Functions of P38 and ERK kinases in zebrafish early development

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CHAPTER III

Characterization and expression analysis of two novel zebrafish P38 isoforms

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Abstract

The P38 MAPK subfamily consists of four isoforms (P38α, P38β, P38γ, and P38δ) that play a role in proliferation, differentiation and stress responses. P38 signaling is found to be deregulated in pathologies like Alzheimer, cancer, rheumatoid arthritis and asthma.

The Zebrafish orthologs of most MAPK genes have recently been identified, however two isoforms of the p38 family remained to be detected. In this study, two novel zebrafish p38 (β, δ) cDNAs were cloned, which translated into 361 and 362 amino acid proteins, respectively, with approximately 56% identity. Comparison of the amino acid sequences of all zebrafish P38 MAPKs by multiple sequence alignment indicated conservation of the dual phosphorylation site TXY motif, DFG site, ED site and CD domain in all zebrafish P38 isoforms. The catalytic region, with the glycine-rich loop, hinge region and α-Helix C, is more than 83% identical between the P38α and P38β isoforms. Mutation prone residues are mainly localized at the MAP Kinase insert and α-Helix G. Semi-quantitative reverse transcriptase-PCR revealed an ubiquitous expression pattern of zebrafish p38β throughout embryogenesis, whereas p38δ levels were variable. Unlike p38δ, p38β was maternally expressed and both genes were expressed in the adult zebrafish. To localize the expression whole mount in situ hybridization was performed from 1- to 5- dpf. p38β mRNA was detectable in the heart, lens, notochord and the telencephalon, diencephalon, presumptive forebrain ventricle, the midbrain tegmentum, mid-hindbrain boundary and rhombencephalon of the brain. P38δ was aslo expressed in the notochord, the telencephalon, diencephalon, midbrain tegmentum and rhombomeres of the hindbrain. In addition p38β mRNA was concentrated in the chondrocranium, pectoral fins, neural retina, the gut and at somite boundaries.

Phylogenetic analysis revealed that zebrafish p38 isoforms cluster with their corresponding orthologues in human, mice, rat, *Xenopus* and medaka. Like in mammals, the zebrafish P38α and P38β are the most similar isoforms.

Altogether, this study reports the identification of two novel zebrafish P38 isoforms and the analysis of their specific spatial and temporal expression patterns during early zebrafish development.

Introduction

The four mammalian P38 MAPKs, α(MAPK14), β(MAPK11), γ(MAPK12) and δ(MAPK13) are representative MAPKs of one of the three main subfamilies of the Mitogen Activated Protein Kinases (MAPK). The other two subfamilies are the extracellular-signal regulated protein kinases (ERK) and the c-Jun amino terminal kinases (JNK). MAPK family is conserved in evolution through the plant and animal kingdom and constitutes one of the major eukaryotic signalling families that transmit extracellular signals from the cell-surface to the nucleus. They contribute to the ability of cells to receive and respond to environmental cues. The repertoire of their substrates includes cytoskeleton proteins as well as transcription factors controlling global gene expression (Johnson, Lapadat, 2002; Kim, Choi, 2010; Roux, Blenis, 2004). These proteins have been implicated in numerous cellular functions including cell growth, migration, proliferation, differentiation, survival and development, and mis-regulated MAPK signaling has been engrossed in many human diseases.

The three subfamilies are distinguished according to the middle amino-acid residue of the conserved Thr-Xxx-Tyr (TXY) dual-phosphorylation motif with TGY being specific for the P38 subfamily. Phosphorylation of the TXY motif activates MAPKs and occurs by upstream MAPKK which in turn are activated by MAPKKK. MAPKK undergo a highly selective interaction with specific MAPKs which could be explained by structural differences of the activation loop containing the TXY motif. MAPKK3 and MAPKK6 interact specifically with the P38 subfamily whereas MAPKK4 binds to the P38α isoform and to JNK MAPKs facilitating crosstalk between these two subfamilies (Zarubin, Han, 2005; Cuadrado, Nebreda, 2010).

The P38 family was initially associated with stress responses since the first identified isoform Hog1/P38α in yeast was rapidly phosphorylated after LPS stimulation or hyper osmotic shock. Additionally, P38α activation in higher eukaryotes is stimulated by stress factors such as heat, pro-inflammatory cytokines, UV irradiation etc (Fearns et al. 2000). In response to these stimuli P38α regulates cellular protective mechanisms including the biosynthesis of pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNFα, COX-2 and IFN-γ) and inhibits the anti-inflammatory and immunosuppressive activities of the glucocorticoid receptor (Lee et al. 1994; Wang, 2004). In addition, the P53 tumor supressor is among the P38α substrates providing a link with the cell cycle checkpoints (Thornton, Rincon, 2009). The P38 subfamily is often found to play a key role in the pathogenesis of disorders such as cancer, rheumatoid arthritis, asthma and Alzheimer, which are a consequence of aberrant functioning of cell cycle progression and immune or inflammatory responses (Coulthard et al. 2009; Cuenda, Rousseau, 2007; Chung, 2011; Munoz, Ammit, 2010) .

Many studies have been performed using the pyridinyl imidazole inhibitors SB203580 and SB202190 which inhibit P38α and β equally, with no significant effect on γ and δ, therefore knowledge on the functional distinction between these two isoforms is elusive. The essential role of the P38α isoform in development was demonstrated in knock-out mice resulting in homozygous embryonic lethality caused by severe defects in placental angiogenesis (Mudgett et al. 2000). In contrast, mice lacking P38β, P38γ and P38δ were viable, fertile and did not show any obvious phenotypes.

Although all P38 isoforms are activated by environmental stress and pro-inflammatory cytokines, P38α is suggested to be the main isoform regulating cytokine production. Mice lacking p38β showed no defects in the cytokine production or immune function after LPS stimulation or after crossing with a mouse line ectopically expressing the systemic cytokine TNFα (Beardmore et al. 2005). Nevertheless a specific metabolic role of P38β has been reported in the regulation of the mTORC1 pathway during energy stress involving intracellular ATP depletion (Kalender et al. 2011). Antagonistic roles of the P38 isoforms were demonstrated, for example P38δ has an opposing role in P38α induced activation of the osmoprotective transcription factor TonEBP/OREBP in HEK293 cells treated with high concentrations of NaCl (Zhou et al. 2008; Pramanik et al. 2003). In addition, P38δ plays a specific role in the regulation of skin homeostasis and tumorigenesis (Eckert et al. 2003; Schindler et al. 2009).

As previously described (Krens et al. 2006a), the zebrafish genome encodes for members of all MAPK subfamily: ERK, JNK and p38 and the functions of ERK1/2 and JNK in embryogenesis are intensively studied (Krens et al. 2006b). However, only a few reports have investigated the role of p38 family in zebrafish. Ectopic expression by injection of a dominant negative (DN) form of p38α indicated that asymmetric distribution of p38α activation contributes to symmetric and synchronous cell cleavage (Fujii et al. 2000). Furthermore, DN p38α phenocopies the *betty boop* (MAPKAPK2) mutant phenotype by inducing premature constriction of the blastoderm margin, in a YSL specific manner in zebrafish pre-gastulation embryos (Holloway et al.2009).

In this study we report the cloning and characterization of novel members of the p38 subfamily p38β and p38δ and describe their spatial and temporal expression patterns during early zebrafish development.

Material & Methods

Zebrafish husbandry

Zebrafish (Danio rerio) wild type line was maintained under standard conditions and guidelines given in the zebrafish book (Westerfield 200). Embryos were kept at 28.5°C and staged in hours post fertilization (hpf) according to (Kimmel et al. 1995).

3.2 cDNA synthesis and Cloning of p38 β/δ genes

Total RNA was isolated from adult Teubingen zebrafish, using Trizol Reagent protocol (GIBCOBRL, Life technologies™). cDNA synthesis was performed with iScript™ cDNA Synthesis Kit (Bio-Rad) and was amplified with Phusion® High-Fidelity DNA Polymerase and gene specific primers of P38β fw: 5' CGGAAAACATGGCGACAAGAC 3' rv: 5' TGCGCTCAGTTTTATTGTTCAACC 3' and p38δ fw: 5' CTAAATGGAGTCTCGGGTGG 3' rv: CTAGGACATGGTGAGTGAGTGTGTT. Subsequently the full CDS flanking sequences were cloned into pCR-BluntII-TOPO (Invitrogen) and transformed in one shot Top10 chemically competent E.coli. Several clones were verified with restriction analysis and DNA sequencing.

3.3 Sequence alignments and phylogenetic analysis

Phylogenetic analysis was performed based on multiple alignments of p38 MAPKs from different species; human (h), rat (r), mouse (m), Xenopus (x), zebrafish (z) and medaka fish (med). ENSEMBLE protein codes: hp38α: ENSP00000229795 hp38β: ENSP00000333685 hp38γ: ENSP00000215659 hp38δ: ENSP00000211287 mp38α:ENSMUSP00000004990 mp38β:ENSMUSP00000132439 mp38γ:ENSMUSP00000086207 mp38δ: ENSMUSP00000004986 rp38α:ENSRNOP00000000617 rp38β: ENSRNOP00000009325 rp38γ: ENSRNOP00000046455 rp38δ: ENSRNOP00000000621 xp38α: ENSXETP00000000926 xp38β: ENSXETP00000048165 xp38γ: ENSXETP00000048167 xp38δ: ENSXETP00000000923 medP38α.1: ENSORLP00000008135 medP38α.2: ENSORLP00000020176 medp38β: ENSORLP00000011217 medp38γ.1: ENSORLP00000020405 medp38γ.2 ENSORLP00000011305 medp38δ: ENSORLP00000000486 zp38αA: ENSDARP00000040361 zp38γ: ENSDARP00000011298 zP38αb.1 ENSDARP00000035686, zP38αb.2 ENSDARP00000040146

RNA isolation and reverse transcriptase PCR

Total RNA was extracted from embryos at different stages of development using Trizol reagent (Invitrogen) phenol-chloroform and phase-lock gel. RNA quality control was performed by agarose gel electrophoresis. Expression patterns during development of P38β and P38δ were determined by RT-PCR with SuperScript™ III

One-Step RT-PCR System with Platinum® Taq DNA polymerase (Invitrogen) and the full CDS-flanking primers used for cloning. β-actin was used as a control fw 5' ggcacccagcacaatgaagat 3' rv 5' aagtcatagtccgcctagaagcat 3'.

In situ hybridization

Anti-sense digoxigenin labeled RNA probes were synthesized from the full length cDNA cloned in pCR-BluntII-TOPO. The constructs were linearized and antisense probes were synthesized using the DIG RNA Labeling Kit (Roche) and T7, sp6 RNA polymerase (Maxiscript kit, Ambion) according to the orientation of the gene in the TOPO construct. Zebrafish embryos were harvested at 24 and 48hpf, dechorionated and fixed overnight in 4% paraformaldehyde in PBS at 4°C. In situ hybridization was performed as described previously (Westerfield 200).

Results & Discussion

Cloning of zebrafish p38β and p38δ

To obtain the cDNA of p38β and p38δ, gene specific primers were designed based on the ensemble predictions zgc:86905 and im:7136778 (Zv8), respectively (Table 1). Several clones were sequenced for both p38β and p38δ. One single p38δ transcript was obtained of 1089bp encoding for a 362 amino acid (a.a.) protein with a 99.4% identical sequence to im: 7136778. Since there was only one transcript found, the splicing of p38δ RNA is most likely to occur uniformly. In contrast, three P38β transcripts were obtained of which two, with a size of 1086bp and 1087bp, are mostly identical. The full protein (361a.a) is translated from the 1086bp transcript. The second transcript contains a single nucleotide insertion that changes the reading frame and results in an early truncation of the protein. The third transcript of 1263bp contains a retained intron of 170bp with an early stop codon, resulting in a truncated protein. The obtained clones were sequenced and the complete coding sequences of the newly identified transcripts p38β.2(1087bp) and p38β.3(1263bp) were submitted to GenBank: JQ513868 and JQ513869 respectively.

Multiple sequence alignment of zebrafish P38 isoforms

After the cloning of the zebrafish P38β and P38δ all isoforms were compared in a multiple sequence alignment (Fig1). Previously, the crystal structures of the human orthologues have been resolved and most functional domains are identified (Wilson et al.1996; Patel et al. 2009). Functional differences between the different P38 isoforms have been assigned to specific amino acids. Comparison of the sequences to other species revealed that these domains and residues are preserved in the analogous positions in zebrafish P38 MAPKs. The comparison of the primary

structure between isoforms, the dual phosphorylation site, catalytic site and protein-protein interaction sites are indicated in the alignment (Fig1).
As observed in most kinases, the N-terminal and C-terminal regions of the protein

Name	Synonyms	LG	Transcripts	Ensemble	Size CDS (bp)	Exons	Size (a.a)
P38αa	MAPK14a SAPK2A	8	1	ENSDART00000040362	1086	12	361
P38αb	MAPK14b SAPK2A	11	1	ENSDART00000030921	1086	12	361
			2	ENSDART00000132768	1047	11	348
			3	ENSDART00000040147	1086	12	361
P38β	MAPK11 PRKM11 SAPK2 zgc:86905	4	1**	ENSDART00000032857	1086	12	361
			2*	_	1087	12	_
			3*	_	1263	13	_
P38γ	MAPK12 ERK6 SAPK3 zgc:101695	18	1	ENSDART00000018502	1092	12	363
P38δ	MAPK13 im:7136778	8	1**	ENSDART00000081341	1089	12	362

Table 1. The zebrafish contains all isoforms of the P38 subfamily of MAPKs.
We confirmed the presence of the first P38β transcript and identified novel alternative transcripts coding for truncated proteins. No additional transcripts of P38δ have been identified. P38αA and P38αB are duplicated genes found in zebrafish. The linkage group corresponds with chromosome number, based on the Ensemble website.* Sequences submitted to Genbank: mapk11_2 JQ513868 and mapk11_3 JQ513869. ** Full coding transcripts of P38β and P38δ.

are folded into two small lobes and the catalytic domain is situated at the interface of these two lobes (Fig1B). The dual phosphorylation motif (TXY) is located on the activation lip at the C-terminal lobe. The position of the activation lip changes upon phosphorylation and induces additional conformational changes of mostly hydrogen interactions between residues. The N' and C' lobe rotate 5º towards each other (P38α) aligning the catalytic residues in the hinge region, glycine-rich loop and α-Helix C. The phenylalanine of the DFG motif, which sterically interferes with ATP binding in the non –phosporylated form, changes its position, thereby enabling the MAPK to become fully active (Zhang et al. 2011).

The dual phosphorylation motif and DFG site are conserved between all zebrafish P38 isoforms and are located in a highly conserved region, as indicated by the use of a blue font color (Fig1A). The existing amino acid differences in the glycine-rich loop, hinge region and α-Helix C are often found between P38α/β and P38γ/δ isoforms. The catalytic site is more than 80% conserved between the P38α and

P38β isoforms. SB203580 and SB202190 inhibitors target the catalytic site and are known to be selective for P38α/β this is likely to be similar in zebrafish. The 175th a.a (histidine in zP38α) is the only highly variable residue in the region of the catalytic and activation domains. However, a possible contribution of this particular residue to the conformation of the activation lip or to the kinase activity is not reported yet. The D177L substitution, two residues upstream, is found to increase the activity of all isoforms except P38δ (Diskin et al. 2007).

The two allosteric docking sites, CD domain and ED site have been found to be important for binding of activators, inactivating phosphatases and substrates (Tanoue et al. 2001). In the folded structure these sites are in close proximity and are part of a hydrophobic docking groove that is recognised by motifs on interacting proteins. Both domains consist predominantly of acidic amino acids and are conserved in zebrafish P38 MAPKs.

In mammals, two MEKK independent mechanisms are known to specifically activate P38α. P38α is capable of auto-activation upon binding with the TGF-β-activated protein kinase 1-binding protein 1 (TAB1) or after phosphorylation of Tyr323 by Zeta-chain-associated protein kinase 70 (ZAP-70), a kinase that is thought to occur specifically in antigen receptor stimulated lymphocytes and natural killer cells (Cuadrado, Nebreda, 2010). TAB1 interacts with several residues in the C-terminal domain inside as well as outside the allosteric docking sites (Ile117, Gln121, Thr219 and Ile276 in zP38α) (Zhou et al. 2006). In zebrafish, TAB1 interacting residues are also conserved in P38α. In contrast, Tyr323 is replaced by phenylalanine in zebrafish P38αA, but is present in P38αB isoform and in other P38 isoforms. Tyr323 is located in loop16 which spans both the C' lobe and the N' lobe. Recently identified activating mutations reveal the importance of this loop for the kinase activity since mutations of phe328 render all isoforms active, except P38δ (Diskin et al. 2007). The F325S substitution increases the activity of P38δ however this residue is not conserved in zebrafish.

The least conserved protein region includes the MAPK insertion, α-Helix G and the L16 loop. The MAPK insertion (MKI), a feature characteristic for MAPKs and cyclin-dependant kinase 2 (CDK2) related kinases, is located at the C'-lobe and consist of two perpendicular positioned α-helixes connected by a short loop. The function of the MKI domain is possibly involved in lipid binding (Diskin et al. 2008).

<u>P38β and P38δ expression during zebrafish development</u>

To determine the expression dynamics of p38β and p38δ genes through the course of zebrafish development, we performed semi-quantitative RT-PCR analysis on RNA isolated from 18 developmental stages (Fig2). The p38β is

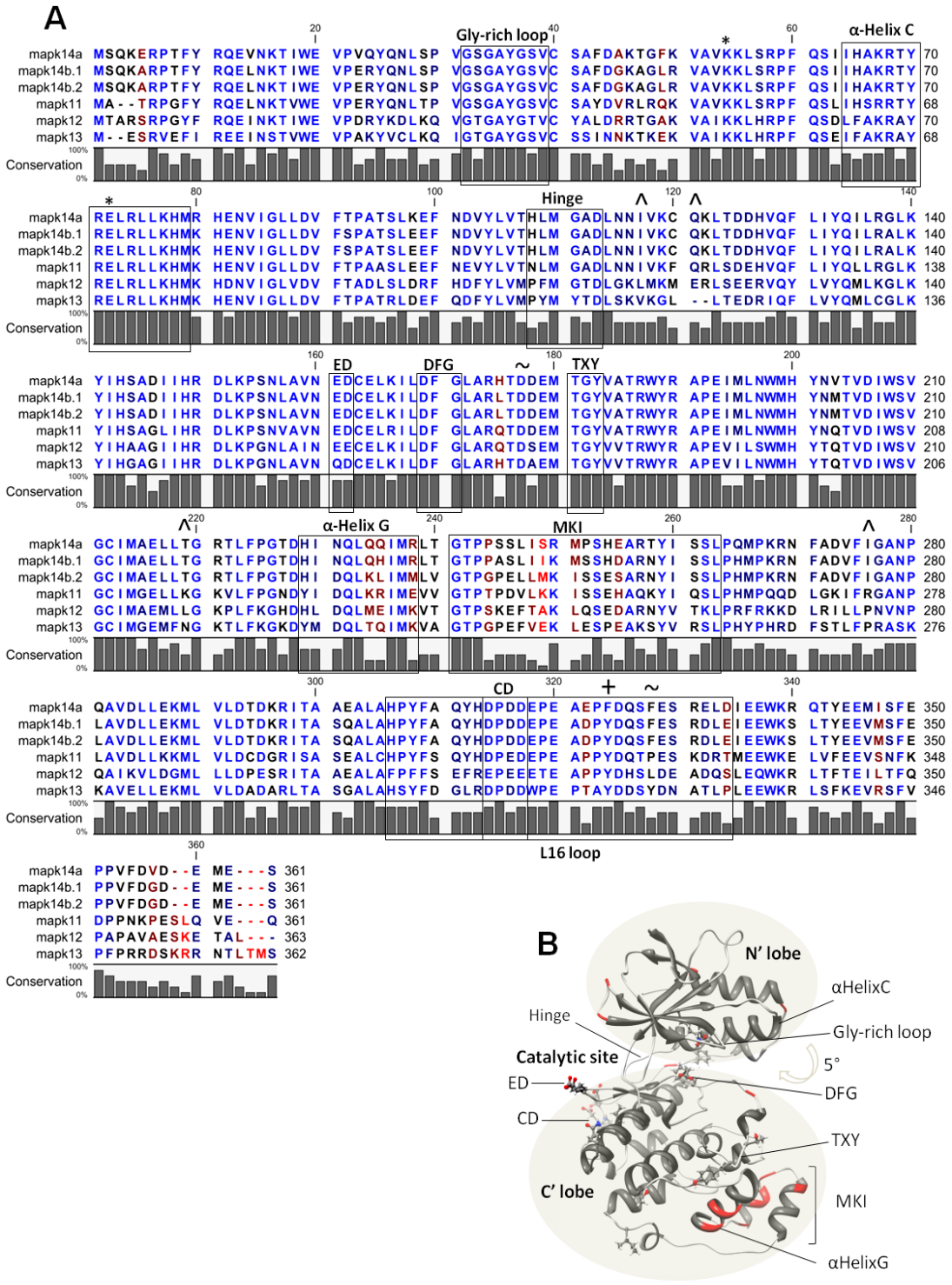
A
Gly-rich loop
α-Helix C
Hinge
ED
DFG
TXY
α-Helix G
MKI
CD
L16 loop
mapk14a
mapk14b.1
mapk14b.2
mapk11
mapk12
mapk13
Conservation
B
N' lobe
αHelixC
Hinge
Gly-rich loop
Catalytic site
5°
ED
DFG
CD
TXY
C' lobe
MKI
αHelixG

Figure 1. Multiple sequence alignment of zebrafish P38 isoforms. A) Amino acid sequence of zebrafish p38 MAPKs were aligned with CLC DNA Workbench 5.7.1. The color gradient marks the conservation: with red the most variable residue and blue highly conserved residues. Functional domains and motifs are boxed and important residues are indicated: * catalytic residues, ^ TAB1 interaction site, + ZAP70 phosphorylation site ~ catalytic activity increasing mutation sites. The same functional domains and residues are displayed on the homology model of zebrafish P38α to illustrate the position in the folded protein (B). Highly variable residues are indicated (red). To obtain the zebrafish P38α folded protein structure homology modeling was performed with Swiss-Model on the web server of the Swiss institute of bioinformatics http://www.isb-sib.ch and based on the crystal structure of the human orthologue of inactive P38α (PDB 1R39). Molecular graphics image were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081).

ubiquitously expressed from the zygote on, throughout zebrafish development and eventually in the adult zebrafish. In contrast, p38δ is not maternally deposited and shows a dynamic expression pattern, as it increases at early blastula stage (16 cells-sphere cells), decreases during gastrulation and early somitogenesis, and increases again until 48hpf, thereafter its expression becomes undetectable. The p38δ is expressed in the adult zebrafish.

Expression of the two p38α genes and p38γ during the same developmental stages was reported previously (Krens et al. 2006a). The p38αA and p38γ have the same ubiquitous expression as p38β while p38αB expression peaks from 16 cells to 30% epiboly.

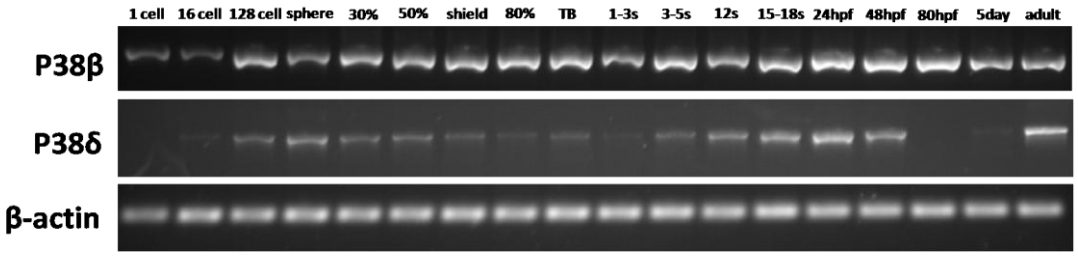

Figure 2. RT-PCR of P38β and P38δ during zebrafish development. Gene specific primers were designed flanking the full coding sequences and used for RT-PCR analysis of p38β and p38δ expression from cleavage until hatching stages. RNA isolated from the zygote and adult tissue was used to determine possible maternal expression and expression in mature adult zebrafish, respectively. β-actin expression was used as a control.

Whole mount in situ hybridization of p38β and p38δ at 24hpf and 48hpf

Whole mount in situ hybridization using antisense digoxigenin labeled RNA probes was performed to localize the expression of p38β (Fig 3) and p38δ (Fig 4) from 1 to 5 dpf. At 1dpf p38β is highly expressed in the in the heart, lens, notochord and specific parts of the brain. In the forebrain p38β is expressed in the dorsal rostral cluster of the telencephalon and in the ventral diencephalon. furthermore the dorsal view of the 1dpf p38β in situ shows an concentrated expression at two perpendicular anatomical lines in the forebrain one on the left-right axis and second one, connected to the first, at the posterior side of the eyes on the left-right axis. Since from 16hpf the forebrain ventricle start to appear at this position p38β could be expressed and perhaps functional in the formation in the presumptive forebrain ventricle. Furthermore p38β is expressed in the midbrain tegmentum of the mesencephalon and although expressed in the entire the rhombencephalon a highly divined expression can be distinguished located at the position of the upper rhombic

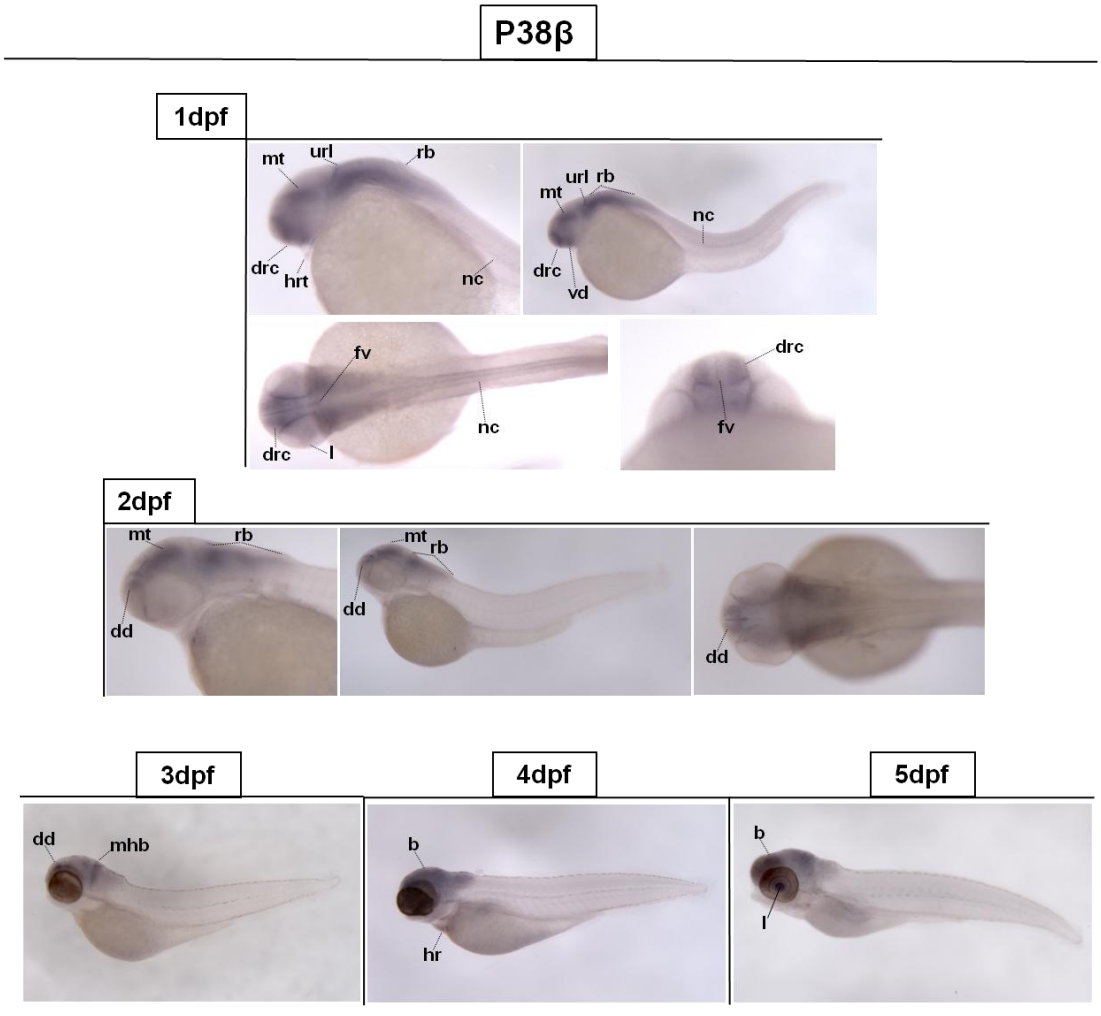

Figure 3. Whole mount in situ hybridisation showing p38β expression in wild-type zebrafish embryos. Presence of p38β RNA was detected in the brain, heart and lens. Antisense In situ probes of the full length coding sequences where used. **1dpf upper panel** Lateral view anterior to left dorsal to top in situ images. p38β expression is detected in the heart(hrt),ventral diencephalon(vd) dorsal rostral cluster of the telencephalon(drc), midbrain tegmentum(mt), the notochord(nc) the upper rhombic lip(url) and the rest of the rhombencephalon(rb)/hindbrain. **1dpf lower panel left** dorsal view anterior to left, p38β expression in the lens(l) **1dpf lower panel right** anterior view dorsal to top, show also expression of p38β in the presumptive forebrain ventricle(fv). **2dpf left and middle image** Lateral view anterior to left dorsal to top in situ **2dpf right image** dorsal view anterior to left. Expression in the dorsal diencephalon(dd), midbrain tegmentum and rhombencephalon(rb)**3,4,5dpf** Lateral view anterior to left dorsal to top. p38β expression in the dorsal diencephalon and mid-hindbrain boundary(mhb) **3dpf**, entire brain and heart **4dpf**, lens(l), telencephalon (te) and mesencephalon(me) **5dpf**.

lip. p38β continues to be expressed in the brain at 2,3,4 and 5 dpf. At 2dpf p38β expression is highest in the dorsal diencephalon, midbrain tegmentum and rhombencephalon. At 3dpf an specific expression is detected in the mid-hindbrain boundary and also dorsal diencephalon. 4 days old zf embryos show p38β expression in the entire brain and heart and at 5dpf a high p38β expression is found in the iris of the embryo.

Like most MAPK p38δ is also expressed predominantly in the brain of the developing zebrafish embryo. At 1dpf p38δ is expressed in the telencephalon and diencephalon of the forebrain and in the notochord. p38δ expression seems to concentrate more specifically in the telencephalon-diencephalon boundary and the mid-hindbrain boundary of the zebrafish brain at 2dpf. In addition, p38δ expression is also detected in the chondrocranium, pectoral fins and pharyngeal pouches. In the tail region p38δ is expressed in the somite boundaries which could possibly be the growing intersegmental veins which appear between 48- and 60 hpf. P38δ expression at the somite boundaries is also detected in 3dpf embryos. furthermore, p38δ is expressed in 3dpf embryos in the dorsal diencephalon, midbrain tegmentum, rhombomeres of the hindbrain, chondrocranium, pharyngeal arches and pectoral fins. At 3dpf p38δ expression is detecteble in the developing gut and this expression will continue to be detected at 4- and 5dpf. At 4 and 5 dpf expression of p38δ in the rhombomeres of the hindbrain and the developed pharyngeal arches becomes more evident.

Expression of the zebrafish p38α and p38γ isoforms have been described previously (Krens et al. 2006a). P38αA is expressed in the tegmentum, hindbrain, otic vesicle, pronephric duct and tail muscle at 24hpf and also in the brachial arches, cerebellum and gut at 48hpf. The p38γ is expressed in the brain at 24hpf and 48hpf. It also shows an expression at the somite boundaries at 24hpf.

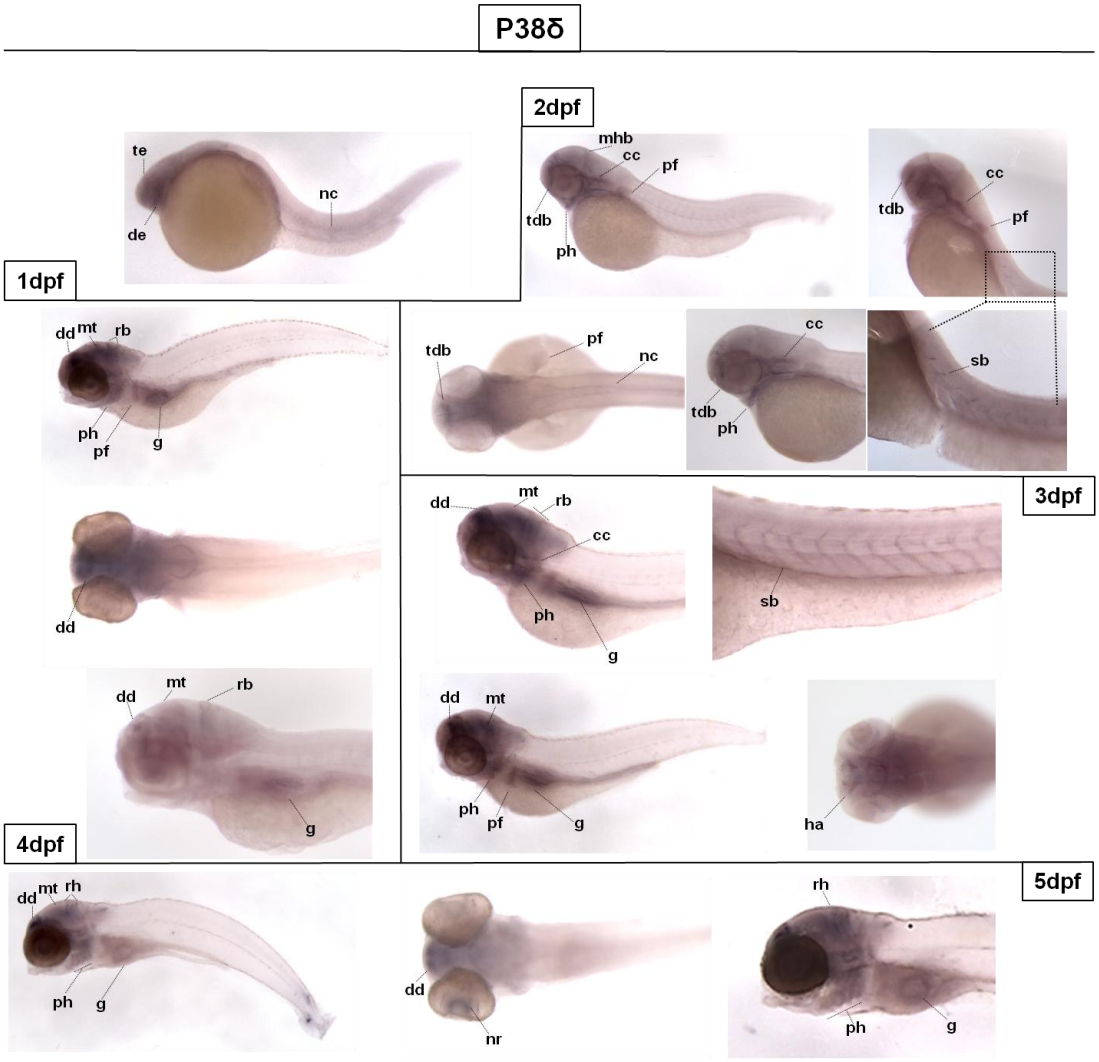

Figure 4. Whole mount in situ hybridisation showing p38δ expression in wild-type zebrafish embryos. 1dpf Lateral view anterior to left dorsal to top in situ image. expression in the telencephalon(te), diencephalon(de) and notochord(nc) **2dpf upper panel left** Lateral view anterior to left dorsal to top in situ image. p38δ expression in the pharyngeal pouches(ph), telencephalon-diencephalon boundary (tdb), mid-hindbrain boundary(mhb), chondrocranium(cc) and pectoral fins (pf). **2dpf upper panel right** Lateral view anterior to top enlargement of the anterior tail region is shown at the **2dpf lower panel right** Lateral view anterior to left dorsal to top, expression at somite boundaries (sb). **2dpf lower panel middle image** Lateral view anterior to left dorsal to top head and trunk region **2dpf lower panel left image** dorsal view anterior to left, shows p38δ expression in the notochord(nc). **3dpf upper panel left** and **3dpf lower panel left** p38δ expression in the dorsal diencephalon(dd), midbrain tegmentum (mt), rhombomeres of the hindbrain (rb), chondrocranium, pharyngeal arches, pectoral fins and the developing gut. **3dpf upper panel right** image of the middle of the tail showing expression of p38δ at the somite boundaries. **3dpf lower panel right** expression in the habenulae(ha) of the diencephalon. **4dpf top and bottom image** p38δ expression in the gut, pectoral fins, pharyngeal arches, dorsal diencephalon, midbrain tegmentum and rhombencephalon. **4dpf middle image** dorsal view anterior to left showing high expression of p38δ in the dorsal diencephalon. **5dpf left and right image** expression in the gut, pharyngeal arches, dorsal diencephalon, midbrain tegmentum and rhombomeres. **5dpf middle image** p38δ expression strongest in the dorsal diencephalon and neural retina (nr).

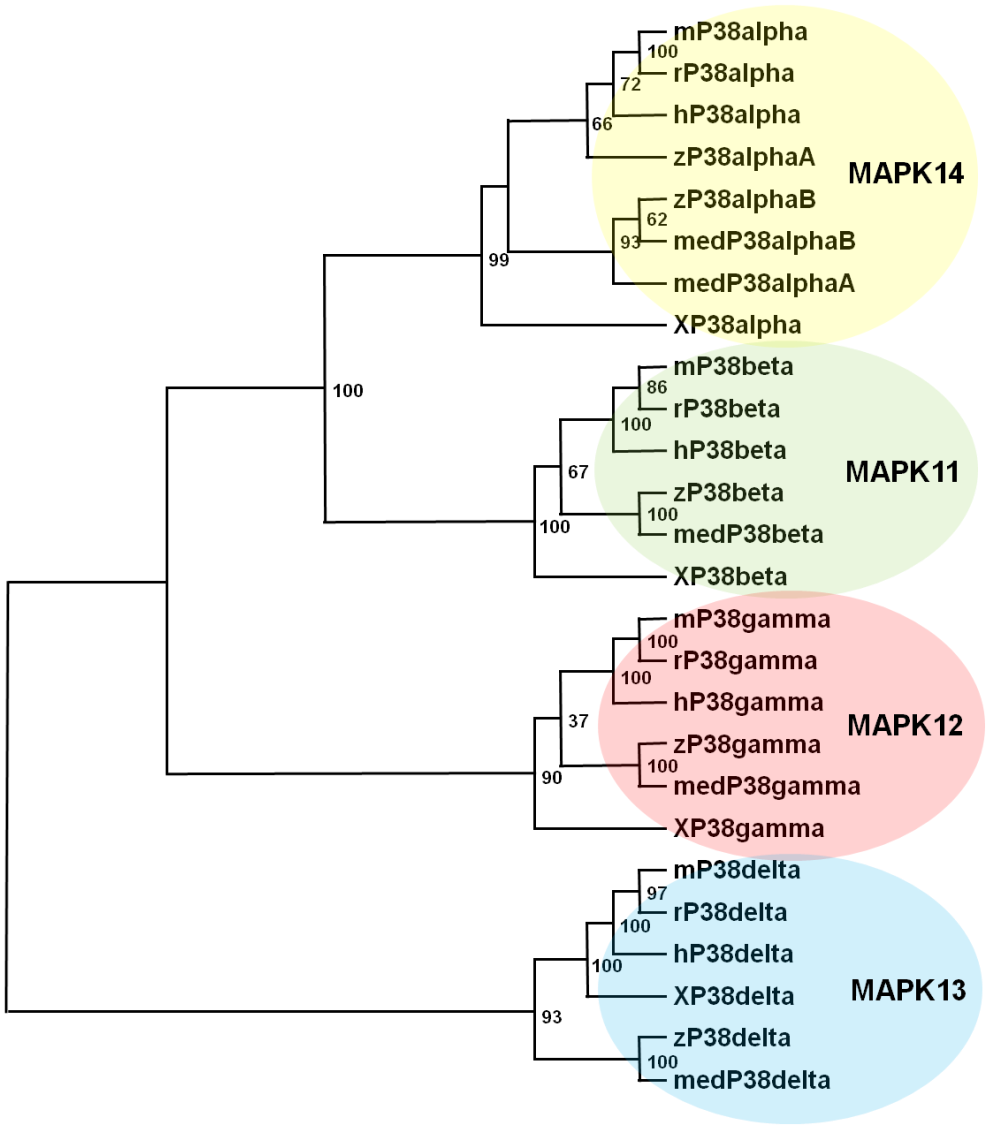

Figure 5. Unrooted phylogenetic tree of the zebrafish to other vertebrates P38 proteins. The phylogenetic tree was created with the ClustalW software from the DNA Data Bank of Japan(DDBJ) and the image was obtained using Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Amino acid sequences of full length sequence predictions (ENSEMBLE) and sequencing verified coding sequences were uploaded for multiple alignment and subsequent neighbor joining analysis using default settings. The numbers indicate the occurrence of nodes during bootstrap analysis. The p-distance values are given of 1000 reiterations. Human (h), rat, (r), Mouse (m), Xenopus tropicalis (x), Zebrafish (z) and Medaka fish (med).

Phylogenetic analysis of P38 MAPKs

The unrooted phylogenetic tree based on the difference in amino acid sequences illustrates evolutionary conservation of the P38 MAPK isoforms between zebrafish, medaka, *Xenopus*, mice, rat and human(Fig5). The four zebrafish p38 isoforms cluster with their corresponding orthologues into separate branches. In addition, zebrafish and medaka are both teleost fish and the phylogenetic tree of P38 kinases shows their closely related origin since each isoform branch separates into three clusters of fish, mammalian and amphibian species.

Like in mammals, zebrafish P38α and P38β are the most similar isoforms with ~75% identity, and the catalytic domains share an even higher similarity (~80%). P38δ shows a slightly higher similarity to P38γ (61%) than when it is compared to the other isoforms: 60% with P38α and 57% P38β.

Acknowledgments

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