined with bioinformatics analysis, we can conclude that *qki2* and *qkia* are similar to *QKI6* and *qkia* with *QKI5* both in sequence and expression patterns. Also, comparison of *in situ* hybridization results of both glia markers and quaking, might indicate a potential role of quaking in myelination process. Taken together, zebrafish is a good model to study the early development of quaking and further studies might reveal essential information about the role of quaking in early neural development and how it can affect the origin of schizophrenia.

ΠΕΡΙΛΗΨΗ

Η σχιζοφρένια είναι μια ψυχιατρική διαταραχή η οποία χαρακτηρίζεται απο διαταραχή στη διαδικασία της σκέψης και στην απουσία τυπικών συναισθημάτων. Μία απο τις αιτιολογίες της σχιζοφρένιας είναι η μη σωστή μυελίνωση και η αποδιοργάνωση της λευκής ουσίας. Ένα απο τα γονίδια που θεωρείται ότι έχει σχέση με τη σχιζοφρένια είναι το KH domain containing, RNA binding (*QKI*). Μελέτες σε ποντίκια έχουν δείξει ότι το *QKI* έχει σημαντικό ρόλο στη μυελίνωση , γεγονός που υποδικνύει το πιθανό ρόλο του *QKI* στη σχιζοφρένια. Στόχος της μελέτης μας είναι να κατανοήσουμε το ρόλο του *QKI* κατά τα πρώημα στάδια της ανάπτυξης, χρησιμοποιόντας το zebrafish σαν μοντέλο. Για τον λόγο αυτό, κατασκευάστηκαν δείκτες που σχετίζονται με την γλιογένεση για μελλοντικό συνεντοπισμό τους με το *qki* στο zebrafish, καθώς και qPCR για να εξετάσουμε την αναπτυξιακή έκφραση των γονιδίων. Τα αποτελέσματα μας δείχνουν παρόμοιο πρότυπο έκφρασης των γονιδίων quaking μεταξύ ανθρώπου, ποντικού και zebrafish. Συνδιάζοντας τα αποτελέσματα μας με αποτελέσματα απο πρωτεϊνική αλληλουχική ανάλυση, μπορούμε να συμπεράνουμε ότι το qki2 και qkia είναι παρόμοια με το QKI6, ενώ το qkia με το QKI5. Σύγκιση των αποτελεσμάτων απο την υβριδοποίηση in situ των γονιδίων quaking και των δεικτών γλιογένεσης, μπορεί να δείξουν πιθανό ρόλο του στη μυελίνωση. Συνδιάζοντας όλα τα παραπάνω, συμπερένουμε ότι το zebrafish είναι ένα καλό μοντέλο για να μελετίσουμε το ρόλο του quaking κατά την πρώΙμη νευρική ανάπτυξη και το πώς το γεγονός αυτό μπορεί να επιρεάσει την έναρξη της σχιζοφρένιας.

INTRODUCTION

Schizophrenia

Schizophrenia (SCZ) is a mental disorder characterized by a breakdown of thinking process and by absence of typical emotions. The first detailed case of schizophrenia was reported in 1809 by Phillipe Pinel (Heinrichs 2003). Typically, the first symptoms occur in young adulthood (age 20-24), with a prevalence of 0.5–1% in general population. Although the primary cause of SCZ is not known, evidence from family, twin and adoption studies indicate a substantial genetic contribution to the disease (McGuffin et al. 1995, Cannon et al. 1998). Many genes such as *DISRUPTED IN SCHIZOPHRENIA1 (DISC1), NEUREGULIN1, DYSTROBREVIN BINDING PROTEIN (DTNBP1), KIF1, KIF17, SHANK3* and *NOTCH4* are implicated in SCZ as risk factors (Tarabeux et al. 2010, Girard et al. 2012). Except of the genetic background, other important contributories of schizophrenia seem to be environmental effects such as diet, parenting style and exposure to toxins or teratogens (Sullivan et al. 2003). Early events that shape the prenatal environment, such as maternal infections during pregnancy, which cause toxic effects on embryo's neurons (Urakubo et al. 2001) and fetal hypoxic or ischemic damage that can be due to complications during pregnancy or delivery are also related to abnormal development of the CNS (Miyamoto et al. 2003). Histopathological studies have shown imperfect neuronal production, migration, guidance and/or integration with target tissue in patients with schizophrenia (Akbarian et al. 1996). Other studies demonstrate abnormal myelination and disorganisation of the white matter suggesting that the function of myelin-forming oligodendrocytes may also be compromised in SCZ (Flynn et al. 2003, Chenard and Richard 2008). These findings suggest that SCZ is likely to arise from abnormal interactions between multiple cell types during early brain development. These genetic differences imply hundreds of different genes which may disrupt the developing brain.

QKI

In 2006, *KH domain containing, RNA binding (QKI*) was proposed as a new candidate gene for schizophrenia (Aberg et al. 2006). QKI is a member of RNA-binding proteins family called STAR proteins (Signal Transduction and activation of RNA) and it is highly expressed in glial cells, including astrocytes and myelin-forming oligodendrocytes. QKI acts by regulating pre-mRNA splicing, mRNA export, mRNA stability and protein translation. Over 1400 potential QKI mRNA targets have been bioinformatically predicted based in the presence of the QKI recognition element (QRE): 5'-NACUAAY-N(1,20)-UAAY-3' (where N is any base and Y is pyrimidine). These transcripts are involved in development, cell adhesion, morphogenesis, organogenesis, transport, cell differentiation, cell growth or/and maintenance and cell communication (Galarneau and Richard 2005). In humans, *QKI*

is located on chromosome 6 and four protein isoforms are generated via alternative splicing: QKI5, QKI6, QKI7 and QKI7b. QKI7b is the most recently identify isoform, and therefore its function is not yet well characterized. All QKI isoforms share a single K-homology (KH) domain, involved in target mRNA binding, and two flanking domains QUA1 and QUA2. QUA1 is essential for homodimerization (Chen et al. 1997, Chen and Richard 1998), while QUA2 is involved, together with KH domain, in RNA biding (Ryder and Williamson 2004). QKI isoforms differ only in their unique C-terminal amino acid sequence as shown in Figure 1. QKI5 is located in the nucleus consistent with the nuclear localization signal present in its C-terminus, while QKI6 and QKI7 are mostly cytoplasmic (Wu et al. 1999).

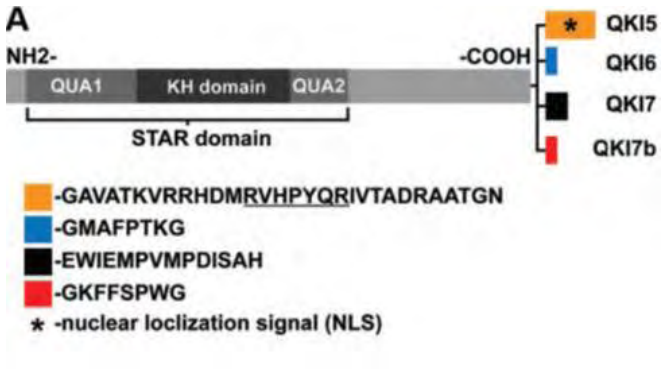

Figure 1. Schematic diagram of human QKI proteins. All QKI isoforms share a single highly conserved K-homology (KH) RNA-binding domain that is flanked by the QUA1 motif responsible for dimerization, and QUA2 region involved in RNA binding. KH together with QUA motifs constitute the STAR domain. Isoform-specific C-termini peptides are shown in yellow (QKI5), blue (QKI6), black (QKI7) and red (QKI7b). Nuclear localization signal (asterisk) is encoded by a unique sequence in QKI5 C-terminus (underlined). From Radomska et al. 2013.

Expression pattern and function

QKI developmental expression pattern has been well characterized in mice. QKI proteins are widely expressed in developing and adult nervous system, but also in other tissues such as lungs, heart and testes (Ebersole et al. 1996). In the central nervous system (CNS) *QKI* is first expressed in the multipotent neural progenitor cells (NPCs) of the ventricular zone. These cells are highly proliferative and generate both neurons and glial cells (including astrocytes and oligodendrocytes). *QKI* expression declines as NPCs differentiate into neurons, but not if they differentiate into glial cells (Hardy 1998) suggesting that QKI may act (in concert with other molecules) to induce NPCs to adopt a glial fate.

QKI5 is the most abundant isoform during the early development, but its expression declines postnatal, during the active phase of myelination (Ebersole et al. 1996). QKI5 is essential for embryogenesis as *Qki* null mice are embryonic lethal (Justice and Bode 1988, Akbarian et al. 1996, Ebersole et al. 1996, Larocque et al. 2002, Noveroske et al. 2002). QKI6 and QKI7 exhibit an expression pick during myelination and expression remains high through adulthood (Hardy et al. 1996, Hardy 1998). Initially, high embryonic expression of QKI5 leads NPCs to proliferate. During early postnatal development, the rising levels of QKI6 and QKI7, as well as the decreasing levels of QKI5 promote NPCs to exit the cell cycle and differentiate. QKI proteins have been shown to regulate expression of factors required for differentiation of myelinating glia (oligodendrocytes in the CNS and Schwann cells in the PNS), including Myelin basic protein (*Mbp*), Myelin associated glycoprotein (*Mag*) and

early growth response protein 2 (*Krox20*) (Nagarajan et al. 2001, Parkinson et al. 2004, Decker et al. 2006). *Qki* viable (qk^{v}) mice, harbouring a large deletion in *qki* regulatory region suffer from severe demyelination in both CNS and PNS (Sidman et al. 1964). Human *QKI* has been recently shown to regulate expression of astrocyte differentiation marker Glial Firbillary Acidic Protein (*GFAP*) (Radomska et al. 2013) supporting the notion of QKI role in glia fate specification.

QKI and schizophrenia

QKI has been proposed as SCZ susceptibility gene based n linkage, association and expression studies (Lindholm et al. 2004, Aberg et al. 2006). Several studies show reduced *QKI* mRNA expression in multiple brain regions of SCZ patients. Particularly, *QKI7* and *QKI7b* are preferentially reduced, suggesting post transcriptional misregulation of QKI as an etiology for SCZ. Moreover, *QKI* deficiency in SCZ is linked with reduced expression of mRNAs involved in oligodendrocyte development and myelination, linking *QKI* hypofunction to myelin deficits and white matter impairment observed in SCZ (Aberg et al. 2006).

Why zebrafish?

Zebrafish offers an excellent model organism to study the genetic, morphological and behavioral significance of altered *qki* expression during early neurodevelopment. The rapid external development of the embryos combined with optical transparency makes possible the *in vivo* visualization for analyzing cell type, cell behavior and subcellular structures in both living and fixed specimens. Moreover, it's known genome with the powerful genetic tools make zebrafish ideal for both gain and loss of function experiments. Critical for this selection is that ethical rules suggest the use of lower vertebrates when it's possible in large scale experiments. High degree of conservation between human *QKI* and zebrafish *qki* was observed by the means of bioinformatics analysis as shown in Figure 2.

Quaking in zebrafish

Three *qki* genes are expressed in zebrafish: *qkia* (chromosome 17), *qkib* (chromosome 13) and *qki2* (chromosome 12). Predicted protein products show striking conservarion with human QKI, especially within the functional HK-domain, as shown in Figure 2. Early developmental expression of *qkia* has been previously described and its function in somite muscle morphogenesis has been characterized (Tanaka et al. 1997, Lobbardi et al. 2011). The function of *qkib* and *qki2* are yet unknown.

Jazin's group has recently studied developmental expression patterns of zebrafish *qki* mRNAs using whole mount in-situ hybridization (Figure 3). During early somitogenesis, *qkia* was highly expressed in the somite muscle precursors (adaxial cells), consistent with previous reports (Lobbardi et al. 2011). *qki2* showed similar pattern during somitogenesis, however, starting from 48hpf, its expression becomes restricted to the nervous system. Similarly, *qkib* was also highly expressed in the developing brain and spinal cord.

AIM

The aim of this study was to characterize developmental expression of *qki* genes in zebrafish (*Danio rerio*) using quantitative real-time PCR. Moreover, in order to better understand the function of *qki*, it was essential to disclose the identity of *qki*-expressing cells previously revealed by hybridization *in situ*. This can be achieved by co-labelling of embryos with *qki* and cell type specific markers. For that reason RNA probes were developed for targeting *in situ* genes expressed at different stages of oligodendroglia and astroglia development. These probes have been validated using whole mount *in-situ* hybridization and they can be further used in double *in situ* hybridization experiments.

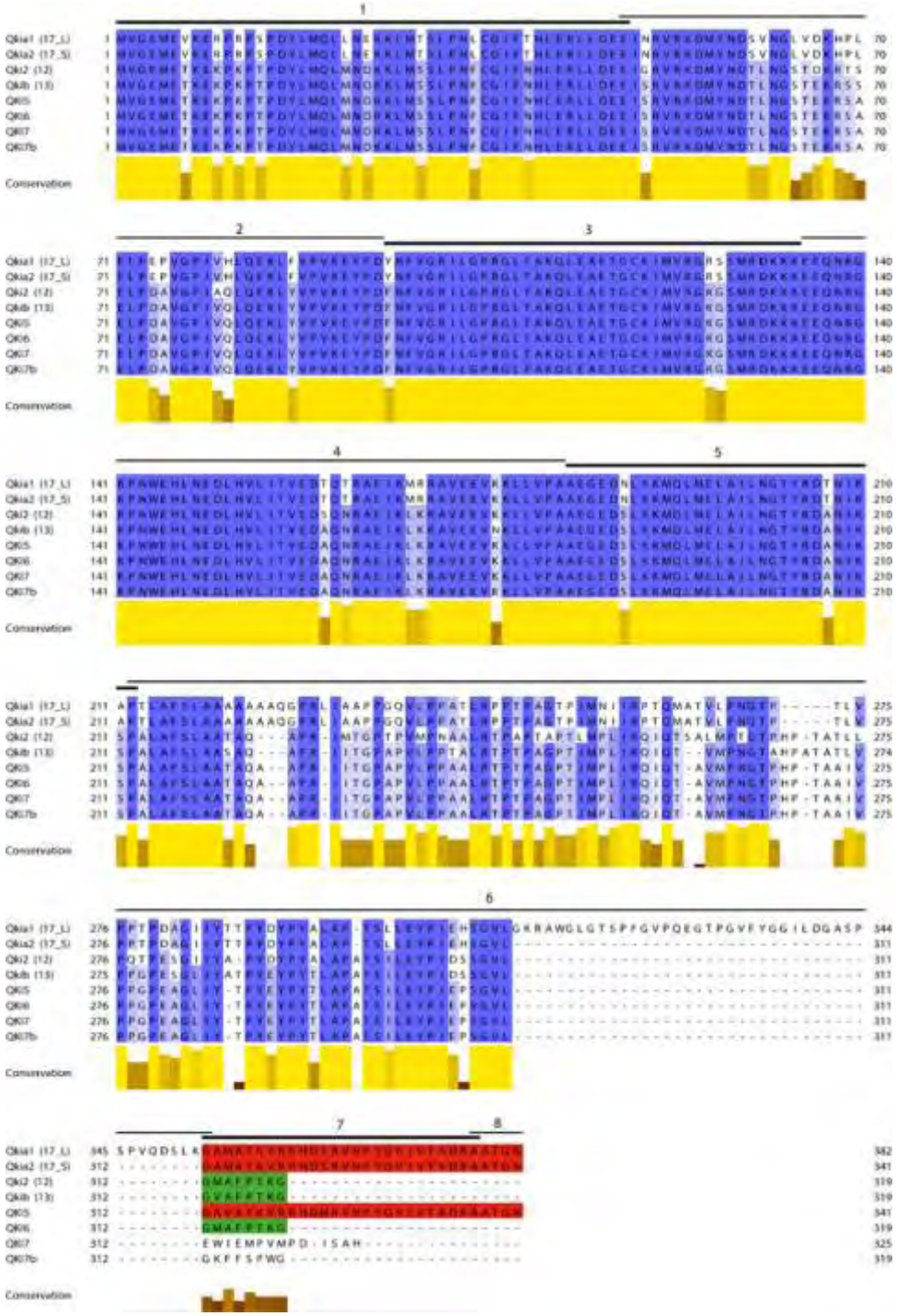

Figure 2. Protein alignment between human and zebrafish quaking genes. Zebrafish Qki proteins show striking conservation with the human QKI, particularly within the functional K-homology (KH) domain (exon 2,3 and 4) involved in target mRNA binding. Zebrafish *qkia* (chr.17) is alternatively spliced giving rise to two protein isoforms Qkia2 (short_S), and Qkia1 (long_L), containing 41aa insertion within exon6. Both, *qki2* (chr.12) and *qkib* (chr.13) encode 319aa products. Four protein isoforms (QKI5, QKI6, QKI7, QKI7b) are derived from a single human *QKI* gene (chr.6). Exons and numbered above the alignment. (Figure prepared by Åsa Tellgren-Roth).

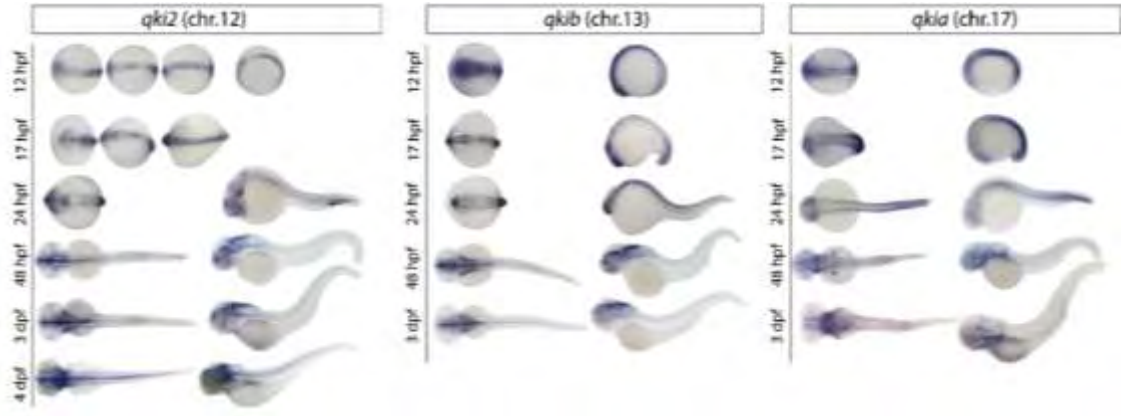

Figure 3. Developmental expression of quaking genes in zebrafish (figure prepared by Katarzyna Radomska).

MATERIALS AND METHODS

Zebrafish maintence

Wild type AB Zebrafish were used as model organism for this study. The fish was maintained in SciLifeLab facility, where also most of the caring as breeding and feeding took place.

Collection of the embryos

Firstly, a couple of males and females fish were placed in breeding tanks the night before eggs were to be collected. It is critical to separate the fish with a plastic separator. Next morning, when the lights went on, the separator was removed and after half an hour there were fertilized eggs ready for harvesting. Then the eggs were placed in Petri dishes (50 embryos each) and remained in the incubator (28.5 ^{o}C) until use. The collection contained the following samples: shield stage, 5 somites, 10 somites, 13-15 somites, 21 somites, 24 hpf, 36 hpf, 48 hpf, 3 dpf, 4 dpf, 5 dpf, 6 dpf, 7 dpf, 10 dpf, 3 weeks. Different embryonic stages were used for different methods.

For *in situ* hybridisation, embryos younder than 48hpf were dechorionated. Dechorionation of 24 hpf embryos were held by adding 60 µl pronase (5 ng/µl) to a Petri dish with water and left the fish for half an hour in 28.5^{o}C. Then the embryos were removed in a new Petri dish with clean water. Dechorionation of embryos in somite stages was performed manually by forceps, using optical microscope. Removal of pigment was necessary for embryos older than 48 hpf. Embryos were treated with bleaching solution and the process was observed under normal optical microscope. After the removal of pigment the reaction was stopped by washes with PBS.

Probe synthesis for in situ hybridization

a. RNA extraction

Initially, *RNA isolation* from different developmental stages of zebrafish embryos was held, using TRIzol reagent and chorophorm/isopropanol protocol. Around 50 zebrafish embryos were collected in a 1.5 ml tube and water was removed. They were immersed in liquid nitrogen and then placed in dry ice. Immediately 250 μl of TRIzol was added, the samples were homogenized by a pestle and 750 μl more TRIzol was added. The samples were incubated for 5 min at room temperature and then 0.2 ml of chloroform was added and mixed for 15 sec. The samples were transferred to a new tube with 100-140 mg of gel lock heavy (5 prime) and incubated for 2 min at room temperature before centrifugation at 12,000 x g for 15min at 4^{o}C. The upper aqueous phase was transferred to a new tube and 0.5 ml isopropanol was added and the samples were incubated for 10 min at room temperature and centrifuged at 12.000 x g for 10 min at 4^{o}C. After that a gel like pellet on the bottom of the tube was visible, the supernatants were removed and replaced with 1ml 75% ethanol. The samples were mixed well and centrifuge at 7,500 x g for 5 min at 4^{o}C. The ethanol was removed and the samples were air dried (around 5 min), 10-30 μl of RNAse-free water was added and the samples were incubated at 55^{o}C for 10 min. RNA concentration was measured on NanoDrop ND1000 Spectrophotometer and RNA integrity was monitored by running ~500 ng of RNA sample on an agarose gel.

b. Reverse transcription

400 ng of RNA was reversely transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems). The reaction mix included: 1 μl of RT-buffer, 2.2 μl of $MgCl_2$, 2 μl of dNTPs, 0.5 μl of random hexamers, 0.2 μl of RNase inhibitor and 0.25 μl of reverse transcriptase, and RNAse free water to a total volume 20 μl. The mix was incubated at 25 °C for 10 min (primer annealing), 48 °C for 1 h (synthesis) and 95°C for 10 min (enzyme inactivation). cDNA samples were diluted with RNAse-free water to a final concentration of 5 ng/μl and stored at −20°C.

c. Polymerase chain reaction (PCR)

cDNA templates for probe synthesis were prepared using PCR-based method. First, an inner PCR reaction was performed. The reaction mixtures are shown in Table 1, the primer sequences are shown in Table 2 and the PCR programs are shown in Table 3. Products of inner PCR were used as templates for nested PCR using primers with SP6 RNA polymerase recognition sites. The reaction mixtures are shown in Table 4, the primer sequences are shown in Table 5 and the PCR programs are shown in Table 3. Concentration and purity of nested PCR products were measured using NanoDrop ND1000 Spectophotometer.

Table 1 Inner PCR reaction mixture

	plp1a,mag **µl/final concentration**	**plp1b** **µl/final concentration**	**mpz** **µl/final concentration**	**krox20** **µl/final concentration**
10x PCRbuffer-MgCl2	2 / [1x]	2 / [1x]	2/ [1x]	2/ [1x]
MgCl2 (50 mM)	1 / [2.5 mM]	0.6 / [1.5 mM]	0.6 / [1.5 mM]	0.6/ [1.5 mM]
dNTPs (2 nm each)	2 / [0.25 mM]	2 / [0.2 mM]	2 / [0.2 mM]	2 / [0.2 mM]
Primer F inner	0.5 / [0.25 mM]	0.5 / [0.25 mM]	0.5 / [0.25 mM]	0.5 / [0.25 mM]
Primer R inner	0.5 / [0.25 mM]	0.5 / [0.25 mM]	0.5 / [0.25 mM]	0.5 / [0.25 mM]
cDNA template	0.5 / 10 ng	2 / 10 ng	4 / 40 ng	0.2 / 10 ng
Platinum Taq polymerase	0.4 / [1 unit]	0.4/ [1 unit]	0.4 / [1 unit]	0.4 / [1unit]
MiliQ water	11.6	12	10	13.8
TOTAL	20	20	20	20

Primer ID	**Gene**	**F**	**R**
plp1a	Proteolipid protein 1a	TGGCATTGCTCTTTTCTGTG	TCCCTTTCCTCCCCAAATAC
Mag	Myelin-associated glycoprotein	GATACGGCAACATGAACACG	TGCGCTGTAGAACATTGAGG
Mpz	Myelin protein zero	TTACATGGCATTACCGTCCA	TTTTCCTTTGCTTTCTTGC
krox20	Early growth factor response protein 2	ACTTTGGAGAAAGCCCCTGT	ATGAGATGGGACAGGTGGAG
plp1b	Proteolipid protein 1b	CTGGCCTCCTTCTTCTTCCT	TGTGGTCCTTTGGTTTGTGA

Table 2 PCR inner primers

Table 3 PCR temperature program

	plp1a, mag, plp1b,mpz **temperature (^{o}C), time (min)**	**krox20** **temperature (^{o}C), time (min)**	
Denature	94^{o}C,02:00	94 ^{o}C, 02:00	
Denature	94 ^{o}C, 00:30	94 ^{o}C, 00:30	40 cycles
Anneal	58 ^{o}C, 00:30	59 ^{o}C, 00:30	
Extend	72 ^{o}C, 01:00	72 ^{o}C, 01:00	
Extend	72 ^{o}C, 07:00	72 ^{o}C, 07:00	
	4 ^{o}C, for ever	4 ^{o}C, for ever	

Table 4 Reaction mixture for nested PCR

	Plp1a,mag **µl/final concentration**	**Plp1** **µl/final concentration**	**mpz** **µl/final concentration**	**Krox20** **µl/final concentration**
10x PCRbuffer-MgCl2	2 / [1x]	2 / [1x]	2/ [1x]	2 / [1x]
MgCl2 (50 mM)	1 / [2.5 mM]	0.6 / [1.5 mM]	1 / [1.5 mM]	0.6/ [1.5 mM]
dNTPs (2 nm each)	2 / [0.25 mM]	2 / [0.2 mM]	2 / [0.2 mM]	2 / [0.2 mM]
Primer F inner	0.5 / [0.25 mM]	0.5 / [0.25 mM]	0.5 / [0.25 mM]	0.5 / [0.25 mM]
Primer R inner	0.5 / [0.25mM]	0.5 / [0.25 mM]	0.5 / [0.25 mM]	0.5 / [0.25 mM]
cDNA template	1 / 50 ng	1 / 50 ng	1/ 40 ng	1 / 10 ng
Platinum Taq	0.4 / [1 unit]	0.4/ [1 unit]	0.4 / [1 unit]	0.4 / [1 unit]
MiliQ water	12.6	13	12.6	13

TOTAL	20	20	20	20

Table 5 Primers for nested PCR

ID	5' - Forward primer -3'	5'- Reverse primer-3'
plpla	CTGTAATACGACTCACTATAGGGTGGCATTGCTCTTTTCTGTG	GGGATTTAGGTGACACTATAGAATCCCTTTCCTCCCCAAATAC
plp1b	CTGTAATACGACTCACTATAGGGCTGGCCTCCTTCTTCTTCTT	GGGATTTAGGTGACACTATAGAATGTGGTCCTTTGGTTTGTGA
mag	CTGTAATACGACTCACTATAGGGGATACGGCAACATGAACACG	GGGATTTAGGTGACACTATAGAATGCGCTGTAGAACATTGAGG
mpz	CTGTAATACGACTCACTATAGGGTTACATGGCATTACCGTCCA	GGGATTTAGGTGACACTATAGAATTTTCCTTTGCCTTTCTTGC
krox20	CTGTAATACGACTCACTATAGGGACTTTGGAGAAAGCCCCTGT	GGGATTTAGGTGACACTATAGAAATGAGATGGGACAGGTGGAG

d. PCR product purification

Nested PCR products we purified using "Mini Elute" PCR purification kit, (QIAGEN) according to the manufacturer's instractions. The concentration of purified products were measured using NanoDrop ND1000 Spectophotometer Samples were stored at -20^{o}C until further use.

e. RNA probe synthesis and DIG labeling

For each probe, the following mixture was made: 200 ng of DNA template, 2 μl of 10x Transcription buffer, 2 μl of DTT (0.1M), 2 μl of DIG-RNA labeling mix, 1 μl of RNAse inhibitor, 2 μl of RNA polymerase SP6 and fill in with RNAse free H2O up to 20 μl. Then, the samples were incubated at 37^{o}C for 2 h.

f. LiCl precipitation

RNA probes were precipitated using LiCl. Initially, 2 μl LiCl 4M (0.1V) and 60 μl 100% pre cooled EtOH (3V) were added to the samples and then they were incubated at -80^{o}C overnight. Next day, the samples were centrifuged at 13.000 x g for 15 min at 2-8^{o}C. Then ethanol was decanted, the pellet was washed carefully with

750 μl cold ethanol and the samples were centrifuged at 13.000 x g for 5 min. Ethanol was decanted again and the pellet left to dry under vacuum. Finally, each sample was dissolved in 30μl sterile, RNAse free double distilled water. To verify the quality of the probes NanoDrop measurements was performed as well as 2% agarose gel electrophoresis. After the electrophoresis 30μl formamide was added to each sample and stored at -20^{o}C.

Whole mount in situ hybridization

Initially, dechorionated and non-dechorionated embryos were fixed in 4% PFA/PBS at 4^{o}C overnight. Next day, the embryos were washed 3 times in PBS for 5 min each. Then, PBS was replaced by 25%, 50%, 75% and 100% MeOH/PBS. The embryos stayed in each solution for 5 minutes and then once again in 100% MeOH. At that time they embryos should stay at -20^{o}C freezer for at least 30 min, or stored for as long as necessary.

Hydration of the embryos with serial incubations of 50% and 30% MeOH for 5minutes each at room temperature was done. Then the fish were washed two times with PBST (PBS+0.1% Tween20) for 5 min each at room temperature. Digestion with 5 μg proteinase K is followed and incubation time depends on the embryonic stage. This step was crucial so the samples should be immediately refixed with 4% PFA for 20 min. Before next step, 3 washes with PBST for 5 min were required. The procedure was followed by prehybridization and hybridization of the embryos. PBST was replaced with 200 μl Hyb- for 5 min at 6^{o}C. Then Hyb- was replaced with 200 μl Hyb+ and the samples were incubated for 2 h at 67^{o}C. Finally, 120 μl of Hyb+ were removed and replaced with 1-2 μl of the RNA probe and then the samples incubated overnight at 67^{o}C.

The probes were removed and washed 2 times for 30 min each with 50%formamide/2x SSCT and then followed 15minutes wash with 2x SSCT and two washes for 30 min with 0.2x SSCT. All the washes occurred at 67^{o}C. Afterwards, the samples were washed 2 times with 1x malate buffer at room temperature and then they stayed for at least one hour at room temperature with blocking buffer. Finally, the blocking buffer was removed and replaced by 500μl alkaline phosphatase (AP) coupled Fab antibody (ROCHE) and the samples were incubated overnight at 4^{o}C.

Four washes of 25 min each with 1x malate buffer were held and followed by 3 washes of 5 min each with staining buffer, at room temperature. Then, 500 μl of BM Purple AP substrate precipitating solution (Roche) was added and the samples were incubated at 37^{o}C for as long as necessary. As the signal was appeared, the reaction stopped by 3 washes in PBST and fixation with 4% PFA at 4^{o}C overnight. After fixation, the embryos were placed in 30% glycerol/PBS until they sunk and eventually in 70% gycerol/PBS, where they could be stored at 4^{o}C forever.

Imagining

Nikon SMZ1500 microscope was used combined with Nis-Elements viewer 4.0 program, for the observation of ISH results.

Quantitative real-time PCR

Embryo collection and cDNA synthesis reaction were performed as described above. All real-time PCR experiments were performed in 7500 Real-Time PCR System (Applied Biosystems) using 96-well optical plates (ABI). The reaction mix included 4 µl of cDNA sample, 9.5 µl of MiliQ water, 0.75 µl of forward primer (0.3µM), 0.75 µl (0.3µM), of reverse primer, 10 µl SYBR Green mix. The thermal cycle was as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min followed by the dissociation step. Sequences of primers are listed in Table 6. Gene expression was quantified using standard curve method and normalized against eef1a1l1 as a housekeeping gene. In order to evaluate the statistical significance of differential gene expression, the two-tailed t-test was used.

Table 6. qPCR primers

Primer ID	Gene	5'-Forward primer-3'	5'-Reverse primer-3'
eef1a1l1	eukaryotic translation elongation factor 1 alpha 1, like 1	AGCAGCAGCTGAG GAGTGAT	CCGCATTTGTAGAT CAGATGG
qkib	quaking (chromosome 13)	TGGAGTATCCCATC GACTCC	TGGGAATGTGACAG GTCTGA
qki2	quaking (chromosome 12)	CCAGAGTCCGGCAT CATCTA	TTTTGTCGGGAAAG CCATAC
qkia1/2	quaking (chromosome 17)	CCCACTGGAGTTAC CAGAGC	CAGTTGCTTCGCTG TGAGTC

Results

Developmental expression of qki genes using quantitative real-time RT-PCR

Expression of *qki* genes was analyzed in zebrafish starting from 7 hpf embryo up to 3-week old larva. As shown in Figure 4A, *qki2* was first detected at 14hpf and

its expression gradually increased reaching the maximum level at 3-4 days. *Qkib* (Figure 4B) showed a very similar expression pattern. On the other hand, the expression of qkia was the highest during early embryogenesis and it slightly declined at about 36 hpf and stayed rather stable during later stages (Figure 4C).

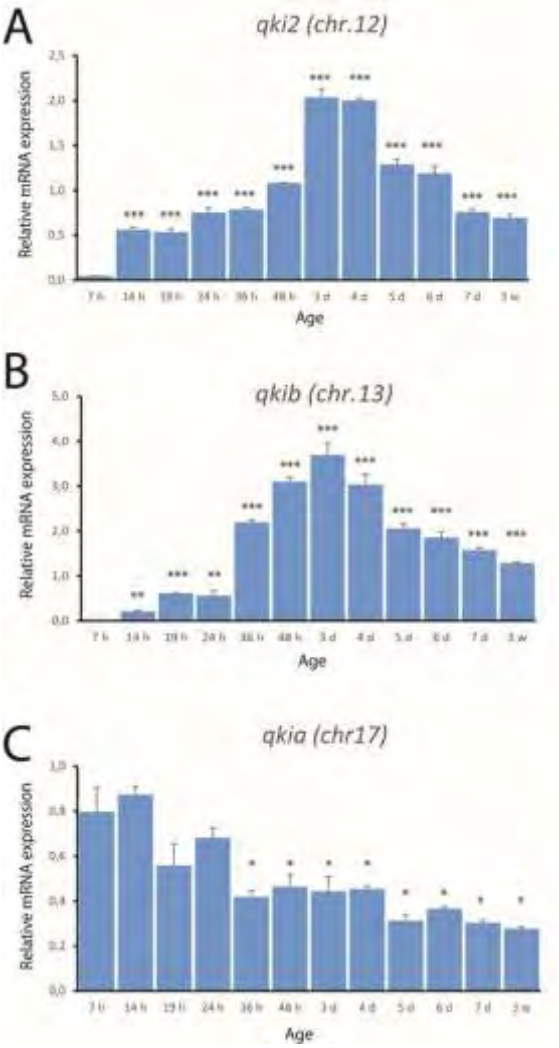

Figure 4 Relative mRNA expression of (A) *qki2*, (B) *qkib* and (C) *qkia* during embryonic and larval zebrafish development. Asterisks indicate significant deviation on the mRNA levels compared with 7hpf embryos (*** p-value<0.001, ** p-value < 0.01, * p-value < 0.05). Mean and standard errors are given based on three biological replicates.

Whole-mount in-situ hybridization

RNA probes targeting the following genes were developed: glia fibrillary acidic protein (gfap), early growth response protein 2 (krox20), myelin associated glycoprotein (mag), myelin basic protein (mbp), Myelin protein zero (mpz), oligodendrocyte lineage transcription factor 2 (olig2), sex deremination reagion-box10 (sox10), proteolipid protein 1a and b (plp1a/plp1b).These probes were tested using whole-mount *in situ* hybridization protocol on different stages of zebrafish development and the representative images are shown in Figure 5. From the total nine glia related probes only plp1a and plp1b did not worked. That could be explained by the fact that the genes are expressed in later stages than the one used. Also, further optimization of the protocol is needed.

Glia fibrillary acidic protein (gfap) is one of the major intermediate filament protein in mature astrocyte and is used to assort astrocytes from other glia cells in developing CNS. In 3dpf embryo gfap is expressed in the dorsal part of the spinal cord. (Figure 5 r, s).

Myelin basic protein (Mbp) has an important role in the formation of myelin sheath by oligodendrocytes in CNS and Schwann cells in PNS. Expression of mbp was first observed in 3dpf embryos (Figure 5 d, e) in hindbrain and spinal cord oligodendrocytes (CNS) and in Schwann cell (PNS)

Sex determination region-box 10 (Sox10) is a transcription factor acting during embryonic development with an important role in neural crest formation and PNS determination. Sox10 is required for cell cycle arrest and the differentiation of Schwann cells. In 24 hpf embryos (Figure 5 p, q) sox10 is expressed in cranial neural crest cells, otic vesicle, trunk neural crest, oligodendrocytes, and Schwann cells

Oligodendrocyte lineage transcription factor2 (olig2) is a transcription factor necessary for development of motoneurons and oligodendrocytes. In 3dpf embryos (Figure 5 m, n, o) olig2 is highly expressed in forebrain and hindbrain, including developing cerebellum and weakly expressed in the splinal cord oligodendrocyte precursors (OPCs).

Early growth response protein 2 (krox20) is sequence-specific DNA-binding transcription factor with important role in PNS myelination and myelin maintenance. Krox20 is required for the cell cycle arrest and the Schwann's cells differentiation. In 13-15 somites embryos (Figure 5 a, b) krox20 is expressed in rhombomere 3 and 5, which will give rise to hindbrain and neural plate respectively. The same pattern is observed at 24hpf (Figure 5 c).

Myelin protein zero (mpz) is a major structural component of the myelin sheath in PNS. In 13-15 somites embryos (Figure 5 i, j) expression of mpz is observed in midbrain and hindbrain and at 48hpf embryos (Figure k, l) mpz is expressed in hindbrain.

For first time (Figure 5 f, g), the expression of *Myelin-associated glycoprotein (*mag) in the migratory oligodendrocyte precursors, along the spinal cord in 3dpf embryo was observed.

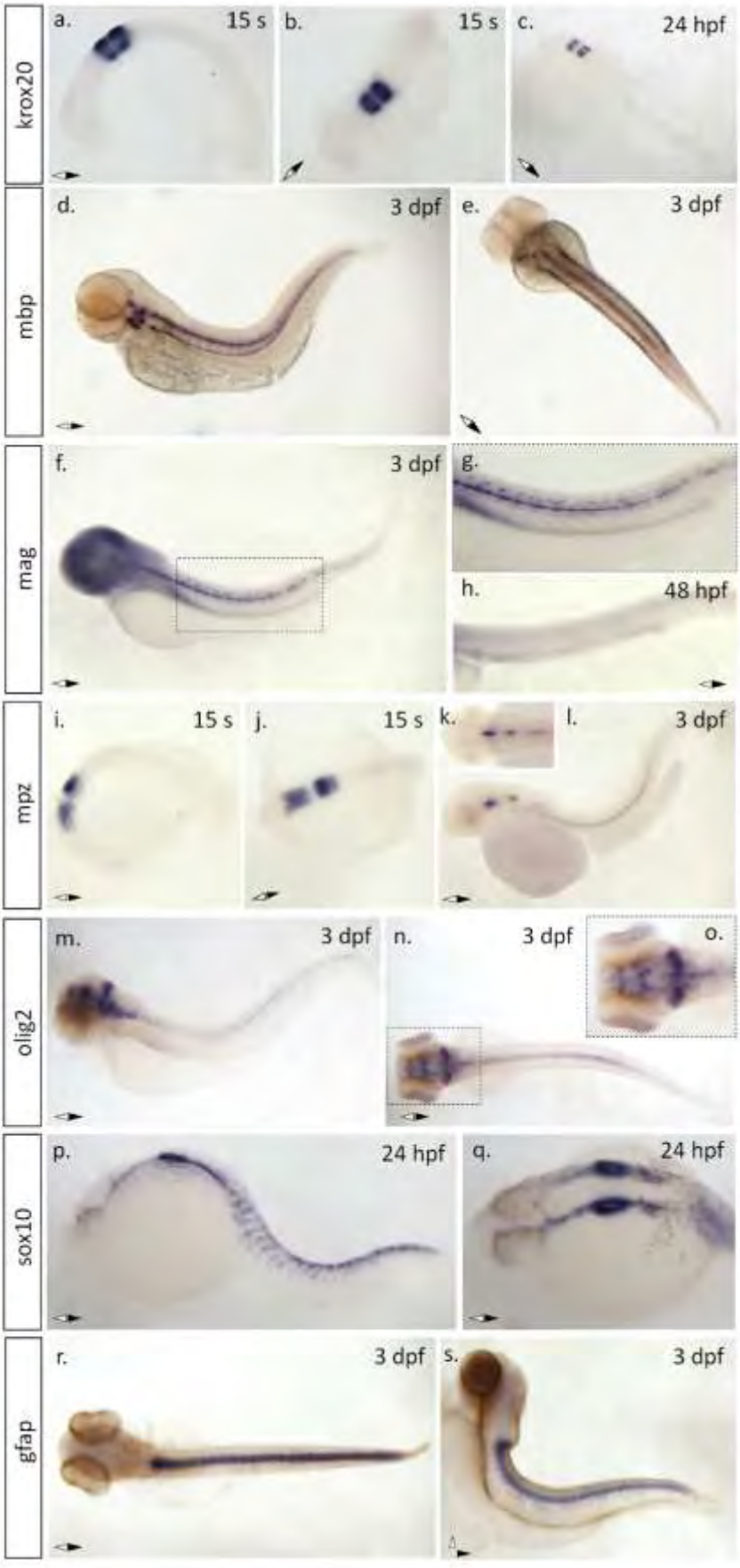

Figure 5 ISH with myelination associated markers.

DISCUSSION

Developmental expression

Our qPCR results suggest that zebrafish quaking genes have similar expression pattern as in humans and rodents. In particular, *qkia* is highly expressed early in development similarly with *qki5* in rodents. Interesting observation is that *qkia,* continues to have high expression levels during development more similar to human *QKI5* (Lauriat et al. 2008) rather than *qki5* in mice, which declines more dramatically during early postnatal life. The high expression of *qki5* is associated with its essential role in embryogenesis as qki null mice (with mutation in *qki5* region) are embryonic lethal with vascular insufficient and heart defects. Early in development the expression of *qki5* leads the cells to proliferate, promoting factors which are involved in myelin production and differentiation of myelin producing cells (such as MBP, MAG, Krox20). Another important role of *qki5* is the regulation of alternative splicing of myelin-specific genes (such as MBP, PLP and MAG). The difference between the expression levels of human *QKI5* and rodent's *qki5* might indicate differences in the signal pathways they are involved. In this case *QKI5* could be involved in late myelination procedures. Also, the high requirement of *QKI5* might indicate that it has an extra role later in the development. Such hypothesis could be supported by the fact that except of nervous system, QKI are expressed in heart, lung, testes, astrocytes, Bergmann glia and neuroectodermal cells during neurulation. As for *qkia,* it has a well characterized role in muscle development by Hh signaling, but also expression of *qkia* has been observed in other structures such as the heart tube, which might indicate the reason why it has continues high expression in later stages. Further functional studies should be held in order to identify why this difference is observed. *Qki2* and *qkib* have a peak of their expression at 3 days post fertilization which coincides with the onset of expression of several myelin associated genes (unpublished data). This might suggests that *qki2* and *qkib* play a role in myelination similarly to *qki6* and *qki7* in rodents. *Qki6* and *qki7* have more similar patterns to each other. They are found in both oligodendrocyte and Schwann cells, fact that indicates an essential role in myelination both in CNS and PNS, acting as positive regulators in myelin production and differentiation of oligodendrocytes and Schwann cells. Combined these data with bioinformatic analysis and *in situ* hybridization, we can assume that *qki2* and *qkib* might indicate similarities with *qki6*.

QKI expression via in situ hybridization analysis

Having the expression pattern of *qki* in zebrafish, the next step was to identify its spatial localization. For this reason we compared *in situ* hybridization results from developmental expression of quaking (Figure 3) and glia probes (Figure 5). All of the probes were previously linked with myelination, glia fate determination and some of them even with dysfunctions in SCZ. We can easily observe similarities in the expression patterns. For instance *Qki2* could be co-localized with *krox20* in 12 to 24 hpf embryos as we observe signal in rhombomere, while *mbp* can be used in 3 dpf

embryos as the signal is appearing in lateral line Schwann cells and oligodendrocytes. *Krox20* controls Schwann cell proliferation and death, as well as influencing hindbrain-craniofacial developmental interactions. *Mbp* has an important role in the formation of myelin sheath by oligodendrocyte in CNS and Schwann cells in PNS. *Olig2* could be used for localization of *qkib* as in both we observe signal in forebrain and hindbrain, including developing cerebellum and weakly expressed in the spinal cord oligodendrocyte precursors. Olig2 has a role in oligodendrocyte fate specification and controls cell proliferation and differentiation. Possible co-localization of *qki2* with *krox20* or *mbp* and *qkib* with *olig2* will give us a hint of its possible function. Still it is difficult to compare results of two in situ hybridization. For this reason double in situ hybridization will give us more precise details of the spatial expression. Even then, we could only assume a potential role of quaking in myelination or glia cells determination, as many post-transcription or post-translation modifications can be occurred. Only after knocking down *qki* we could know the exact function of them. Nevertheless, *qki2* and *qkib* are mainly expressed in nervous system, making them good targets for functional studies aiming to further our understanding of neurodevelopmental basis of schizophrenia.

Future prospective

In a next level, co-localization of glia markers and quaking genes by double in situ hybridization will reveal more information about the spatial localization of quaking. Moreover, morpholinos experiments are necessary, to show the phenotype of the genes after silencing, as well as rescue experiments are necessary controls. Furthermore, behavior studies will reveal the role of quaking in the fish, and consequently in hums, during the early development. Finally, phylogenetic analysis will reveal information about the evolution of quaking, which may help us understand possible differences in the function between the species.

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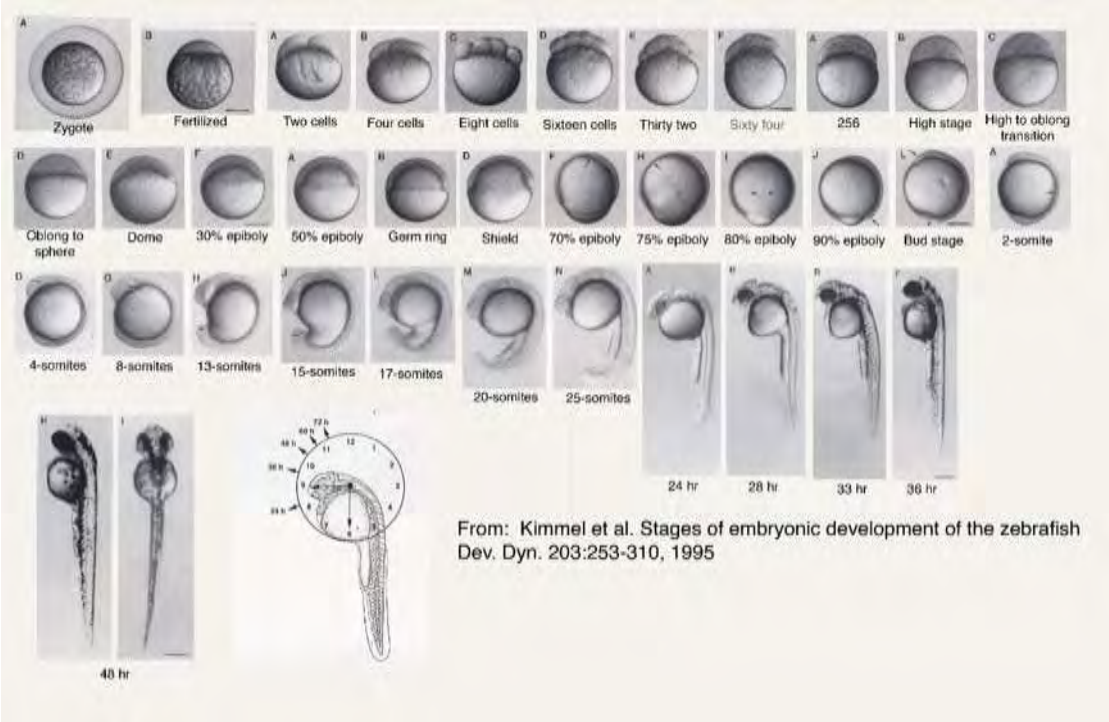
Complimentary files

1. zebrafish developmental stages

(http://hutsonlab.org/resources.htm)



2. Zebrafish Developmental Staging Series

Period	**Stage**	**Begins**	**Developmental Landmarks**
Zygote (0 - 0.75 h)	1-cell	0.00 h	Cytoplasm streams toward animal pole to form blastodisc
Cleavage (0.75 - 2.25 h)	2-cell	0.75 h	Partial cleavage
	4-cell	1.00 h	2 X 2 array of blastomeres
	8-cell	1.25 h	2 X 4 array of blastomeres

	16-cell	1.50 h	4 X 4 array of blastomeres
	32-cell	1.75 h	4 X 8 array of blastomeres
	64-cell	2.00 h	3 regular tiers of blastomeres
Blastula (2.25 - 5.25 h)	128-cell	2.25 h	5 blastomere tiers; cleavage planes irregular
	256-cell	2.50 h	7 blastomere tiers
	512-cell	2.75 h	9 blastomere tiers; YSL forms
	1k-cell	3.00 h	11 blastomere tiers; single row of YSL nuclei; asynchronous cell cycle
	High	3.33 h	> 11 blastomere tiers; blastodisc flattening begins; YSL nuclei in two rows
	Oblong	3.66 h	Blastodisc flattening; multiple rows of YSL nuclei
	Sphere	4.00 h	Spherical shape; flat border between blastodisc and yolk
	Dome	4.33 h	Yolk cell bulging toward animal pole as epiboly begins
	30%-epiboly	4.66 h	Blastoderm an inverted cup of uniform thickness
Gastrula (5.25 - 10.33 h)	50%-epiboly	5.25 h	Blastoderm remains of uniform thickness
	Germ-ring	5.66 h	Germ ring visible from animal pole; 50%-epiboly
	Shield	6.00 h	Embryonic shield visible from animal pole; 50%-epiboly
	75%-epiboly	8.00 h	Dorsal side distinctly thicker; epiblast, hypoblast, evacuation zone visible
	90%-epiboly	9.00 h	Axis and neural plate; brain and notochord rudiments
	Bud	10.00 h	Tail bud prominent; early polster; 100%-epiboly
Segmentation (10.33 - 24 h)	1-4 somites	10.33 h	First somite furrow
	5-9 somites	11.66 h	Polster prominent; optic vesicle, Kupffer's vesicle, neural keel
	10-13 somites	14 h	Pronephros forms
	14-19 somites	16 h	EL (embryo length) = 0.9 mm; otic placode, brain neuromeres
	20-25 somites	19 h	EL = 1.4 mm; lens, otic vesicle, hindbrain neuromeres
	26+ somites	22 h	EL = 1.6 mm; blood islands, otoliths, midbrain-hindbrain boundary
Pharyngula	Prim-5	24 h	EL = 1.9 mm; early pigmentation, heartbeat

(24 - 48 h)	Prim-15	30 h	EL = 2.5 mm; early touch reflex, retina pigmented
	Prim-25	36 h	EL = 2.7 mm; early motility, tail pigmentation
	High-pec	42 h	EL = 2.9 mm; rudiments of pectoral fins
Hatching (48 - 72 h)	Long-pec	48 h	EL = 3.1 mm; elongated pectoral fin buds
	Pec-fin	60 h	EL = 3.3 mm; pectoral fin blades
Larval	Protruding-mouth	72 h	3.5 mm total body length
	Day 4	96 h	3.7 mm total body length
	Day 5	120 h	3.9 mm total body length; 6 teeth
	Day 6	144 h	4.2 mm total body length
	Days 7-13	168 h	4.5 mm total body length; 8 teeth
	Days 14-20	14 d	6.2 mm total body length; 10 teeth
	Days 21-29	21 d	7.8 mm total body length
Juvenile	Days 30-44	30 d	10 mm total body length; adult fins/pigment
	Days 45-89	45 d	14 mm total body length; 12 teeth
Adult (90 d - 2 y)		90 d	Breeding adult