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## Characterization and expression patterns of the MAPK family in zebrafish

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### Abstract

The mitogen activated protein kinases (MAPK) family pathway is conserved in evolution through the plant and animal kingdoms. These proteins have been implicated in diverse cellular processes including cell growth, proliferation, differentiation, survival and development. In this study we annotated and cloned members of the zebrafish MAPK gene-family, containing the ERK, JNK and p38 subfamilies. Their sequences were compared to orthologs of other vertebrates (human, mouse and rat) and the temporal and spatial expression levels of the zebrafish *mapk* genes were determined during early zebrafish development. Semi-quantitative reverse transcriptase-PCR analysis revealed that most *mapk* genes are expressed throughout zebrafish development. *Erk2,3* and *p38a* were expressed at a constant level throughout zebrafish embryogenesis, whereas *erk1,4,5,6,7* and *p38b* showed specific temporal expression patterns. The spatial expression patterns were obtained by whole mount in situ hybridization at 24 h post fertilization (hpf) and 48 hpf embryos. The expression patterns were localized in specific regions at both stages and were tightly regulated during embryogenesis. For *p38b*, no staining was detected at 24 and 48 hpf. However, its expression was demonstrated at blastula-stage. Together, we identified the zebrafish orthologs of the zebrafish MAPK gene family and determined their specific spatial and temporal expression and distribution patterns during zebrafish embryogenesis.

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**Keywords:** BMK1; ERK1; ERK2; ERK3; ERK4; ERK5; ERK6; ERK7; JNK1; p38a; p38b; MAPK1; MAPK3; MAPK4; MAPK6; MAPK7; MAPK8; MAPK12; MAPK14a; MAPK14b; MAPK15; p42MAPK; p44MAPK; p63MAPK; p97MAPK; MAPK; SAPK; Expression patterns; Zebrafish

Cells recognize and respond to extra-cellular stimuli by engaging specific intracellular responses, like the signaling cascade that lead to the activation of Mitogen activated protein kinases (MAPK). All eukaryotic cells contain multiple MAPK pathways, which coordinately regulate diverse cellular activities such as: gene expression, metabolism, motility, proliferation and differentiation. MAPKs are also crucial factors for the balance between survival and apoptosis. (Marshall, 1995; Ballif and Blenis, 2001) The MAPK signaling module is composed of three evolutionary conserved sequentially acting kinases: MAPK kinase kinase (MKKK), MAPK kinase (MKK) and MAPK (Widmann

et al., 1999). The MKKKs are activated through phosphorylation as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extra-cellular stimuli (Chang and Karin, 2001). MKKK activation leads to phosphorylation and activation of a MAPKK, which subsequently activates MAPK by a dual-phosphorylation event on threonine and tyrosine residues, separated by one amino acid (TxY consensus). Once activated, MAPKs translocate into the nucleus and phosphorylate specific target molecules on serine or threonine residues.

The MAPK family is divided in three subfamilies; extracellular signal-regulated kinases (ERK), c-Jun amino-terminal kinases (JNK) and the p38-subfamily. This subdivision is based on the consensus of the dual-phosphorylation site. The ERK-MAPK subfamily members contain a TEY activation domain, where JNKs have a TPY

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activation domain and p38-isoforms have a TGY activation domain (Johnson and Lapadat, 2002).

Currently, six different MAPK cascades are characterized in mammals: ERK1/2, JNK1/2/3, p38-isoforms  $\alpha/\beta/\gamma/\delta$ , ERK3/4, ERK5 and ERK7 (Bogoyevitch and Court, 2004; Johnson et al., 2005; Roux and Blenis, 2004; Abe et al., 1999).

MAPK play crucial roles in vertebrate embryogenesis, since gene-disruption of *erk2* (Saba-EI-Leil et al., 2003), *erk5* (Yan et al., 2003) and *p38 $\alpha$*  (Allen et al., 2000) are lethal during mouse development. *Erk1*  $-/-$  mice are viable, fertile and of normal size, but showed affected proliferation and maturation of the thymocytes (Gilles Pagès et al., 1999). Single *jnk* mutant mice survived normally, but mutants lacking *jnk1* and *jnk2* genes were embryonic lethal and showed severe affects in regulation of apoptosis in the brain (Kuan et al., 1999).

In this study we have identified, cloned and characterized the zebrafish MAPK gene family and determined their expression patterns during zebrafish development.

## 1. Results and discussion

### 1.1. Cloning of the zebrafish MAPK genes

The cDNA sequences of the zebrafish MAPK were predicted by performing blast searches with the human MAPK sequences against the zebrafish genome database of the Sanger Institute. The obtained hits were analyzed and Genscan-predictions were used to determine 5'-utr, intron-, exon- structures and 3'-utr. The obtained mRNA sequence predictions were used for a blast search against the human genome. Only one zebrafish *jnk1* ortholog and two isoforms of *p38 $\alpha$*  were found, in contrast to the human genome, that contains three *jnk* genes and four *p38* orthologs. Furthermore, to determine if the smaller number of *mapk* genes is specific for zebrafish, or is common to the other Teleost species *Fugu rubripes* and *Tetraodon nigroviridis*, genome blast searches were performed in these species. A second *jnk*-gene was predicted in the *Fugu* and *Tetraodon nigroviridis* genomes. The *Fugu rubripes* genome predicts an additional *p38 $\beta$*  gene. The results show that a smaller number of the *mapk* genes is apparently not specific for the zebrafish, but more general for other Teleost fish.

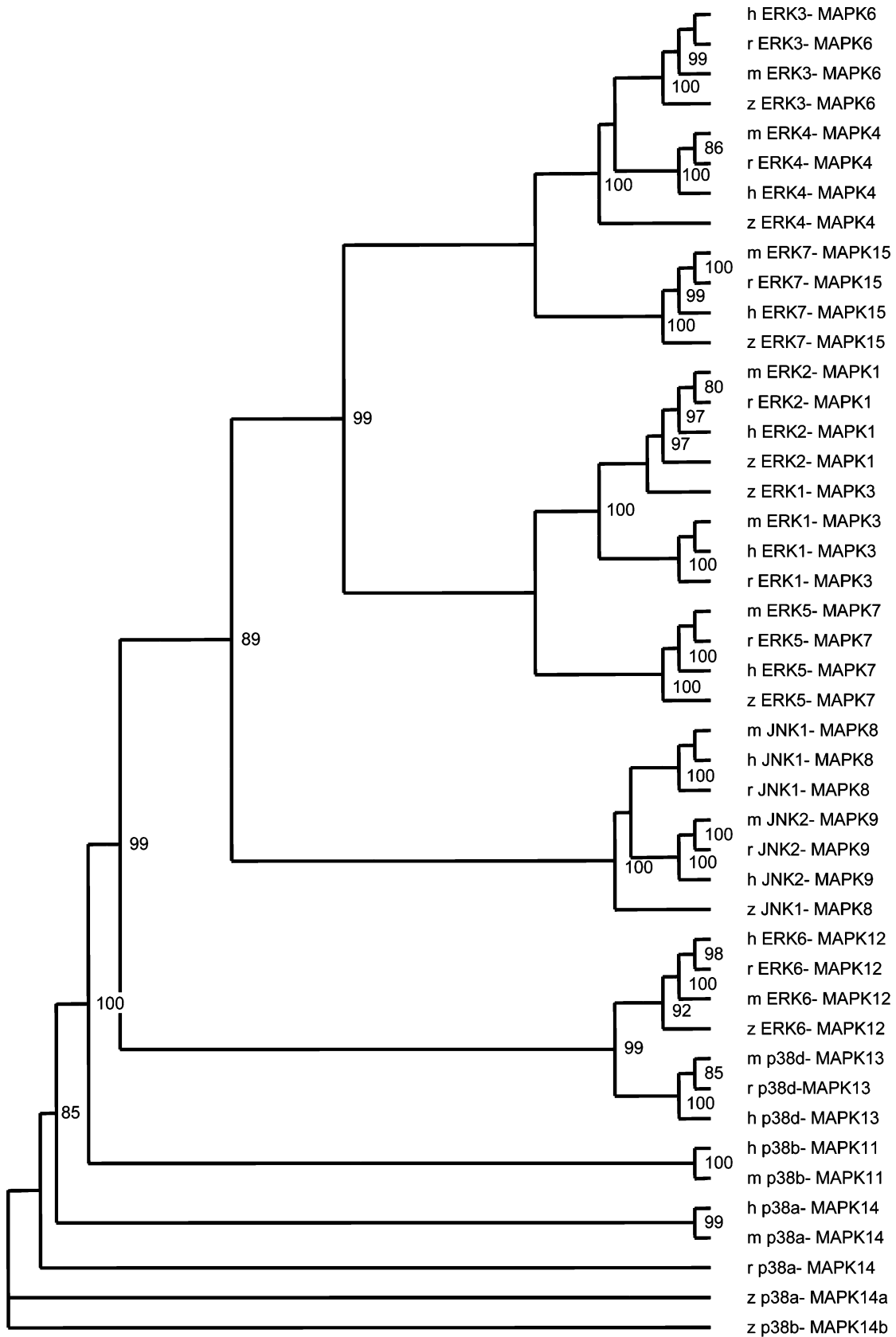
Based on the predicted sequences, gene specific primers were designed to obtain and clone the complete coding sequences. The obtained clones were sequenced and the predicted annotations were corrected. Complete coding sequences for the zebrafish MAPKs were submitted to GenBank: ERK3 (DQ360074), ERK4 (DQ360073), ERK5 (DQ360072), ERK6 (SAPK3) (DQ360071) and ERK7 (DQ360070). Zebrafish ERK1 (AY922319) and ERK2 (AY922320) were previously submitted by us (Krens et al., unpublished data). The zebrafish JNK1, p38a and p38b were not annotated to the genome, since ESTs of these genes containing full coding sequences, were already published; NM\_131721, AB030897 and AB030898, respectively (Fujii et al., 2000).

The derived amino acid sequences of the cloned and sequenced zebrafish MAPKs were used to construct an unrooted phylogenetic tree to determine the sequence similarities between all MAPK proteins identified in the zebrafish genome, compared to the human, rat and mouse orthologs (Fig. 1). All the zebrafish MAPKs cluster with mammalian orthologs, but show less identity and branch off earlier. Alignment of ERK1 and ERK2 to those of other vertebrates, showed that zebrafish ERK1 and ERK2 display approximately 80% and 90% identity to the respective subgroups of human, rat and mouse ERK1 and ERK2. The zebrafish and mammalian ERK2 proteins form a well-defined cluster. Zebrafish ERK1 forms an intermediate branch with low bootstrap value (lower than 67.9%) between the mammalian ERK1 and ERK2 groups. However, analyses of exon-intron patterns and chromosomal microsynteny show zebrafish *erk1* to be a genuine ortholog of the mammalian *erk1* family. With respect to microsynteny, zebrafish *erk1*, on chromosome 3 and human *erk1*, on chromosome 16, are surrounded by the same set of various corresponding orthologs, showing a strong evolutionary conservation of this area of the genome. This was also observed for zebrafish *erk2* on chromosome 5, compared to human *erk2* located on chromosome 22. Zebrafish JNK1 shows an identity to human, mouse or rat JNK1 of 87%; 77% and 80%, respectively. The identity of zebrafish JNK1 to JNK2 is 71% for the human, mouse and rat orthologs.

Both zebrafish p38a and p38b cluster with the p38 $\alpha$  cluster and showed an identity of approximately 86% and 84% compared to human, mouse and rat p38 $\alpha$ . The identity to p38 $\beta$  of zebrafish p38a and p38b were approximately 69% and 66%, respectively. This indicated that p38a and p38b are both p38 $\alpha$  isoforms. In rat also no ortholog for p38 $\beta$  (MAPK11) is found to date. The amino acid sequence alignment of the area around the dual-phosphorylation site also shows that all the zebrafish MAPKs contain the correct amino acid residue within the specific dual-phosphorylation site, designating them to the corresponding MAPK subfamily (Fig. 2). Although the ERK3 and ERK4 proteins contain an alternative dual-phosphorylation domain SEG, this domain still contains an intermediate glutamic acid (E) residue, designating them to the ERK MAPK subfamily. An overview of the MAPK names, classification, specific dual-phosphorylation consensus (TxY) in the genome, protein size and location on the zebrafish genome is summarized in Table 1.

### 1.2. Expression patterns of the MAPK genes during zebrafish development

The expression dynamics of all the *mapk* genes during zebrafish development was first examined by semi-quantitative RT-PCR analysis (Fig. 3). We confirmed that nearly all the predicted *mapk* genes were expressed in adult fish, except for *p38b*. During embryogenesis, the expression patterns showed different temporal regulation during the tested developmental stages. Maternal expression was



TRICHOTOMY

Fig. 1. Unrooted phylogenetic tree of the zebrafish MAPK family to other vertebrates. The tree was constructed by neighbor-joining analysis based on an alignment of the amino acid sequences of the complete predicted and sequencing verified coding sequences. The numbers indicate the occurrence of nodes during bootstrap analysis. The bootstrap values are given as percentages of 10,000 reiterations and only values above 80 are shown. h, human; m, mouse; r, rat; z, zebrafish.



Fig. 2. Amino acid alignment of the proteins-region around the characteristic MAPK dual-phosphorylation domain of the zebrafish MAPK orthologs. Identical residues are indicated with black boxes, strongly conserved in dark gray and similar residues are boxed light grey. The phosphorylated amino acids of the dual-phosphorylation site and the intermediate amino acid, designating the mapk to a subfamily, are indicated with ▼ and \*, respectively.

Table 1  
The zebrafish MAPK gene-family

Protein Name	Synonyms	Orthologs	Subfamily classification	Phosphorylation domain	Size (aa/kDa)	linkage group	exons
ERK1	MAPK3 p44-MAPK	<i>h_erk1</i> <i>m_erk1</i> <i>r_erk1</i>	ERK	TEY	392/44.1	LG:3	8
ERK2	MAPK1 p42-MAPK	<i>h_erk2</i> <i>m_erk2</i> <i>r_erk2</i>	ERK	TEY	369/42.1	LG:5	8
ERK3*	MAPK6 p97-MAPK p55MAPK (rat)	<i>h_erk3</i> <i>m_erk3</i> <i>r_erk3</i>	ERK	SEG	729/83.2	LG:18	6
ERK4*	MAPK4 p63-MAPK ERK3-related MNK2 (rat)	<i>h_erk4</i> <i>m_erk4</i> <i>r_erk4</i>	ERK	SEG	674/75.9	–	7
ERK5	MAPK7 BMK1	<i>h_erk5</i> <i>m_erk5</i> <i>r_erk5</i>	ERK	TEY	862/95.0	LG:12	9
ERK6	MAPK12 <i>p38-γ</i> SAPK3	<i>h_erk6</i> <i>m_erk6</i> <i>r_erk6</i>	p38	TGY	363/42.0	LG:18	12
ERK7	MAPK15	<i>h_erk7</i> <i>h_erk8</i> <i>m_erk7</i> <i>r_erk7</i>	ERK	TEY	534/60.2	LG:25	14
JNK1	MAPK8 SAPK1	<i>h_jnk1</i> <i>m_jnk1</i> <i>r_jnk1</i>	JNK/SAPK	TPY	384/44.1	LG:13	6
p38a	MAPK14a <i>p38-α</i> SAPK2A	<i>h_p38α</i> <i>m_p38α</i> <i>r_p38α</i>	p38/SAPK	TGY	361/41.6	LG:8	12
p38b	MAPK14b <i>p38-α</i> SAPK2A	<i>h_p38α</i> <i>m_p38α</i> <i>r_p38α</i>	p38/SAPK	TGY	348/39.9	–	–

The table represents an overview of the characterization on the zebrafish MAPK gene family. Their names, synonyms and orthologs (human (h), mouse (m) and rat (r)) and classification to specific subfamily are shown. The dual-phosphorylation sites, number of amino acids and molecular weight (kDa) are based on the translations of the coding sequence. ERK3 and ERK4 are indicated with an asterisk (\*), because of their distinct SEG dual-phosphorylation site. The linkage group corresponds with chromosome-number, based on blast analysis of the Sanger Institute zebrafish genome sequencing website (release Zv5). From these analyses the numbers of exons were also predicted.

detected for *mapk* genes, except for *p38b* and *erk7*. Constant expression levels were observed for *erk2*, *erk3*, and *p38a* throughout zebrafish development. The expression level for *erk1* was close to the detection limit at 1-cells stage

and *erk1*-expression in adult fish seem to be higher than during development. The temporal expression profile for *erk6* was almost constant as it showed a small decrease in expression early epiboly stages (30–50% epiboly) only. *Jnk1*

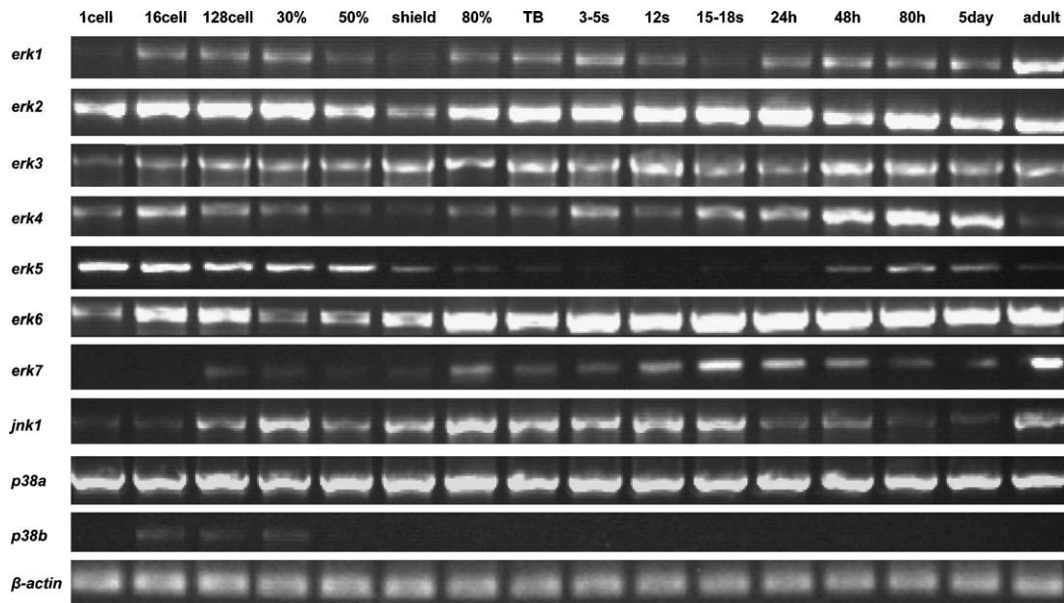


Fig. 3. Temporal expression patterns of zebrafish MAPKs by RT-PCR analysis through zebrafish development. RNAs used for amplification were isolated from indicated stages. To obtain specific expression patterns, primers flanking the full coding sequences were used, but for *erk7*, where primers specific for the distinct c-terminus of the *erk7* gene were used. *Erk4*, *erk5*, *erk7*, *jnk1* and *p38b* showed dynamic expression levels through zebrafish development compared to *erk1*, *erk2*, *erk3*, *erk6* and *p38a*.  $\beta$ -Actin ( $\beta$ -ACT) was used as a control for constitutive expression.

was weakly expressed in early developmental stages, but show higher expression at late gastrula-stages to segmentation (80% epiboly to 18 somites). From 24 to 5 dpf the expression-level of *jnk1* was low, and reached a high expression-level again in adult fish. *P38b* show a short and defined expression during blastula stage. After this, expression diminishes at the onset of gastrulation and remains undetectable. Expression level of *erk4* was lower during mid-gastrula (shield), but increases slowly during the later gastrula stages (80% epiboly) to a maximum around 4 dpf (48–5 dpf). In adult fish, expression of *erk4* is hardly detectable. *Erk5* is strongly maternally expressed. Expression levels slowly decrease to a barely detectable level at mid-somitogenesis (12-somites), after which *erk5*-expression levels increase again with a peak at 80 hpf. Expression in adult fish was detected to a lower extent again. The onset of *erk7*-expression is during mid-blastula, thereafter *erk7*-expression levels increased to a maximum at 15–18 somite stage and after lowered again. Later in adult fish, *erk7* showed high expression. Surprisingly, four of the tested genes showed higher levels of mRNA at early blastula stage (16 cells 128 cells) than at one cell stage. As the majority of zebrafish genes are not transcribed up to midblastula transition (MBT) this apparent accumulation of *erk1*, *erk7*, *jnk1* and *p38b* was unexpected, although array studies revealed evidence for pre-MBT transcription accumulation. These genes did not show transcript accumulation during the one-cell, four-cell and eight-cell stages, however transcripts for these genes increased from the 64-/128-cell stages onward (Mathavan et al., 2005).

To localize the expression, we performed whole mount in situ hybridization at 24 and 48 hpf for all *mapk*, using antisense digoxigenin labeled RNA probes encompassing

the full lengths of the *mapk* coding sequences (Fig. 4). Expression was localized ubiquitously for all *mapk* genes during blastula and gastrulation stages. This was also mainly the case during segmentation, except for *erk5*, *erk7* and *p38b*. *Erk5* was already specifically expressed in the pronephric ducts and *erk7* expression was detected in the notochord. For *p38b* no expression was detected, as also no expression was detected by rtPCR (supplemental data). For both stages a lateral view of the embryo is showed. A dorsal view of the head, anterior to left, is also demonstrated for 48 h-stage. All *mapk* showed expression at 24 and 48 hpf, except for *p38b*.

At 24 hpf, the expression of *erk1* was detected in the telencephalon, diencephalon, tectum, hindbrain, tail muscle and proctodaeum. At 48 hpf the expression-domains were more spatially distributed. Expression of *erk1* in telencephalon, diencephalon and hindbrain were pronounced and weaker expression was detected in the hypothalamus. A dorsal view of the head clearly demonstrates the *erk1*-expression in the telencephalon.

*Erk2* showed more localized expression domains at 24 hpf, compared to *erk1*. Expression of *erk2* was found in diencephalon, tegmentum, cerebellum (mid-hindbrain boundary: MHB), hindbrain and somites boundaries. At 48 hpf, *erk2*-expression remained in the diencephalon, cerebellum, hindbrain, but was also found in hypothalamus, branchial arches, the area behind the otic vesicle, and ventral tail mesoderm. The dorsal head image clearly reveals *erk2*-expression is also localized in telencephalon and olfactory placodes. More patches of expression were observed surrounding the branchial arches.

*Erk3* showed specific expression in tegmentum, tectum, hindbrain and otic vesicle at 24 hpf. These expression



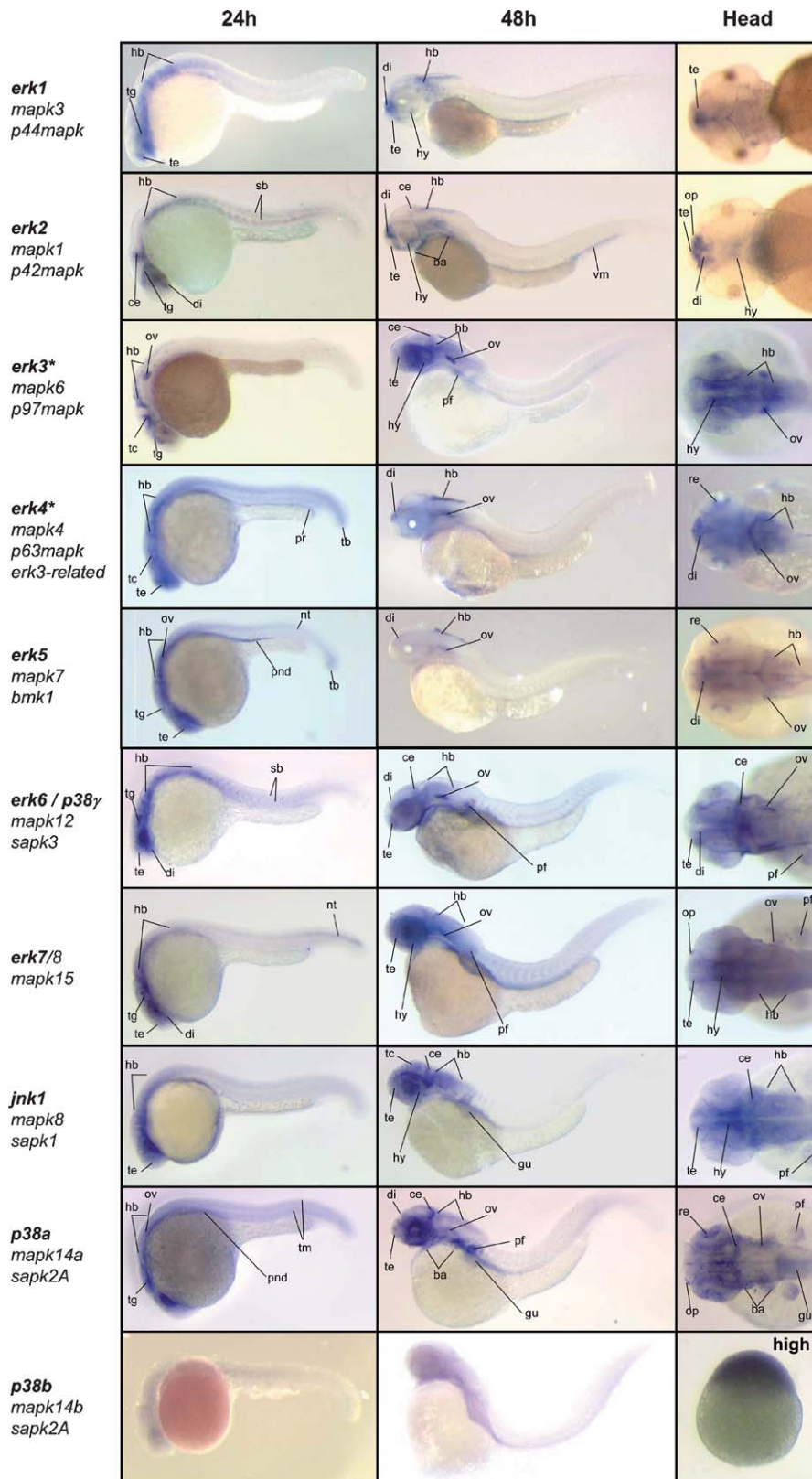


Fig. 4. Expression patterns of the zebrafish MAPK genes by in situ hybridization. The first two columns show 24 and 48 hpf old zebrafish, lateral view, anterior to left, dorsal to top. The third column shows a dorsal view of the head region, anterior to left, at 48 hpf. All MAPK genes are indicated with all known names. MAPKs *erk3* and *erk4* are indicated with an asterisk (\*) because of their alternative dual phosphorylation site (SEG). All MAPK were expressed and showed distinct expression patterns at 24 and 48 hpf, but *p38b*. Therefore the dorsal head-image is replaced for an of the expression patterns at high stage (~3.5 hpf), where *p38b* was expressed in the whole animal pole. ce, cerebellum; di, diencephalon; gu, gut; hb, hindbrain; hy, hypothalamus; nt, notochord, op, olfactory pit / placode; pf, pectoral fin; pnd, pronephric ducts; pr, proctodaeum re, retina; sb, somite boundary; tb, tail bud; tc, tectum; te, telencephalon; tg, tegmentum; tm, tail muscle.

domains expand through all regions of the head, at 48 hpf, resulting in a completely stained head. After short staining, specific *erk3*-expression was detected in the diencephalon (top-view), hypothalamus, cerebellum, hindbrain, otic vesicle and pectoral fin.

More global expression was obtained for *erk4* at 24 hpf, but enhanced signal was detected in the telencephalon, tectum, hindbrain (rhombomeres), otic vesicle, proctodaeum and tail. At 48 h, *erk4*-expression remains in the telencephalon, hindbrain, but is also detected in the otic vesicle and the presumptive branchial arches. In the dorsal view of the head expression of *erk4* was detected in the retina in the eye.

Next to specific *erk5*-expression in telencephalon, diencephalon, tegmentum, otic vesicle, pharyngeal arches and hindbrain, *erk5* is also distinctly expressed in the pronephric ducts and in the notochord in the tail at 24 hpf. Some enhanced signal is also observed in the tail-bud. The expression in the diencephalon, otic vesicle and hindbrain is still detected at 48 hpf. The dorsal view of the head showed *erk5* is also expressed in the retina, very close to the lens of the eye.

*Erk6* was expressed in diencephalon, tegmentum and hindbrain (rhombomeres). Some light expression in the tail reveals the segmented pattern of the somite boundaries. Later at the 48 h-stage, the expression pattern becomes more defined to telencephalon, diencephalon, cerebellum, otic vesicle and the pectoral fin (better visible in the dorsal head image). *Erk6* might also be expressed at the branchial arches, just anterior to the pectoral fin. The expression domain from the cerebellum shows a gradient-like pattern in the dorsal direction, also staining in anterior hindbrain structures. The dorsal head image reveals enriched expression at left side of the lateral body axis, next to the pectoral fin at the presumptive gut.

At 24 hpf, a clear *erk7* expression-domain in the tail notochord is detected, which is the strongest in the tail tip. *Erk7* expression was also detected in the diencephalon, tegmentum and the hindbrain (rhombomeres). *Erk7*-expression remains in these domains at 48 hpf, but is also present in the hypothalamus and otic vesicle. The dorsal image of the head clearly demonstrated expression of *erk7* specifically in the telencephalon, olfactory placode, and the pectoral fin.

Expression of *jnk1* at 24 hpf was localized in the telencephalon boundary, near the diencephalon. The *jnk1*-expression domain expands ventral-dorsal to the midbrain and hindbrain. At 48 hpf, the spatial pattern of expression becomes more defined and enhanced expression was observed in telencephalon and hypothalamus. The *jnk1*-expression gradient expanded dorsally from the cerebellum to the anterior region of the hindbrain structures. Expression in telencephalon and the pectoral fin was better visualized in the dorsal image of the head. Expression of *jnk1* was also detected in the presumptive gut.

*P38a* and *p38b* are both orthologs of  $p38\alpha$  and showed complete different expression in time. *P38a* is expressed at

24 hpf in the diencephalon, tegmentum, otic vesicle, tail muscle and the pronephric ducts. The expression at 48 h was localized in the diencephalon, cerebellum, hindbrain, otic vesicle, branchial arches and the pectoral fins. The dark spot anterior of the pectoral fin represents the gut. These expression domains were also clearly detected in the dorsal view of the head. This image also reveals expression of *p38a* in the olfactory placodes and the eye retina. *P38b* isoforms were not detected by rtPCR nor by in situ hybridization at 24 and 48 hpf. The third panel of Fig. 4 represents an image of the *p38b*-expression at  $\sim 3.5$  hpf (high stage) showing that expression was detected in all blastula cells.

The *mapk* gene family are tightly regulated during zebrafish embryogenesis and showed spatial and temporal dynamics in their expression patterns. All *mapk* were expressed during zebrafish development and localized predominantly in the brain structures at 24 and 48 hpf, except for *p38b* which is only expressed in earlier blastula stage.

## 2. Experimental procedures

### 2.1. Animals

Zebrafish embryos were raised according to standard procedures (Westerfield, 1989) and staged in hours post fertilization (hpf) according to (Kimmel et al., 1995).

### 2.2. Sequence alignments and phylogenetic analysis

The amino acid alignment of zebrafish MAPKs (Fig. 2) was made using the program Vector NTI version 8.0. The phylogenetic tree of zebrafish sequences and the phylogenetic tree were constructed by the neighbor-joining method (Saitou and Nei, 1987) using ClustalW (<http://hypernig.nig.ac.jp>) at the web server of the DNA Data Bank of Japan (DDBJ). Clustal W analysis was done with default settings, without Kimura's correction. Bootstrap sampling was reiterated 10,000 times. For the matrix table 'blosum' was used. The gap extension penalty was set at 0.2 and the gap distance was set at 8. Trees were printed using the program Treeview (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

Phylogenetic analysis was performed by multiple alignments with MAPK subfamily members from different species; human (h), rat (r) and mouse (m) sequences. Accession numbers; hERK1, XM\_055766.6; hERK2 NP002736; hERK3, AAH35492; hERK4, NP\_002738; hERK5, Q13164; hERK6 (= p38gamma), NP\_002960; hERK7, AAH28034; hJNK1, NP\_002741; hJNK2, P45984; hP38alpha, Q16539; hP38beta, Q15759; hP38delta, NP\_002745; mERK1, Q63844; mERK2, P63085; mERK3, NP\_056621; mERK4, NP\_766220; mERK5, NM\_011841.1; mERK6 (= p38gamma), O08911; mERK7, AAH48082; mJNK1, AAH53027; mJNK2, NP\_997575; mP38alpha, NP\_036081; mP38beta, Q9WU11; mP38delta, NP\_062104; rERK1, NP\_059043; rERK2, AAA41124; rERK3, NP\_113810; rERK4, Q63454; rERK5, XP\_340814; rERK6 (= p38gamma), NP\_068514; rERK7, AAD12719; rJNK1, P49185; rJNK2, NP\_059018; rP38alpha, NP\_112282; rP38delta, NP\_062104 zERK1 AY922319; zERK2, AY922320; zERK3, zERK4, zERK5, zERK6 (= p38gamma), zERK7, zJNK1, BAB11810; zP38alpha, BAB11807; zP38beta, BAB11808.

### 2.3. Cloning of MAPK gene family in zebrafish

Total RNA was isolated from adult Tuebingen zebrafish, using TRI-ZOL<sup>®</sup> Reagent protocol (GIBCOBRL, Life technologies TM). The complete ORFs of all the zebrafish MAPKs were amplified based on the predicted sequences (see above) using flanking PCR on cDNA from adult



zebrafish, and cloned into pCR<sup>®</sup>4Blunt-TOPO<sup>®</sup> or pCRII-TOPO<sup>®</sup> (Invitrogen). To verify the predicted ORFs and orientation of the predicted MAPK, the genes were checked by restriction analysis and DNA sequencing. SuperScript™ One-Step RT-PCR with Platinum<sup>®</sup> Taq System (Invitrogen) was used to study expression during development on rtPCR level. P38b was cloned from the pooled products from the reactions at 16 cell-128 cell and 30% epiboly.

#### 2.4. Reverse transcriptase-PCR

Total RNA was isolated from embryos at various stages of development using Trizol reagent (Invitrogen). SuperScript™ One-Step RT-PCR with Platinum<sup>®</sup> Taq System (Invitrogen) was used to study expression during development on rtPCR level. Full CDS-flanking primers were used in most cases. Amplification of zebrafish  $\beta$ -actin (AF057040) was used as a control for constitutive expression.

#### 2.5. Whole-mount *in situ* hybridization

Full coding sequence anti-sense digoxigenin labeled RNA probes were synthesized using the pCR<sup>®</sup>4Blunt-TOPO<sup>®</sup> and pCRII-TOPO<sup>®</sup> constructs contain the zebrafish *erk1,2,3,4,5,6,7 jnk1* and *p38a* and *p38b*. The constructs were linearized with EcoRV. Anti-sense probes were synthesized with T7 RNA polymerase. *In situ* hybridization was performed as described previously (Thisse et al., 1993).

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#### Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.modgep.2006.04.008](https://doi.org/10.1016/j.modgep.2006.04.008).

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