The function of mitogen activated protein kinases in zebrafish development

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Functions of the MAPK family in vertebrate development

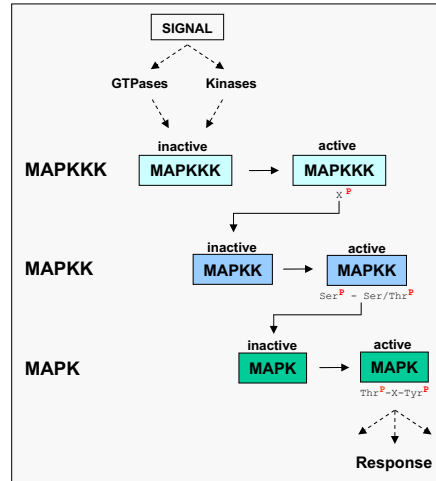S.F. Gabby Krens, Herman P. Spaink, B. Ewa Snaar-Jagalska

Abstract

The mitogen activated protein kinase (MAPK) family, consisting of the extracellular signal regulated protein kinase, c-Jun amino terminal MAPK and p38 subfamilies, is conserved in evolution throughout the plant and animal kingdoms. These proteins have been implicated in diverse cellular processes including cell growth, migration, proliferation, differentiation, survival and development. Gene-targeting approaches in mice, chickens, frogs and zebrafish revealed crucial roles of MAPK in vertebrate development. Gene-disruption or –silencing often lead to lethal effects, therefore the zebrafish *ex utero* development provides an excellent *in vivo* model to study the function of MAPK in early embryogenesis. In this review, we summarize the current understanding of the MAPK family function in vertebrate development and place this into the perspective of possibilities for future research.

1. Introduction

The mitogen activated protein kinase (MAPK) family is conserved in evolution and is involved in diverse cellular processes including cell growth, proliferation, differentiation, survival, innate immunity and development (Johnson et al., 2005; Bogoyevitch and Court, 2004; Roux and Blenis, 2004). MAPKs transmit signals in the form of sequential phosphorylation events. The phospho-relay system is composed of three kinases: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. Phosphorylation of the MAPKs occurs on a conserved dual-phosphorylation domain (Thr-Xxx-Tyr) leads to activation of the protein (Fig.1) and the subsequent formation of dimers which translocate into the nucleus to activate downstream targets (Johnson et al., 2005; Bogoyevitch and Court, 2004; Roux and Blenis, 2004). Three major subfamilies of MAPK proteins have been defined: extracellular signal regulated kinases (ERK), the c-Jun amino-terminal kinases (JNK), and the p38 MAP kinases. The middle amino-acid residue of the conserved Thr-Xxx-Tyr dual-phosphorylation domain designates a MAPK protein to one of these subfamilies. In general, the ERK subfamily (TEY) is mainly activated by growth factors, p38 (TGY) by stress factors and JNK (TPY) are activated by stress-, differentiation- and growth-factors (Weston and Davis, 2002; Nebreda and Porras, 2000; Kolch, 2005). It should be stressed here that scaffold proteins play an important role in the spatial–temporal organization of signaling complexes leading to activation of a specific cascade (Johnson et al., 2005; Garrington and Johnson, 1999; Kolch, 2005).

To illustrate the evolutionary conservation of the MAPK family, we construct-

Figure 1. The mitogen activated protein kinase module. The MAPK module consists of three kinases: a MAPKKK, a MAPKK and a MAPK. Different MAPK cascades can be activated by various signals. Upon activation the upstream kinase activates the downstream kinase by phosphorylation and leads to a response.

ed a phylogenetic tree of the vertebrate MAPKs by the neighbor-joining method (Fig.2) (Saitou and Nei, 1987). This analysis was performed by multiple alignments with the amino acid sequences of the different vertebrate MAPKs from human, rat, mouse, *Xenopus* and zebrafish. As expected from the evolutional point of view the different vertebrate MAPKs cluster with their corresponding orthologs, which are also indicated by their MAPK-family number (MAPK1–15). Currently, based on phosphorylation consensus, sequence identity, signaling profile and functions, six different MAPK cascades have been identified in mammals: ERK1/2, ERK3/4, ERK5, ERK7/8, JNK1/2/3, and p38-isoforms $\alpha/\beta/\chi$(ERK6)/δ (Johnson et al., 2005; Bogoyevitch and Court, 2004; Roux and Blenis, 2004) (Fig.3). The best studied ERK1 and ERK2 are activated by the upstream MAPKKs MEK1 and MEK2, while MEK1-2 are in turn activated by their upstream MAPKKKs or Raf protein kinases. Interestingly, ERK3 is a ubiquitously active MAPK and its activity is regulated by protein stability. The mechanisms of regulation for ERK4 are still largely unclear. In addition, ERK5 is exclusively activated by MEK5, which can be phosphorylated by the MAPKKKs MEKK2 and MEKK3. ERK7 is similar to ERK3 in that it is constitutively activated, presumably by the C-terminus of the protein. ERK7 activity is not regulated by extracellular stimuli. Despite intensive efforts, the activators for ERK8 are yet unknown, although ERK8 is shown to be activated after long

stimulations with serum and in cells expressing the oncogene v-src, suggesting that ERK8 is involved in long term signaling. Similar to ERKs, the p38-MAPK module includes a range of MAPKKKs such as MEKK1-4, MLK2-3, apoptosis signal-regulating kinase 1 (ASK1) and TGFβ activated kinase (TAK1). These activate the MAPKKs MEK3 and MEK6, resulting in the activation of the p38 α, β, γ, δ isoforms. The JNK MAPKs are activated by the MAPKKs MEK4 and 7, which are in turn activated by the MAPKKKs MEKK1-4, MLK2-3, TAO1-2, TAK1 and ASK1-2 (Bogoyevitch and Court, 2004; Garrington and Johnson, 1999; Kuida and Boucher, 2004; Kyriakis and Avruch, 2001). Details of the molecular mechanisms governing the developmental functions of different MAPK-cascades in vertebrate models are beginning to emerge and will be discussed in the following sections (Fig.2 and 3).

ERK1/2

ERK1 (MAPK3, p44MAPK) was the first identified MAPK (Boulton et al., 1990). Until now, homologs for *erk1* have been reported for human, mouse, rat and zebrafish genomes. ERK1 and ERK2 are the most intensively studied MAPKs in developmental processes. Detailed immuno-histochemical analysis revealed localized spatio-temporal patterns of ERK1/2 phosphorylation during mouse (Corson et al., 2003), chicken and zebrafish development, with FGF as most predominant activator during development. *Erk1*-/- mice are viable, fertile and of normal size (Pagès et al., 1999). The proliferation and maturation of the thymocytes is affected, despite expression of ERK2. Mice lacking ERK1 also manifest abnormal signaling responses, which are linked to an upregulation of ERK2 activity in the brain. ERK1 has a critical regulatory role in brain long-term adaptive changes underlying striatum-dependent behavioral plasticity and drug addiction (Mazzucchelli et al., 2002). Furthermore, ERK1 is an important modulator of synaptic plasticity. The existence of the distinct scaffold MP1 for ERK1-MEK1 indicates specific functions and location for MEK1/ERK1 complex (Schaeffer et al., 1998). The MP1 scaffold was initially identified by its capability to bind to the proline-rich region of MEK1 and to be involved in the activation of ERK1, but not ERK2 signaling (Johnson et al., 2005; Schaeffer et al., 1998).

ERK2 (MAPK1, p42MAPK) is found in human, mouse, rat, frog, chicken and zebrafish genomes. Importantly, mouse embryos lacking exon 2 of the *erk2* gene die in utero before embryonic day (E) 8.5 due to a defect in trophoblast development (Saba-El-Leil et al., 2003). *Erk2*-deficient mice fail to form the ectoplacental cone and the extra-embryonic ectoderm, which gives rise to
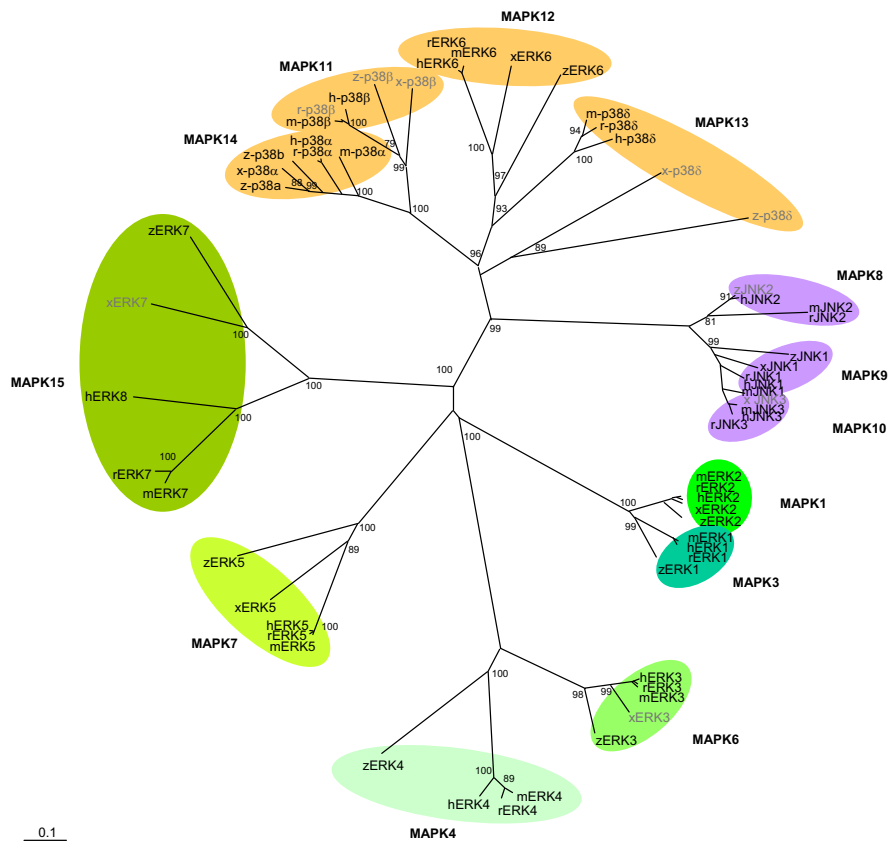
mature trophoblasts in the fetus. In addition *erk2-/-* embryos also fail to form mesoderm, based on histological criteria at E6.5 and E7.5 (Yao et al., 2003; Ornitz and Itoh, 2001). Significantly, ERK1 is incapable of compensating for ERK2 function in *erk2-/-* mice, suggesting that the observed effect is ERK2 specific and cannot be rescued by ERK1.

Despite effort, in frog and chicken no *erk1* gene has been found until now. However, the ERK1 protein has been detected by Western-blot analysis in chicken, where p-ERK1/2 expression was observed in motor axons, but not in sensory axons. In a follow up study, Kato and co-workers have also demonstrated specific activation of ERK1/2 in growing motor axons suggesting that p-ERK1/2 may be involved in outgrowth and/or guidance of this subset of axons (Kato et al., 2005). In contrast, modulation of ERK2 activity affects mesoderm differentiation in *Xenopus* embryos, whereas inhibition of the ERK2 activation prevents animal caps to differentiate into mesoderm tissues (Gotoh et al., 1995). Elevated ERK activation is also detected by immunohistochemistry during segmentation in mouse, chicken and zebrafish (Sawada et al., 2001).

In zebrafish, the presence of the ERK-MAPK cascade was first shown by Western-blot analysis, where insulin-like growth factors (IGFs) stimulates zebrafish cell proliferation by activating MAPK and PI3-kinase signaling pathways (Pozios et al., 2001). Subsequently it was shown that chitin oligosaccharides, activate ERK1 and ERK2 in zebrafish cells, via the Ras-Raf-MEK module (Snaar-Jagalska et al., 2003). The developmental roles of ERK1 and ERK2 in zebrafish development have mostly concentrated on the functions of the FGF/MAPK pathway, which also contains the inhibitors Sef (Furthauer et al., 2002; Tsang et al., 2002), Sprouty2/4 and the MAPK phosphatases MKP1 and MKP3 (Shinya et al., 2001). Overactivation of the FGF/ERKpathway leads to dorsalized embryos by inhibiting expression of BMP genes (Furthauer et al., 2004). Overexpression of ERK-MAPK phosphatase MKP3 or injection of a high dose of mRNA of the inhibitor Sef also results in an opposite ventralization (Furthauer et al., 2002; Tsang et al., 2004). This implies that manipulation of ERK-MAPK activation affects zebrafish development. It can therefore be suggested that ERK1/2 in zebrafish are regulated via canonical pathways, but precise regulation and distinguished developmental function for ERK1 and ERK2 remain to be defined. Recently, a developmental role for ERK1 in axial mesoderm formation was demonstrated and the absence of active ERK2 in the blastula-margin blocked the initiation of epiboly cell migration, disturbed the microtubule organization and led to an arrest of embryogenesis, preventing further differentiation of epiblast and hypoblast (unpublished data). Similarly to mice also in zebrafish ERK1 is not able to rescue developmental phenotypes caused by ERK2 knock-down.

Figure 2. Phylogenetic tree of the vertebrate MAPKs. The phylogenetic tree was constructed by the neighbor-joining method of the amino acid sequences of the different vertebrate MAPKs using Clustal W, available at the web server of the DNA Data Bank of Japan (DDBJ, http:// hypernig.nig. ac.jp). Clustal W analysis was done using default settings, without Kimura's correction. Bootstrap sampling was reiterated 10000 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). The phylogenetic tree illustrates the evolutionary conservation of the MAPK family members in human (h), rat (r), mouse (m), *Xenopus laevis* (x) and zebrafish (z). The different MAPKs cluster together and are indicated by their MAPK-family number (MAPK1–15). Black text = previously annotated and shown to be expressed; gray text = translation of genomic prediction. The predicted sequences were found in the following versions of the genomes of the Sanger Ensembl: Rattus norvegicus = v38, *Xenopus* = v38; zebrafish = Zv6.

Introduction

ERK3/4

Interestingly, ERK3 and ERK4 are the only ERK-MAPKs that lack the characteristic TEY activation motif, but display a SEG activation domain (Fig.3). Only the serine residue in this SEG-motif can be phosphorylated. ERK3 has a characteristic 400 amino acid C-terminus, which is partly homologous to ERK4 (170 amino acids). The stimulants for ERK3 and ERK4 have not been identified, so far.

ERK3 (MAPK6, p97MAPK) has been reported for human, mouse, rat, zebrafish and gene-predictions were also found in the frog and chicken genomes (Fig.2). ERK3 seems to be mainly regulated by its protein stability and autophosphorylation. Human ERK3 interacts with MAPK-activated protein 5 (MK5 or PRAK) and spatio-temporal expression of ERK3 and MK5 suggest co-expression of both kinases (Seternes et al., 2004). In mice, deletion of *mk5* leads to strong reduction of ERK3 protein levels and is lethal around E11, the time-point where expression levels of *erk3* are maximal in wildtype mouse embryos (Seternes et al., 2004; Schumacher et al., 2004). This suggests an universal role of this MK5-ERK3 module in development. *Erk3*-/- mice have been made, although their phenotypes are not described in literature yet (Turgeon B and Meloche S, manuscript in preparation; Seternes et al., 2004).

ERK4 (MAPK4, ERK3-related, ERK3-beta, p93MAPK), is closely related to ERK3 (Turgeon et al., 2002), was first identified in human (Gonzalez et al.,1992) and subsequently described for mouse, rat, and zebrafish. A predicted ORF was also found in the chicken. Its spatio-temporal expression-pattern is predominantly localized in the brain during zebrafish development (Krens et al., 2006). However, a developmental function of this MAPK remains to be determined.

ERK5 (MAPK7, BMK1)

ERK5, also referred to as big MAP kinase 1 (BMK1), is found in human, mouse, rat, frog and zebrafish. Activation of ERK5 is mediated by MEK5. The ERK5 pathway is activated by oxidative stress, hyper-osmolarity and growth factors (Wang and Tournier, 2006). ERK5 has a unique carboxy-terminal domain, which interacts with the transcription factor myocyte enhancer factor 2 (MEF2). Genetic studies with ERK5- or MEK5-deficient mice revealed that the MEK5–ERK5 pathway is essential for blood vessel development and cardiovascular development (Regan et al.,2002; Nishimoto et al., 2005; Hayashi and Lee, 2004; Yan et al., 2003; Sohn et al., 2002; Wang et al., 2005). Mice that lack ERK5 or MEK5 die around E.10 due to defects in placental develop-

ment, angiogenesis and cardiovascular development. Studies with conditional knockout, using the inducible promoter Mx1 to direct Cre expression, revealed that ablation of ERK5 in adult mice was lethal within 2–4 weeks after induction of the Cre recombinase (Hayashi et al., 2004). Histological and in vitro analysis revealed that endothelial cells lost their integrity, became round and eventually apoptotic. The loss of these functional endothelial cells resulted in abnormally leaky blood vessels and hemorrhages in multiple organs. These findings indicate that survival of endothelial cells is MEK5 and ERK5 dependent, and that the cardiovascular defects observed in *erk5-/-* and *mek5-/-* embryos are due to the loss of endothelial cells (Hayashi et al., 2004).
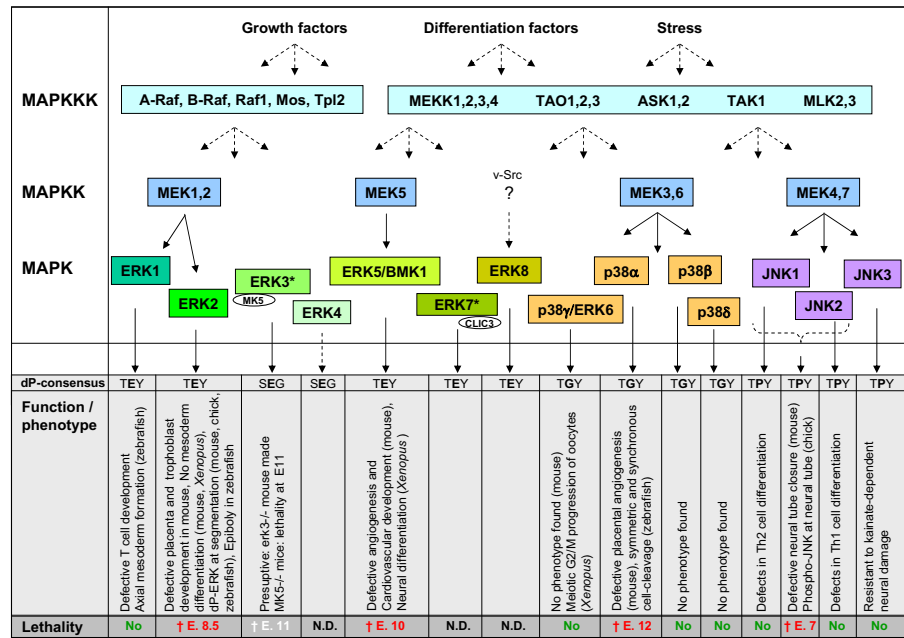
In *Xenopus* knockdown of ERK5 by antisense morpholino injection inhibits neural differentiation and leads to growth retardation in the head and eventually to reduced head structures (Nishimoto et al., 2005). In this system the activation of the MEK5–ERK5 pathway is necessary for neural differentiation in early embryonic development. It is likely that the observed inhibition of neural differentiation may be one of the reasons for growth retardation in the head region. Controversially, studies with conditional brain specific knockout mice, with Cre-expression controlled by the neuron specific synapsin I or nestin promoters revealed that these mice develop normally (Hayashi et al., 2004). This might be due to species differential functions for ERK5, the use of an inappropriate promoter in the mouse model, or that a more global effect is responsible for the observed phenotypes in the brain after ERK5 knockdown in the *Xenopus*. Furthermore, expression of *erk5* is also mainly localized in the zebrafish brain and possibly a follow-up study in zebrafish will clarify developmental functions of ERK5 (Krens et al., 2006).

ERK7, ERK8 (MAPK15)

ERK7 (MAPK15) is found in rat, mouse and zebrafish and recently the *Dictyostelium erkB* (ERK2) has been characterized as an *erk7*-ortholog (Ray et al. 2005; Abe et al., 1999). Genome-based analyses revealed that human *erk8* and rodent *erk7* are orthologs (Saelzler et al., 2006). Furthermore, gene-predictions for *erk7* were found within the chicken and *Xenopus* genomes.

Like ERK3 and ERK5, the size of ERK7 and ERK8 is significantly larger than ERK1 and ERK2. Presumably, ERK7 is kept in a constitutively active conformation by its characteristic C-terminus (Saelzler et al., 2006). Studies to determine their function in vertebrate development have not yet been reported.

Growth factors · **Differentiation factors** · **Stress**

MAPKKK: A-Raf, B-Raf, Raf1, Mos, Tpl2 | MEKK1,2,3,4 | TAO1,2,3 | ASK1,2 | TAK1 | MLK2,3

MAPKK: MEK1,2 | MEK5 | v-Src ? | MEK3,6 | MEK4,7

MAPK: ERK1, ERK2 (MK5), ERK3*, ERK4 | ERK5/BMK1 | ERK8 | ERK7* (CLIC3) | p38α, p38γ/ERK6 | p38β, p38δ | JNK1, JNK2, JNK3

	dP-consensus	Function / phenotype	Lethality
ERK1	TEY	Defective T cell development Axial mesoderm formation (zebrafish)	No
ERK2	TEY	Defective placenta and trophoblast development in mouse, No mesoderm differentiation (mouse, Xenopus), dP-ERK at segmentation (mouse, chick, zebrafish), Epiboly in zebrafish	† E. 8.5
ERK3*	SEG	Presuptive erk3-/- mouse made MK5-/- mice: lethality at E11	† E. 11
ERK4	SEG		N.D.
ERK5/BMK1	TEY	Defective angiogenesis and Cardiovascular development (mouse), Neural differentiation (Xenopus)	† E. 10
ERK8	TEY		N.D.
ERK7*	TEY		N.D.
p38α	TGY	No phenotype found (mouse) Meiotic G2/M progression of oocytes (Xenopus)	No
p38γ/ERK6	TGY	Defective placental angiogenesis (mouse), symmetric and synchronous cell-cleavage (zebrafish)	† E. 12
p38β	TGY	No phenotype found	No
p38δ	TGY	No phenotype found	No
JNK1	TPY	Defects in Th2 cell differentiation	No
JNK2	TPY	Defective neural tube closure (mouse) Phospho-JNK at neural tube (chick)	† E. 7
JNK3	TPY	Defects in Th1 cell differentiation	No
	TPY	Resistant to kainate-dependent neural damage	No

Figure 3. The mitogen activated protein kinase module and the developmental functions of the MAPK proteins. MAPK-modules can be activated by various stimuli: mitogens (growth factors, cytokines), differentiation and stress factors (UV, osmolarity). The MAPK module consists of a MAPKKK, a MAPKK and a MAPK. Two MAPKs (ERK3 and ERK7) are not activated by an upstream MAPKK, but are constitutive active and are regulated by protein stability (indicated by an asterisk *). The MAPKs are subdivided into their corresponding subfamily, based on their dual phosphorylation domain (*dP-consensus*). The functions, obtained by gene-targeting or -silencing studies, but also immuno-histochemistry, are described for each MAPK. The lethality-index indicates if gene-disruption in mice resulted in a lethal developmental phenotype and at what day of development (*†* = lethal, *No* = not lethal, *ND* = not determined). For *erk3*-/- mouse the results are not published yet. The indicated lethality shown for ERK3 (in white) is for *mk5*-/- mice, a direct target of ERK3.

JNK1,2,3 (MAPK9,10,11; SAPKγ,α,β,)

Activated JNKs phosphorylate the N-terminus of the c-Jun protein and increases activity and stability of c-jun as a transcription factor (Fuchs et al., 1996; Derijard et al., 1994). Both JNK activation and c-Jun phosphorylation regulate cell growth, whereas sustained JNK and c-Jun activation following

stress induces cell apoptosis, indicating that the role of JNK in cell survival and death is complex (Ip and Davis, 1998). Mouse *jnk1* and *jnk2* are expressed ubiquitously during development, whereas *jnk3* is primarily expressed in the brain and to a lesser extent in the heart and testis. Mice lacking individual members of the *jnk* family are viable (Dong et al., 1998; Yang et al.,1998). The *jnk1-/-* knockout mice exhibited an affected T helper type-2 response, while T-cells from *jnk2-/-* mice showed impaired T helper type-1 differentiation. Both knockouts demonstrated defects in T cells activation and apoptosis of thymocytes (Sabapathy et al., 2001). Mice lacking both of the ubiquitously expressed *jnk* isoforms (*jnk1* and *jnk2*) die during mid-gastrulation (around E.7) with neural tube closure and brain defects (Kuan et al., 1999). Recently, it was shown that mice with a single allele of *jnk2* (*jnk1-/- jnk2-/+*), can survive to birth, but fail to close the optic fissure (retinal coloboma), a morphogenetic process that resembles dorsal and thorax closure in *Drosophila* by regulating BMP expression (Weston et al., 2003). Localization of phospho-JNK in the spinal cord changes dramatically from cell-axons to the cell nuclei during development in the chicken, suggesting physiological functions of JNK during neuronal development (Kuan et al., 1999).

In *Xenopus* oocytes initially two JNK isoforms, p40 JNK and p49 JNK, were shown (Bagowski et al., 2001), but until now only one *jnk* gene (*jnk1*) is cloned. The ensemble genome project of *Xenopus tropicalis* does predict a *jnk3*-gene. JNK activity increases abruptly just prior to germinal vesicle breakdown and is shown to be involved in the non-canonical Wnt pathway to regulate *Xenopus* convergence extension movements (Yamanaka et al., 2002). Furthermore, the active JNK signaling complex formed by the scaffold protein POSH (Plenty Of SH3s) and the JNK-module is essential for the expression of anterior neural genes and apoptosis in *Xenopus* anterior development (Kim et al., 2005).

In zebrafish also only one *jnk*-gene is described, which is expressed throughout development and shows distinct temporal and spatial expression patterns (Krens et al., 2006), but the latest release of the zebrafish genome (*Zv6*) predicts a second *jnk* gene (Fig.2).

p38α,β,γ,δ (MAPK14,11,12,13)

The p38 family includes p38α (MAPK14, SAPK2a, CSBP), p38β (MAPK11, SAPK2b), p38γ (MAPK12, ERK6, SAPK3) and p38δ (MAPK12, SAPK4). Both *p38α* and *p38β* are widely expressed isoforms that are involved in regulation of cell proliferation, differentiation, development, and response to stress.

The $p38\alpha$ knockout mice are lethal due to defects in placental angiogenesis (Mudgett et al., 2000; Adams et al., 2000). In some genetic backgrounds, $p38\alpha$ deletion results in a decrease of erythropoietin (Epo) production, leading to anemia (Tamura et al., 2000).

In *Xenopus*, the p38 MAPK signaling pathway is essential for skeletal muscle differentiation in tissue culture models. Knockdown of p38 MAPK causes distinct defects in myogenesis in *Xenopus laevis*, showing that p38 MAPK is involved in myogenesis during early development (Keren, et al., 2005). The zebrafish $p38\alpha$ otholog, p38a (MAPK14a), is asymmetrically activated on one side of the blastodisc during the early cleavage period in zebrafish embryos. The use of a dominant negative form of p38a revealed that asymmetric p38a activation is required for symmetric and synchronous cleavage, and may be regulated by the same machinery that controls the initiation of dorsalization signals (Fujii et al., 2000).

By screening with the rat *erk3* gene, an *erk6*-clone was isolated from a human skeletal muscle cDNA library, that appeared to function as a signal transducer during differentiation of myoblasts to myotubes (Lechner et al., 1996). Later it was found that SAPK3 was identical to ERK6 (Li et al., 1996; Mertens et al., 1996). Based on the phosphorylation domains and function, ERK6 is now classified as p38γ MAPK.

Mouse lacking p38β, p38γ or p38δ survive normally and do not show any obvious phenotypes (Kuida and Boucher, 2004; Beardmore et al., 2005; Sabio et al., 2005). Also the $p38\gamma$ and $p38\delta$ double knockout mice were viable and fertile and had no obvious health problems (Sabio et al., 2005). Despite the suggested role for p38 MAPKs in inflammatory responses, these knockout mice do not show pathological changes, indicating dispensable physiological functions for p38β, p38γ and p38δ.

In frog, overexpression of a constitutively active mutant of the p38 activator MKK6 accelerates progesterone-induced maturation of *Xenopus* oocytes and was therefore suggested to be involved in the meiotic maturation. Phosphorylation of Cdc25C by p38γ/SAPK3 is important for the meiotic G2/M progression of *Xenopus* oocytes (Perdiguero et al., 2003).

Information has been particularly limited regarding the functional role of p38δ (SAPK4). Eckert and coworkers describe p38δ as a regulator of surface epithelia differentiation and apoptosis (Eckert et al., 2003). Until now p38δ is found to be expressed in human, mouse and rat, but is not yet found in other vertebrates. However, a genome search in zebrafish (Zv6) and *Xenopus* (v38) does predict a possible *p38δ*-gene.

Conclusions and perspectives

There is an increased understanding about the different MAPK pathways and their crucial roles in vertebrate development. One of the striking observations is that some MAPKs (ERK3 and ERK7) are not regulated by the dogmatic MAPK module, but by protein stability. A major unresolved question is why such a variety of MAPKs is needed. Gene-disruption and -silencing experiments already showed central roles for most of these proteins. These approaches often resulted in early lethal effects, but also revealed redundancy (Fig.3).

The occurrence of redundancy can be addressed and overcome by targeting multiple genes at the same time. Also further conditional and tissue specific gene-targeting experiments will help to understand the functions of the different MAPKs. The use of different vertebrate model organisms and their specific beneficial characteristics will be helpful to achieve this goal. The recent characterization of the zebrafish *mapk* gene-family (Krens et al., 2006) and its advantage to study early embryogenesis *ex utero* provides an excellent system for further investigation of the functions of MAPKs in early development *in vivo*. The transparency of zebrafish embryos is of particular advantage to explore the link of MAPKs to cell migration processes. This has already resulted in the identification of the role for ERK2 in developmental cell migration, additive to the well established proliferation and differentiation functions.

Aim of thesis

The mitogen-activated protein kinase (MAPK) signaling pathway is evolutionary conserved in the plant and animal kingdoms and its function has been implicated in cell growth, proliferation, differentiation, survival, and vertebrate development. Most of the studies on MAPK signaling were performed using *in vitro* cell line model systems. The crucial roles of the different MAPKs during developmental processes have been mainly addressed by gene targeting approaches. However, the lethality of mice knock-outs and *in utero* embryogenesis makes developmental studies on MAPKs difficult and therefore their mechanistic role in development of the vertebrate body plan remains elusive. This thesis aims to further elucidate the role of different MAPK, using both *in vitro* and *in vivo* zebrafish systems.

The zebrafish model offers exciting novel research opportunities because of the optical transparency of its embryos allowing easy bio-imaging, and its amenability to forward and reverse genetics. Zebrafish are small (2-3 cm), vertebrate animals that can be easily cultured under laboratory conditions, with each female capable of producing each week hundreds of transparent embryos

that undergo rapid develop *ex utero*. Importantly, the almost complete genome sequence reveals that most genes are highly conserved between zebrafish and humans, making the zebrafish ideal as a biomedical model for studying the function of genes and pathways that regulate vertebrate development.

This thesis focuses on mechanisms of activation of the MAPKs in zebrafish cell lines and the distinct roles of ERK1 and ERK2 MAPKs in different developmental processes, like cell migration and differentiation.

In chapter 2 compounds which activate MAPK signaling cascade in embryonic zebrafish cell lines are described. It is shown that growth factors and chitin oligosaccharides (CO) specifically activate MAPKs in embryonic zebrafish cell line via the canonical Raf, Mek and ERK pathway.

To study the role of the different MAPK in zebrafish, first the zebrafish *mapk* genes were cloned and characterized (chapter 3). The zebrafish orthologs of all *mapk* gene family members were found and their specific spatial and temporal expression patterns during zebrafish embryogenesis were determined. Subsequently the focus of this thesis was to identify the possible distinct roles of ERK1 and ERK2 MAPKs in different developmental processes, since this hasn't been clarified by the use of other vertebrate models yet (chapter 4 and 5).

To this aim the morpholino antisense oligonucleotide knockdown approach was applied in order to transiently block the translation of (maternal and zygotic) mRNA into protein. The knockdown phenotypes showed distinct effects for ERK1 and ERK2 morphants on cell movements during gastrulation. The observed cell migration effects were considered to be primary changes in gastrulation cell movements and not caused by altered cell fate specification, as the expression of patterning genes was not significantly altered. More stringent knockdown conditions demonstrated that the absence of activated ERK2 from the blastula margin blocked initiation of epiboly and arrested embryogenesis, whereas ERK1 knockdown had only a mild effect on epiboly progression. In order to address which processes were affected by the depletion of ERK1 or ERK2 and identify specific target genes for ERK1 and ERK2, micro-array based gene expression profiles of ERK1 and ERK2 knockdown embryos were compared (chapter 5).

Distinct gene expression profiles for ERK1 and ERK2 knockdown were obtained. Analysis of the obtained expression profiles revealed that ERK1 knockdown possibly represses genes that are expressed at the ventral side. This might lead to a dorsalization of the embryo. Nevertheless, also genes involved in gastrulation cell migration were repressed, supporting the findings described in chapter 4. Knockdown of ERK2 affected key signaling pathways involved in initiation (Nodal) and maintenance of mesoderm (FGF and Wnt), but also

endoderm differentiation was perturbed. This indicates that the initiation of differentiation mesendoderm progenitor cells was still present, but mesoderm maintenance was defected. In addition, also the patterning of the mesoderm was affected, possably due to altered BMP signaling and the disturbance of the expression of dorsal- and ventral- specific genes. Combined, this indicates that active ERK2 is essential for differentiation and patterning and subsequently for epiboly progression. These results provide further evidence for distinct roles for ERK1 and ERK2 in vertebrate embryogenesis. The observed results are summarized and discussed in chapter 6.

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