The function of mitogen activated protein kinases in zebrafish development

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Contents

I

Functions of the MAPK family in vertebrate development

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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-

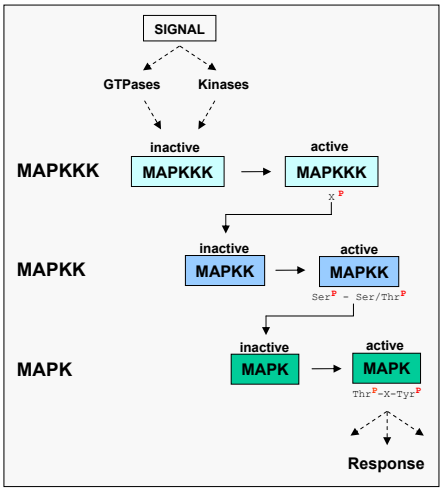

Figure 1. The mitogen activated protein kinase module. The MAPK module consists of three kinases: a MAPKKK, a MAPKK and a MAPK. Different MAPK cascades can be activated by various signals. Upon activation the upstream kinase activates the downstream kinase by phosphorylation and leads to a response.

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.

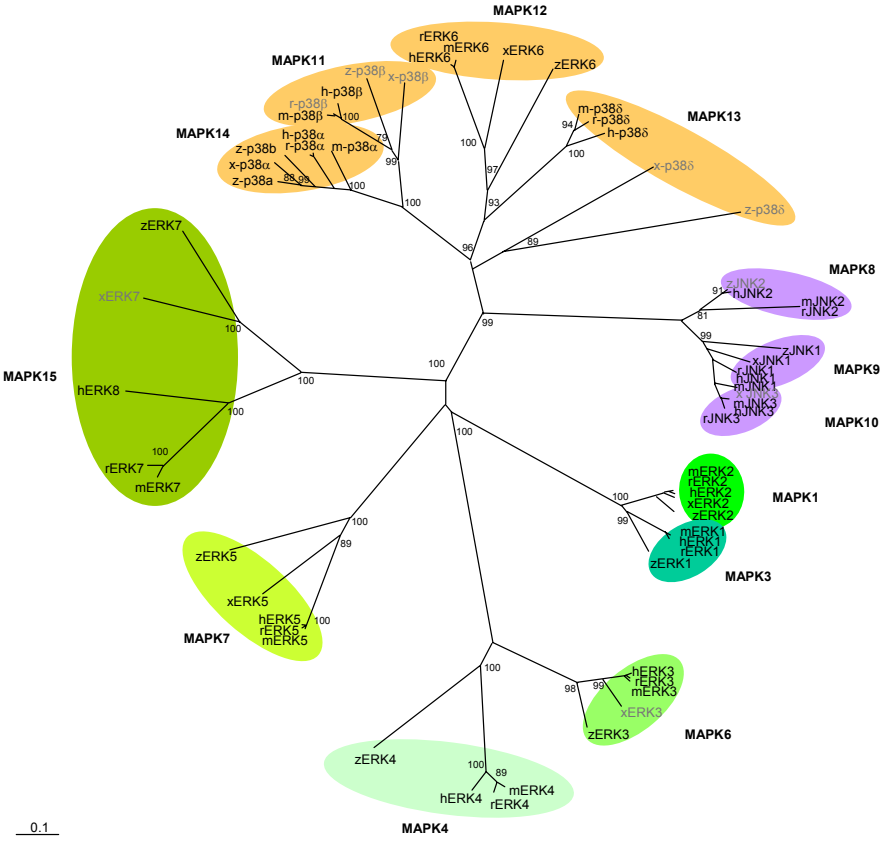

Figure 2. Phylogenetic tree of the vertebrate MAPKs. The phylogenetic tree was constructed by the neighbor-joining method of the amino acid sequences of the different vertebrate MAPKs using Clustal W, available at the web server of the DNA Data Bank of Japan (DDBJ, http:// hypernig.nig.ac.jp). Clustal 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.

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 timepoint 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.

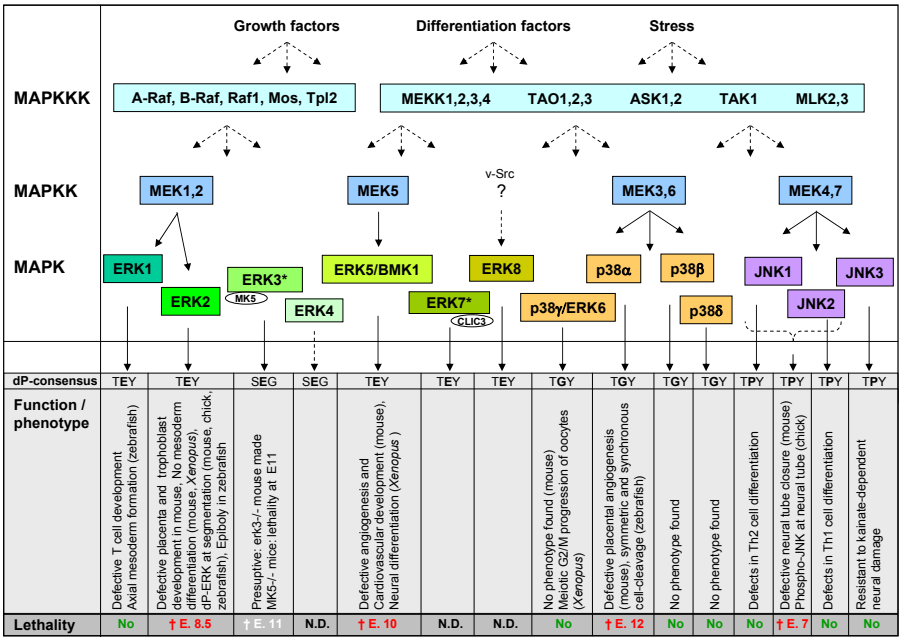

Figure 3. The mitogen activated protein kinase module and the developmental functions of the MAPK proteins. MAPK-modules can be activated by various stimuli: mitogens (growth factors, cytokines), differentiation and stress factors (UV, osmolarity). The MAPK module consists of a MAPKKK, a MAPKK and a 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; SAPK*γ,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).

*p38*α,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 *p38*α and *p38*β are widely expressed isoforms that are involved in regulation of cell proliferation, differentiation, development, and response to stress.

The *p38*α knockout mice are lethal due to defects in placental angiogenesis (Mudgett et al., 2000; Adams et al., 2000). In some genetic backgrounds, *p38*α 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 p38α 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*γ and *p38*δ 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 *p38*δ-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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II

Specific activation of ERK pathways by chitin oligosaccharides in embryonic zebrafish cell lines

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Abstract

Chitin oligosaccharides (COs) play a role in plant development and are presumed to affect body plan formation during vertebrate embryogenesis. The mechanisms of COs recognition and cellular processes underlying embryonic development are still not understood. We analyze the possible link with the mitogen-activated protein kinase pathway that is conserved in evolution through the plant and animal kingdom and has been implicated in diverse cellular processes, including cell growth, proliferation, differentiation, survival, and vertebrate development.

We show that *in vivo* stimulation of embryonic zebrafish cells ZF13 and ZF29 with chitin tetrasaccharides at 10^{-9} M concentration transiently induced activation/phosphorylation of extracellular regulated kinases (ERKs), with a maximum after 15 minutes. Furthermore the biological specificity of chitin tetrasaccharides and various derivatives was examined. The replacement of one or two GlcNAc residues of the chitin backbone by glucose and fucosylation of chitin tetrasaccharides at the reducing terminus caused a complete loss of their activity. We also tested a chitin tetrasaccharide analogue in which the oxygen atoms in glycosidic linkages were replaced by sulfur atoms. This analog, which could not be enzymatically hydrolyzed, was as potent an inducer as chitin tetrasaccharide. These results suggest that the observed activation of ERKs is chitin tetrasaccharide-specific and does not require further enzymatic processing. We examined possible signaling pathways leading to ERK activation by COs by use of phosphospecific antibodies and inhibitors. We conclude that a high-affinity CO receptor system exists that links to the Raf, MEK, and ERK pathway in zebrafish cells.

Introduction

Chitin, a repeating $\beta(1\rightarrow4)$-linked homopolymer of the monosaccharide N-acetylglucosamine (GlcNAc), is one of the most widespread and abundant molecules in the biosphere (Varki, 1996). Chitin oligosaccharide (CO) derivatives play a role in the leguminous plant development and possibly also in vertebrate embryogenesis (Bakkers et al., 1997). Rhizobial NodC protein, responsible for CO synthesis, is homologous to the developmentally regulated DG42/HAS protein of vertebrates (Bulawa and Wasco, 1991; Semino and Robbins, 1995). Members of the DG42/HAS family have been shown to be involved in the biosynthesis of both hyaluronan and COs (DeAngelis and Acbyuthan, 1996; Meyer and Kreil, 1996; Semino et al., 1996; Yoshida et al., 2000; Van der

Holst et al., 2001). A possible biological function for COs was elucidated by microinjection studies of fertilized zebrafish eggs with anti-DG42 antiserum and the rhizobial NodZ fucosyltransferase (Bakkers et al., 1997). These treatments led to severe defects in trunk and tail development, suggesting that COs may function as signaling molecules in cell growth, differentiation, and development of vertebrates (Semino et al., 1998; Semino and Allende, 2000).

Mammalian cells respond to a variety of extracellular stimuli via activation of specific mitogen-activated protein kinases (MAPKs) that orchestrate the transduction of the signal from receptors at the cell surface to the nucleus and play a major role in the integration of multiple biological responses. Three main classes ofMAPK are recognized: the classic MAPK, also known as extracellular-regulated kinases, ERK1 and ERK2, and c-Jun N-terminal kinase (JNK) and p38 MAPK, which are activated by dual phosphorylation at neighboring threonine and tyrosine residues in the activation loop. Dephosphorylation of either residue results in MAPK inactivation (Johnson and Lapadat, 2002). The general scheme of ERK activation involves a cascade of phosphorylation events initiated by stimulation of the Ras proto-oncogene following activation of growth factor receptors. The cascade starts with the activation of one or more Raf family kinases, which phosphorylate and activate the MAPK kinases (MEK1/2). MEK in turn catalyzes dual phosphorylation and activation of ERKs (Chang and Karin, 2001). Once activated, ERKs accumulate in the nucleus by as-yet undefined mechanisms that seem to involve the dissociation of ERK from MEK (Fukuda et al., 1997) and/or a blockade of its export from the nucleus by neosynthesized nuclear anchors (Lenormand et al., 1998).

The ERK pathway has been implicated in diverse cellular processes, including cell growth, proliferation, differentiation,and survival (Marshall, 1995; Ballif and Blenis, 2001). Hence, finely tuned regulation of ERK activation is essential in conveying appropriate signals. The intensity, duration, and subcellular localization of ERK activation are well regulated. Scaffolding proteins and docking sites provide the means to avoid cross-activation between MAPK signaling pathways and permit precise and even cell-specific subcellular localization of ERKs (Pouysségur et al., 2002). The variable responses elicited by this cascade in different cell types are also presumably determined by the cellspecific combination of downstream substrates.

Activated ERK phosphorylates numerous substrates in all cellular compartments (Lewis et al., 1998). More than 50 different ERK substrates have been identified so far. These include ubiquitous or lineage-restricted transcribed factors, the kinases RSK and MNK and proteins involved innucleotide biosynthesis, cytoskeleton organization, ribosomal transcription, and membrane traffic (Sturgill et al., 1988; Marais et al., 1993; Treisman, 1996; Fukunaga

and Hunter, 1997; Lewis et al., 2000; Stefanovsky et al., 2001). RSKs, 90-kDa ribosomal S6 serine/threonine kinases family (also known as MAPK-activated protein kinase-1,MAPKAP-K1) are activated by ERKs *in vitro* and *in vivo* via phosphorylation (Sturgill et al., 1988; Dalby et al., 1998).

The presence and function of MAPK pathways in zebrafish has been recently described. The insulin-like growth factor stimulates zebrafish cell proliferation by activating MAPK and PI3-kinase-signaling pathways (Pozios et al., 2001), while FGF/MAPK signaling is required for development of the subpallial telencephalon and somite boundary formation in zebrafish embryos (Shinya et al., 2001; Sawada et al., 2001). Hence components upstream and downstream of the specific MAPK cascades in zebrafishare not known yet.

Here we show that chitin tetrasaccharides rapidly and specifically activate the ERK pathway in embryonic zebrafish cells. These findings provide strong evidence that CO signaling is initiated on the plasma membrane via activation of high-affinity oligosaccharide receptor system, whichtransmits the signal to nucleus probably using the Ras, Raf, MEK, ERK cascade.

Results

Activation of ERK by chitin tetrasaccharides

To investigate a possible role of COs as signaling molecules in vertebrate development, the embryonic zebrafish cell lines ZF13 and ZF29 were used as a model (Peppelenbosch et al., 1995) and the canonical MAPK pathway was selected as a well-characterized junction between extracellular signals and integrated biological responses. EGF was used as a control of functionality in the system.

Zebrafish cells were serum starved for 24 h, stimulated with $5x10^{-6}$ M chitin tetrasaccharides and EGF for the indicated times, lysed, and processed for western blotting. The activated/phosphorylated form of MAPKs, ERKs was monitored by specific dual phospho-p44/42 MAP kinase antibodies (dp ERK), which detect p44 and p42 MAP kinase (ERK1 and ERK2) only when catalytically activated by phosphorlation at Thr202 and Tyr204. The antibody does not appreciably cross-react with the corresponding phosphorylated threonine and tyrosine residues of either JNK/SAPK or p38 MAPK homologs. It does not cross-react with up to 2 mg non-phosphorylated p44/42 MAPK. The total protein level in all performed experiments was control by ERK1 antibody, which reacts with ERK1 (p44) and to a lesser extent ERK2 (p42), by western blotting. Bands intensities were quantified by densitometry, and ERK activity was com-

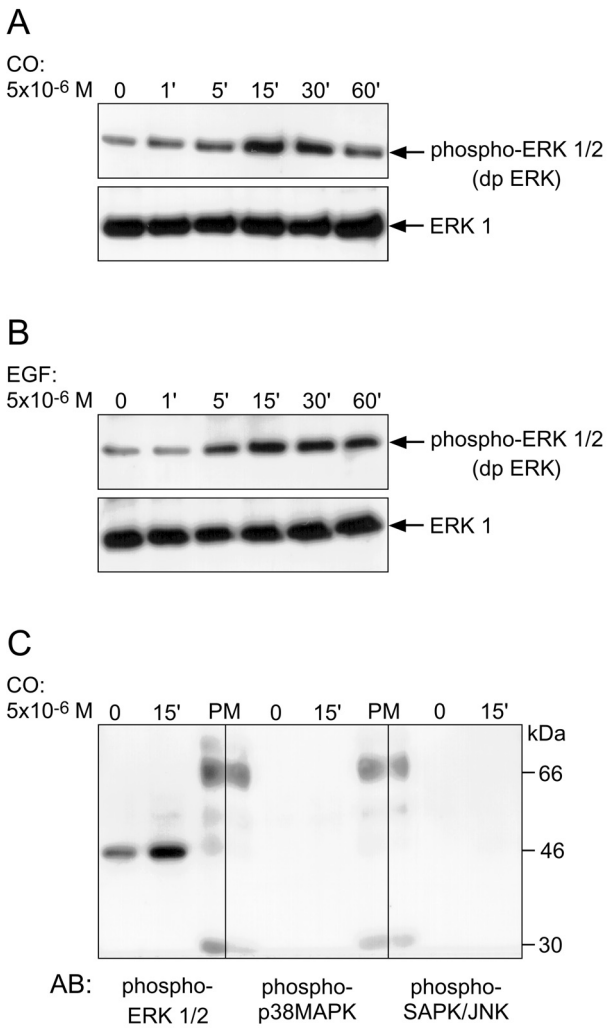

Figure 1. Time-dependent and specific activation of ERKs by COs and EGF. Serum-starved zebrafish cells (ZF13) were stimulated with $5x10^{-6}$ M COs (A) and EGF (B) for increasing periods of time (0-60 min). ERK activation were monitored by immunoblotting of total cell lysates with specific phospho-MAPK (Thr202/Tyr204) antibody (dpERK). The total amount of ERK was visualized using an ERK1 antibody. These results are representative examples of five experiments and were identical for ZF29 cells (data not shown). The lysates obtained from not stimulated (0) and 15 min stimulated (15') cells with $5x10^{-6}$ M CO and protein marker (PM) were subjected to western blotting. The specificity of ERK phosphorylation was determined with anti-phospho-ERK1/2, anti-phospho-p38 MAPK, and phospho-SAPK/JNK antibodies (C).

pared to the basal level at t=0.

In vivo stimulation of ZF13 and ZF29 with chitin tetrasaccharide and EGF transiently induced the 8-15 fold activation/phosphorylation of ERKs, with a maximum after 15 min (Fig.1). The half maximal response was obtained at 10^{-9} M concentration (Fig.3). Although dp ERK antibodies are able to recognize both phosphorylated ERK1 and ERK2 in all experiments, one band of 44 kDa was preferentially detected, suggesting that ERK1 is mainly activated by COs and EGF. The anti-phospho-p38 MAPK and anti-phospho-SAPK/JNK antibodies showed absence of cross-reaction in the CO activation process observed with phospho-ERK1/2 antibody, ensuring the specificity in the western blots (Fig.1C).

ERK activation is specific for chitin tetrasaccharides, CO, and thio-CO

We examined the biological specificity of chitin tetrasaccharides and various derivatives (Fig.2). The replacement of one or two GlcNAc residues of the chitin backbone by glucose and fucosylation of chitin tetrasaccharides by NodZ protein abolished the biological activity of CO(2glc)-MP and CO-fuc in the tested concentration of 10^{-7} M (Fig.3A). This loss of activity was apparently not due to modifications at C1 position of the chitin tetrasaccharide molecule because para-nitrophenyl chitin (CO-PNP) that is structurally very similar to paramethoxy- phenyl (MP) modified at this position showed normal activation of ERK (Fig.2 and 3A). The C2 and C3 position of CO molecule seem to be essential for its biological property. We also tested a chitin tetrasaccharide analog, thioCO, in which the oxygen atoms in glycosidic linkages were replaced by sulfur atoms. This analog, which cannot be enzymatically hydrolyzed (Wang and Lee, 1995) was as potent an inducer as chitin tetrasaccharide. The half-maximal responses, induced by 15 min treatment with CO and thioCO, were obtained at 10^{-9} M concentration for both compounds (Fig.3A). These results suggest that the observed activation of ERKs is chitin tetrasaccharide± specific and does not require further enzymatic processing.

Signaling pathway to ERK activation by chitin tetrasaccharides

In general, ERK activation involves a cascade of phosphorylation events initated by stimulation of the Ras protooncogene following activation of growth factors receptors. The cascade starts with the activation of Raf, which phosphorylates MAPK kinase (MEK) on two serine residues, and then ERK is dually phosphorylated on a tyrosine and threonine residues by MEK. Activated ERK phosphorylates specific serines and threonines of target protein substrates.

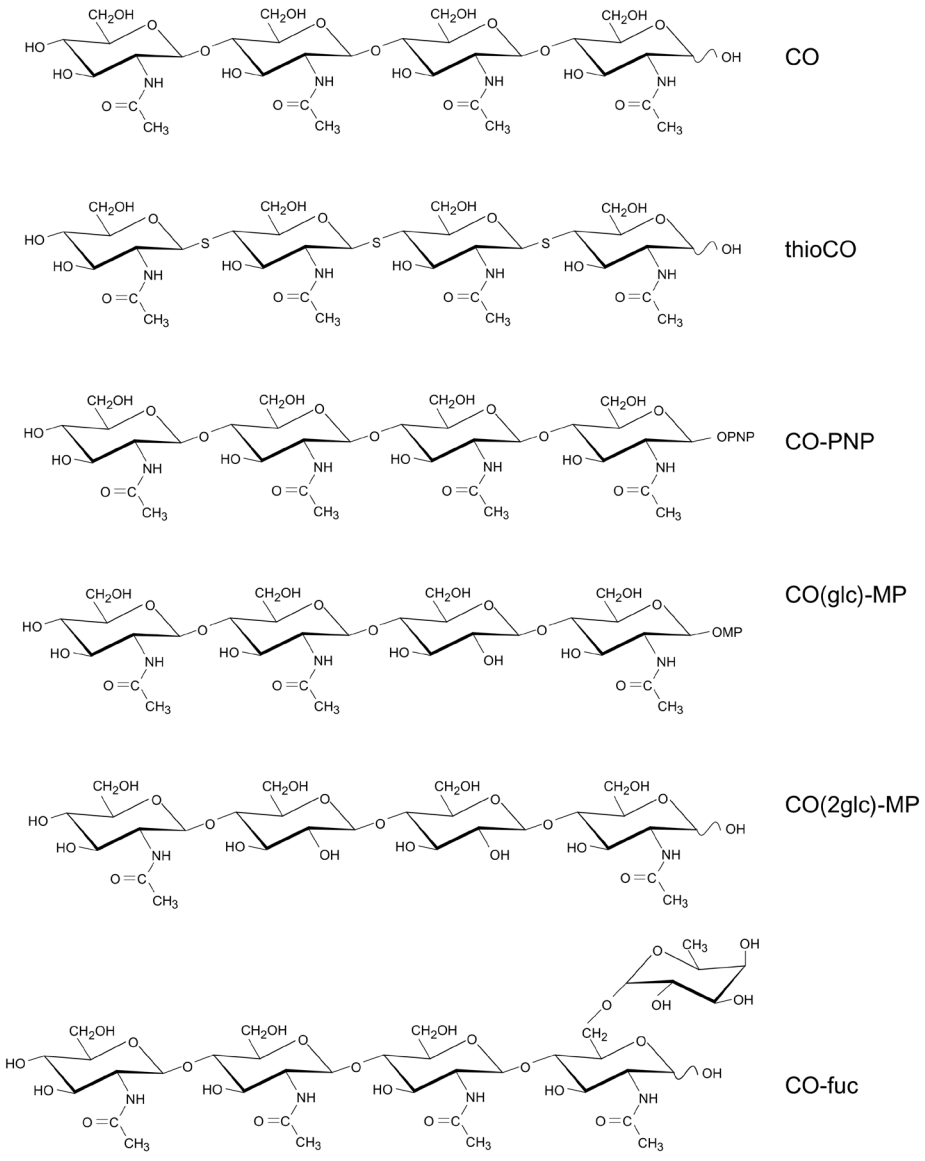

Figure 2. Structures of CO derivatives. CO: chitin oligosaccharide; thioCO: CO in which the oxygen atoms of the β(1→4) linkages are substituted by sulfur atoms; CO-PNP: CO with PNP modification at C1 position; CO(Glc)-MP and CO(2Glc)-MP: one or two GlcNAc residues are replaced by glucose, respectively, and with MP modification at C1 position; CO-fuc: fucosylated CO.

Several downstream targets of ERK1/2 have been identified, for instance, p90RSK is activated by ERK1/2 via phosphorylation at Ser380 (Dalby et al., 1998). To examine a possible signaling pathway to ERKs activation by chitin tetrasaccharides, we made use of phospho-Raf (Ser259), phospho-MEK1/2 (ser217/221), and phospho-p90RSK (Ser380) antibodies to follow expected phosphorylation events in ERK activation cascade. The activation of MEK1/2 and subsequently ERK were blocked by a highly selective inhibitor of MEK1 and MEK2, UO126, at 10^{-5} M concentration (Crews et al., 1992). The result in figure 4A show that CO, thioCO, and EGF induced phosphorylation/ activation of Raf, MEK1/2, and p90RSK with the same kinetics and specificity observed for ERK activation. Again CO-fuc and CO(2glc)-MP were found to be not active (Fig.3A and 4A). Preincubation of cells for 1 h with MEK1/2 inhibitor before stimulation prevented stimulation of ERK phosphorylation by CO and EGF (Fig.4B). From this we conclude that chitin oligosaccharides are recognized by a high-affinity receptor system that activates Raf, MEK, and ERK pathway in these zebrafish cell lines.

Discussion

Many human diseases, including the formation of tumors and their subsequent metastatic spread through the body, are caused by defects in carbohydrates recognition (Schuster and Nelson, 2000). Furthermore, all human pathogens also naturally produce a great variety of carbohydrates and use an unknown number of these molecules to modulate the innate immune response of the host (Aderem and Ulevitch, 2000). Most of these carbohydrates are polymers either in free form or linked to proteins. However, evidence exists that also small oligosaccharides, such as COs, play a crucial role in animal development. The genetic and cellular basis of the CO recognition mechanisms underlying their function in embryonic development, tumorigenesis, or infectious diseases is still not understood. The zebrafish model system offers novel opportunities to study the interplay between signal perception, transmission, and development. In this study we address whether COs are able to function as signaling molecules to activate the canonical ERK pathway in embryonic zebrafish cell cultures. The MAPK/ERK pathway was chosen as biological tool in this project because it plays a major role in the integration of multiple biological responses controlling development (Pouysségur et al., 2002). The activation of ERKs and downstream components was monitored by phosphospecific antibodies because the general scheme of activation involves a cascade of phosphorylation events following activation of specific receptors

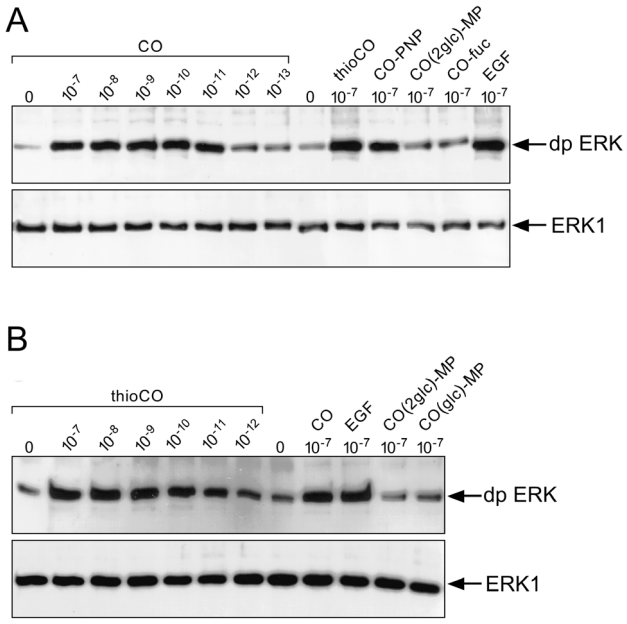

Figure 3. Dose dependence and specificity of ERK activation by CO and derivatives. Serum-starved zebrafish cells (ZF13) were stimulated with various concentrations of CO, CO(2glc)-MP or CO-fuc (A) and thioCO (B) for 15 min. Phosphorylated and nonphosphorylated forms of ERK were detected by dp ERK and ERK1 antibodies. These results are representative of three experiments. 10^{-7} M EGF was used as a control in A and B.

by appropriate signal.

First, we found that chitin tetrasaccharides transiently induced phosphorylation of ERKs, probably ERK1, with a maximum after 15 min stimulation, at 10^{-9} M concentration. This response was very specific for the structure of the glycan backbone. The replacement of one or two GlcNac residues by glucose and fucosylation by the NodZ protein resulted in a loss of their biological activity. In contrast, an analog of CO, thioCO, which could not be enzymatically hydrolyzed, was as potent an inducer as COs, excluding a role of possible degradation products. Furthermore, we also examined the signaling pathway to ERK activation by COs and concluded that a high-affinity oligosaccharide receptor system exists that transduces the signal to Raf, MEK, and ERK. Once activated/phosphorylated ERK phosphorylates p90RSK kinase, known as MAPK-activated protein kinase-1, MAPKAP-K1. Phosphorylated RSKs in turn activate transcription factors, such as CREB, and are involved in cell cycle control, memory formation, and suppression of apoptotic cell death (Nebreda and Gavin, 1999). Moreover, inactivation mutations in the gene en-

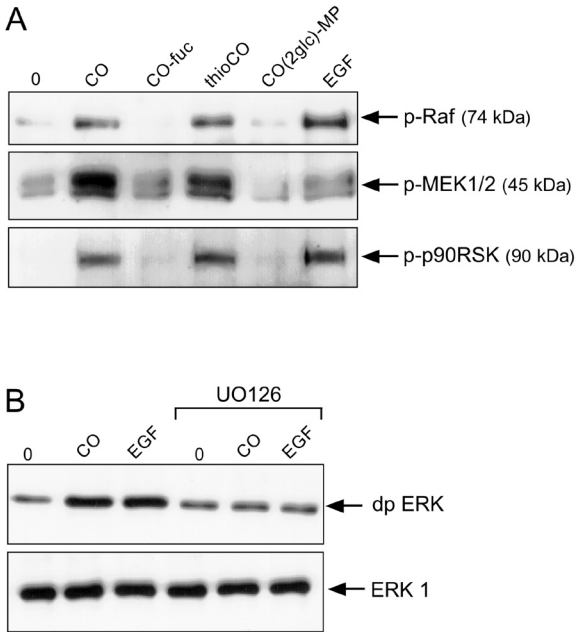

Figure 4. Signaling pathway involved in ERK activation by CO and CO derivatives. Serum-starved zebrafish cells (ZF13) were stimulated with 10^{-7} M of CO, CO derivatives, and EGF for 15 min. Lysates were subjected to western blotting with phosphospecific p-Raf, p-MEK1/2, and p-p90RSK antibodies (A). Inhibition of CO and EGF induced activation of ERK by MEK inhibitor. Serum-starved zebrafish cells (ZF13) were pretreated for 1 h with 10^{-5} M of a highly selective MEK1/2 inhibitor (UO126) prior to 15 min stimulation of ERK phosphorylation by 10^{-7} M CO and EGF (B). Phosphorylated and nonphosphorylated forms of ERKs were detected by dp ERK and ERK1 antibodies.

coding human RSK2 are associated with Coffin-Lowry syndrome, a disease that results in severe mental retardation as well as progressive skeletal deformation (Treisman, 1996). The recently discovered RSK4 is deleted in patients with X-linked mental retardation (XLMR) and may be a candidate XLMR gene (Yntema et al., 1999).

Our results strongly support the model (Fig.5), but final genetic identification of all components of CO signaling remain to be determined. Several lines of evidence in different cell types indicate that p90RSKs are activated by MAPKs *in vivo* via a Ras-dependent protein kinase cascade that is triggered by growth factors or tumor-promoting phorbol esters (Alessie et al., 1995). Moreover, other physiological substrates of p90RSK have been identified with different effects in development, cell cycle, suppression of apoptotic cell death, and progressive skeletal deformation (Treisman, 1996; Nebreda and Gavin, 1999). These findings promote future studies of p90RSK and its physiological

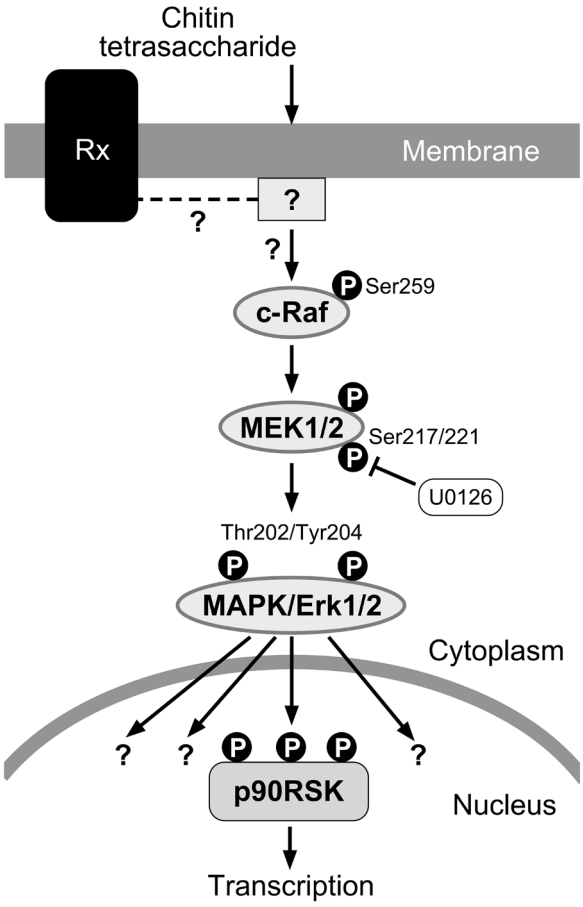

Figure 5. Model for specific activation of the ERK pathway by COs in embryonic zebrafish cells. Chitin tetrasaccharides probably activate a high-affinity receptor system (Rx) that transduces the signal to phosphorylation of Raf, MEK1/2, ERK1/2, and p90RSK. An arrow indicates an alternative path through the membrane.

substrates in zebrafish cells and embryos.

We recently cloned two zebrafish ERK genes in which the phosphorylation consensus is identical to the mammalian species and forms the expected epitope recognized by the dpERK antibody. With a morpholino knock-down approach we plan to find specific CO targets and study their function in zebrafish cells and embryo development. Recent studies in plants have identified leucine-rich repeat (LRR) receptors involved in recognizing carbohydrate microbial factors, such as the Nod factors (Asai et al., 2002; Stracke et al., 2002; Endre et al., 2002). LRR domains are also found in plants as part of disease

resistance cascades, where they function as receptors important for innate immunity. In animals, the relatives of the plant receptors, so called Toll-like receptors, are also involved in the recognition of microbes, meaning that a similar carbohydrate-recognition mechanism could underlie the plant and animal innate immune system (Beutler, 2002; Spaink, 2002). Recently we identified ~20 Toll-like receptors in the zebrafish database from Sanger Centre (unpublished data). Currently we study their expression during embryogenesis and in response to treatment of cell lines with different carbohydrates to elucidate their role in CO signaling cascade.

In future research we aim to identify CO receptors and link them to downstream pathways, such as the ERK pathway, to fully understand CO function in vertebrate development.

Materials and methods

Materials

Polyclonal phospho-p44/42MAP kinase antibody, phospho-ERK1/2 pathway sampler kit, phospho-p38 MAPK antibody, phospho-SAPK/JNK antibody, and MEK1/2 inhibitor (UO126) were purchased from Cell Signaling Technology (Leusden, The Netherlands). Control ERK antibody K-23 was from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody, enhanced chemiluminescence-kit, and rainbow molecular weight markers were from Amersham Life Science (Roosendaal, The Netherlands). Murine EGF was obtained from Invitrogen (Breda, The Netherlands). Leibovitz L-15 medium (L-15) and fetal calf serum were purchased from Invitrogen.

Chitin tetrasaccharides were purchased from Seikagaku (Tokyo). Replacement of one or two central GlcNac residues of the chitin tetrasaccharide by Glc unit was received as described (Wang and Lee, 1995; Robina et al., 2002; Southwick et al., 2002). Fucosylation of COs by NodZ protein was performed as described previously (Bakkers et al., 1997).

The syntheses of thioCO

The syntheses of di-N-acetyl-4-thiochitobiose (TG_2), α-methyl di-*N*-acetyl-4-thiochitobioside (MTG_2), α-methyl tri-*N*-acetyl-4,4'-dithiochitotrioside (MTG_3), α-methyl tetra-*N*-acetyl-4,4',4''-trithiochitotetraoside (MTG_4), and β-methyl tri-*N*-acetyl-4,4'-dithiochitotrioside (β-MTG3) were reported in Wang and Lee (1995). The reducing thiochito-oligomers, tri-*N*-acetyl-4,4'-dithiochitotriose

(TG_3) and tetra-*N*-acetyl-4,4',4''-trithiochitotetraose (TG_4), were prepared as follows.

TG_3. The peracetylated 4,4'-dithiochitotriose (20 mg) (Wang and Lee, 1995) was de-*O*-acetylated with sodium methoxide in methanol (10 mM, 5 ml) at room temperature for 6 h. Water (1 ml) was added and the solution was neutralized with Dowex 50w-x8 resin (H^+ form). After removal of the resin by filtration, the filtrate was concentrated. The trisaccharide was dissolved in a small amount of water and applied to a Sephadex G-10 column (1.5 x 90 cm), which was pre-equilibrated and eluted with 50 mM acetic acid. Fractions containing the trisaccharide were pooled and lyophilized to give 15.5 mg TG_3 (yield: 81%). ^{1}H- nuclear magnetic resonance (NMR) of TG_3 (500 MHz, D_2O, 60°C): δ 5.248 (d, *J* = 3.4 Hz, 0.35 H, H-1α), 4.776 - 4.732 (two pairs of d, *J* = 10.2 Hz, 2 H, H-1',1''), 4.695 (d, *J* = 8.4 Hz, 0.65 H, H-1β), 4.057 - 3.470 (m, 16 H, sugar protons), 2.957 - 2.856 (two pairs of t, 2 H, *J* = 10.4 Hz, H-4,4'), 2.059 - 2.049 (singlets, 9 H, 3 N-acetyl).

TG_4. The α-methyl 4,4',4''-trithiochitotetraoside (4.5 mg) (Wang and Lee, 1995) was per-*O*-acetylated with acetic anhydride±pyridine (1:1, 1.5 ml) at room temperature for 5 h. Ice water (2 ml) was added, and the solution was evaporated to dryness. Trace of volatiles was removed by coevaporation of the residue with toluene (3 x 2 ml) to give 7.5 mg of the peracetylated α-methyl 4,4',4''-trithiochitotetraoside. The compound obtained was subjected to acetolysis with Ac_2O-AcOH-H_2SO_4 (8:2:0.1, 2 ml) to selectively remove the 1-*O*-methyl group. After acetolysis at room temperature for 12 h, the solution was cooled to 0°C, and a cold solution of aqueous sodium acetate (0.2 M, 3 ml) was added. The mixture was evaporated to dryness and the residue was partitioned between $CHCl_3$ (10 ml) and water (2 ml). The organic layer was separated and successively washed with 5% hydrochloric acid (1 ml), saturated sodium bicarbonate (1 ml), and water (2 x 1 ml), dried with anhydrous sodium sulfate, and filtered. The filtrate was evaporated to afford the peracetylated 4,4',4''-trithiochitotetraose as a white solid, which was subsequently de-*O*-acetylated with sodium methoxide in methanol (10^{-2} M, 5 ml). The product was purified by gel filtration with a Sephadex G-10 column the same way as described for the preparation of TG_3 to give 3.5 mg TG_4 (overall yield in three steps: 79%). ^{1}H-NMR of TG_4 (500 MHz, D_2O, 60°C): δ 5.246 (d, *J* = 3.4 Hz, 0.39 H, H-1α), 4.769-4.725 (3 pairs of d, *J* = 10.3 Hz, 3 H, H-1',1'',1'''), 4.693 (d, *J* = 8.0 Hz, 0.61 H, H-1β), 4.059- 3.467 (m, 21 H, sugar protons), 2.945-2.872 (three pairs of t, 3 H, *J* = 10.3 Hz, H-4,4',4''), 2.050-2.047 (singlets, 12 H, 4 *N*-acetyl).

Cell lines

Cell lines ZF13 and ZF29c-1 were obtained from the Hubrecht laboratory. These cells have been derived from dechorionated, disaggregated, 20 h-old zebrafish embryos. They have a fibroblast-like morphology (Peppelenbosch et al., 1995). For our experiments cells were grown at 25°C in 2 ml 67% L-15 medium supplemented with 10% fetal calf serum in 24-well plates (Greiner, Alphen aan de Rijn, The Netherlands) until the plates became 50% confluent. Before stimulation experiments, the medium was changed to serum-free medium (SFM) for 18-24 h to reduce the basal level of phosphorylation. This SFM was then replaced with 1 ml SFM plus indicated stimulus for various times. Stimulation was terminated by quick replacement of SFM for 600 μl, 65°C sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, 3% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue). The cells were scraped off the dish, and the cell lysates were transferred to a new tube, boiled for 3 min, and separated on 10% polyacrylamide slab gels (10 μg protein per lane).

Western immunoblotting

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). After electrophoresis, proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Den Bosch, The Netherlands) by western blotting. The membranes were blocked in 5% w/v nonfat dry milk in Tris-buffered saline-Tween 20 (TBST). The blots were incubated with a 1:1000 to 1:2000 dilution of the indicated antibody in TBST with 3% bovine serum albumin (Sigma, St. Louis, MO) for 1 h at room temperature or overnight at 4°C. Signal was detected using a 1:5000 to 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit antibodies and the enhanced chemiluminescence method (Amersham).

Acknowledgments

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III

Characterization and expression patterns of the MAPK family in zebrafish

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Gene Expression Patterns 6 (2006) 1019 - 1026

Abstract

The mitogen activated protein kinases (MAPK) family 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.

Introduction

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 aminoterminal 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 activation domain and p38-isoforms have a TGY activationsdomain (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-El-Leil et al., 2003), *erk5* (Yan et al., 2003) and *p38*α (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 patters during zebrafish development.

Results and discussion

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*α 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*β gene. The results show that a smaller number of *mapk* genes is not specific for the zebrafish, but is general for Teleost fish.

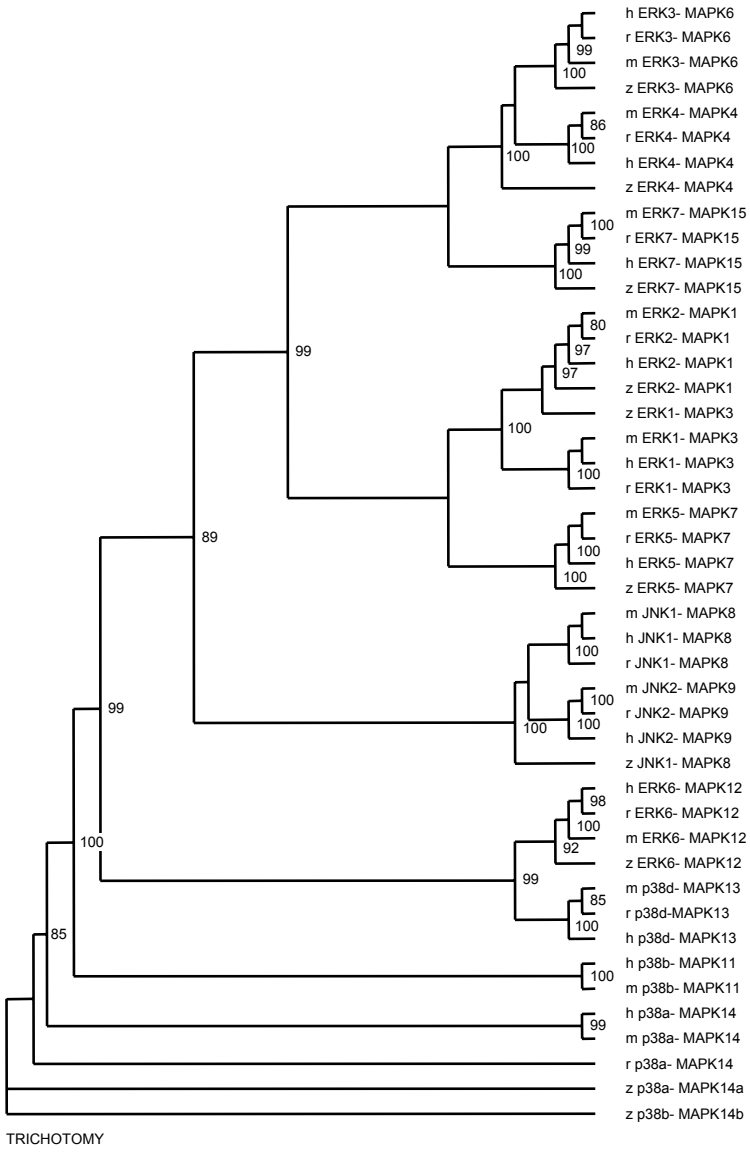

Figure 1. Unrooted phylogenetic tree of the zebrafish MAPK family to other vertebrates. The tree was constructed by neighbor-joining analysis based on an alignment of the amino acid sequences of the complete predicted and sequencing verified coding sequences. The numbers indicate the occurrence of nodes 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.

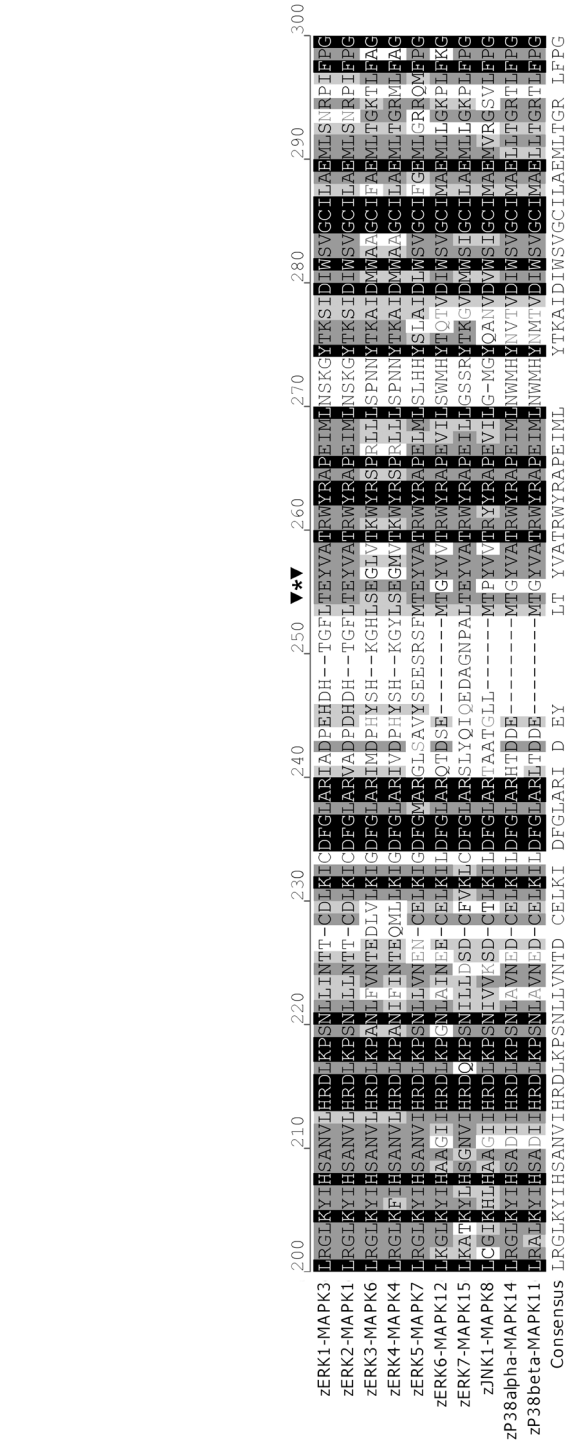

Figure 2. Amino acid alignment of the protein-region around the characteristic MAPK dual-phosphorylation domain of the zebrafish MAPK orthologs. Identical residues are indicated with black boxes, strongly conserved in dark gray and similar residues are boxed light grey. The phosphorylated amino acids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

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 chomosomal 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 orthologues. Both zebrafish p38a and p38b cluster with the p38α cluster and showed an identity of approximately 86% and 84% compared to human, mouse and rat p38α. The identity to p38β of zebrafish p38a and p38b were approximately 69% and 66%, respectively. This indicated that p38a and p38b are both p38α isoforms. In rat also no ortholog for p38β (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.

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 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 seems 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* was weakly expressed in early developmental stages, but show higher expression at late gastrula-stages to segmentation (80% epiboly to 18 somites). From 24 hpf to 5 dpf the expression-level of *jnk1* was low, and reached a high expression-level again in adult fish. *P38b* showed 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 hpf – 5 dpf). In adult fish, expression of *erk4* is hardly detectable. *Erk5* is strongly maternal expressed. Expression levels slowly decrease to a barely detectable level at mid-somitogenesis (12-somites), after which *erk5*-expression levels increases 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

Table1

The zebrafish MAPK gene-family

Protein name	Synonyms	Orthologs	Subfamily classification	Phosphorypation domain	size (aa/kDa)	linkage group	Exons
ERK1	MAPK3 p44-MAPK	*h_erk1* *m_erk1* *r_erk1*	ERK	TEY	392/44.1	LG:3	8
ERK2	MAPK1 p42-MAPK	*h_erk2* *m_erk2* *r_erk2*	ERK	TEY	369/42.1	LG:5	8
ERK3*	MAPK6 p97-MAPK 055-MAPK (rat)	*h_erk3* *m_erk3* *r_erk3*	ERK	SEG	729/83.2	LG:18	6
ERK4*	MAPK4 p63-MAPK ERK3 related MNK2 (rat)	*h_erk4* *m_erk4* *r_erk4*	ERK	SEG	674/75.9	-	7
ERK5	MAPK7 BMK1	*h_erk5* *m_erk5* *r_erk5*	ERK	TEY	862/95.0	LG:12	9
ERK6	MAPK12 p38γ SAPK3	*h_erk6* *m_erk6* *r_erk6*	p38	TGY	363/42.0	LG:18	12
ERK7	MAPK15	*h_erk7* *h_erk8* *m_erk7* *r_erk7*	ERK	TEY	534/60.2	LG:25	14
JNK1	MAPK8 SAPK1	*h_jnk1* *m_jnk1* *r_jnk1*	JNK	TPY	384/44.1	LG:13	6
p38a	MAPK14a p38α SAPK2A	*h_p38*α *m_p38*α *r_p38*α	p38	TGY	361/41.6	LG:8	12
p38b	MAPK14b p38α SAPK2A	*h_p38*α *m_p38*α *r_p38*α	p38	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.

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*.

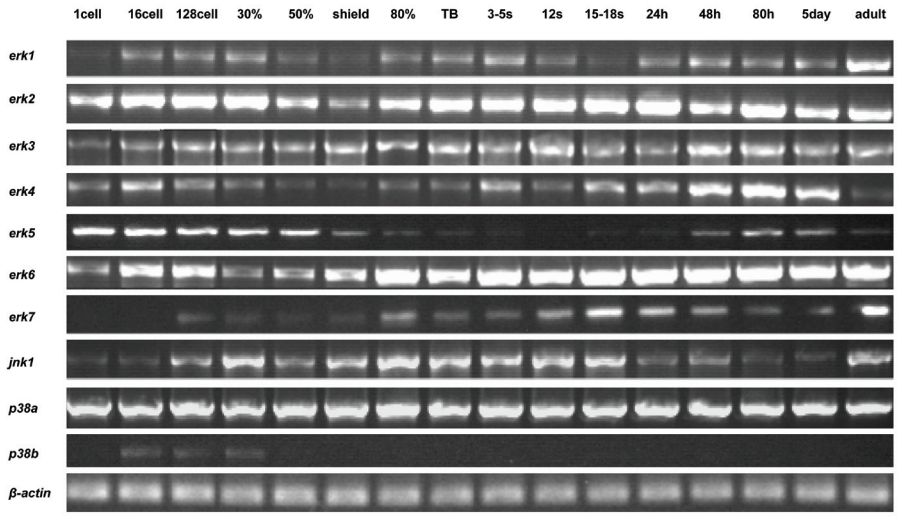

Figure 3. Temporal expression patterns of zebrafish MAPKs by RT-PCR analysis through zebrafish development. RNAs used for amplification were isolated from indicated stages. To obtain specific expression patterns, primers flanking the full coding sequences were used, but for *erk7*, where primers specific 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*. β-Actin (β-ACT) was used as a control for constitutive expression.

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 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

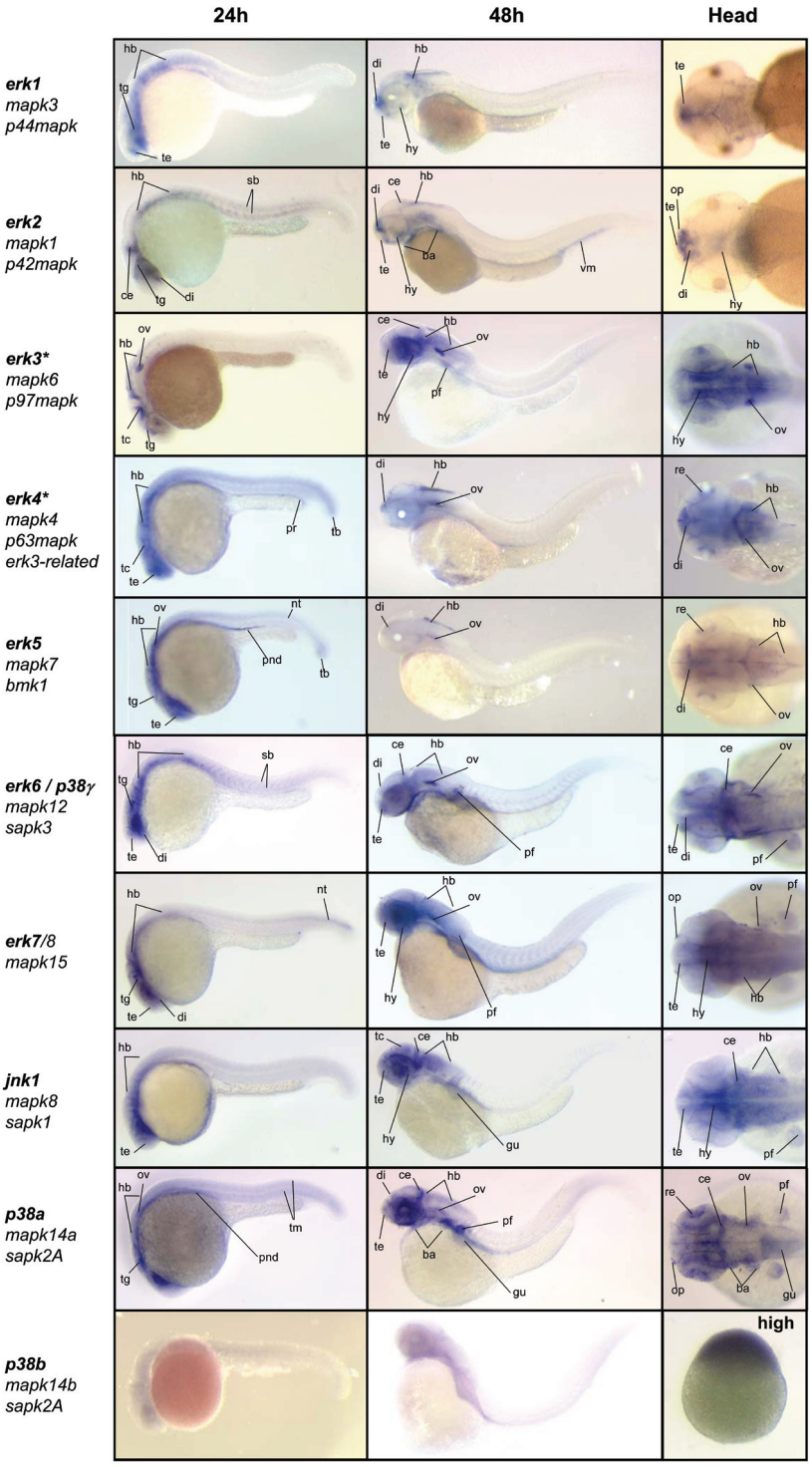
24h
48h
Head
erk1
mapk3
p44mapk
erk2
mapk1
p42mapk
erk3*
mapk6
p97mapk
erk4*
mapk4
p63mapk
erk3-related
erk5
mapk7
bmk1
erk6 / p38γ
mapk12
sapk3
erk7/8
mapk15
jnk1
mapk8
sapk1
p38a
mapk14a
sapk2A
p38b
mapk14b
sapk2A
high

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 p38α 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 ~3.5 hpf (high stage) showing that expression was detected in all blastula cells.

Figure 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 astrix (*) 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; ov, otic vesicle; 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.

The expression of the *mapk* genes 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.

Experimental procedures

Animals

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

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 Clustal W (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, Q9WUI1; 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.

Cloning of MAPK gene family in zebrafish

Total RNA was isolated from adult Tuebingen zebrafish, using TRIZOL® Reagent protocol (GIBCOBRL, Life technologies™). 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®4Blunt-TOPO® or pCRII-TOPO® (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® 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.

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® 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 β-actin (AF057040) was used as a control for constitutive expression.

Whole-mount in situ hybridization

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

Acknowledgement

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Supplementary data

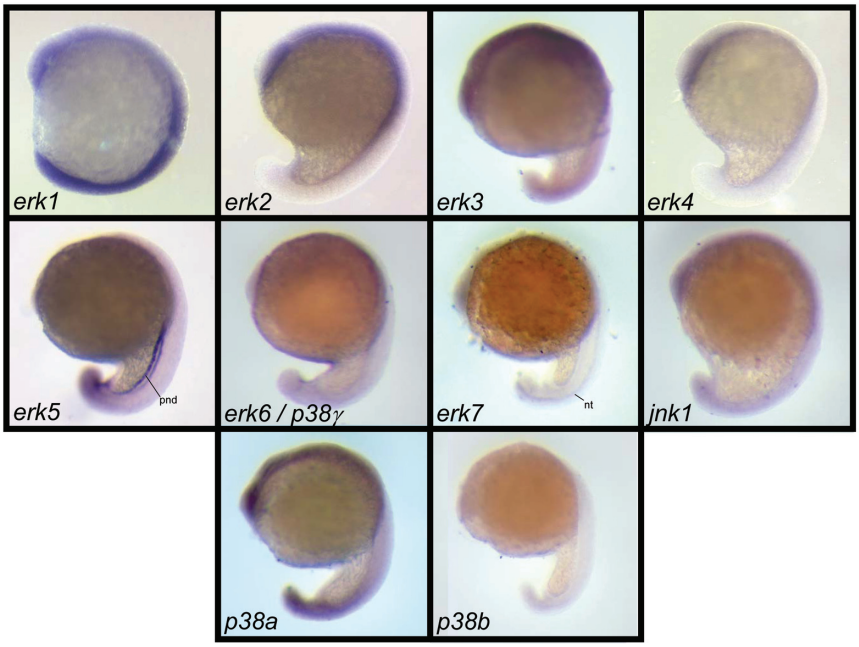

Figure. S1 Expression patterns obtained for the zebrafish mapk genes by in situ hybridization at 15–17 hpf. Lateral view, anterior to top, dorsal to right. Almost all mapk showed a ubiquitously expression, however expression for *erk5* was already enriched in the pronephric ducts and *erk7* expression was enriched in the notochord. *p38b* was not expressed at this stage. nt, notochord; pnd, pronephric ducts

IV

Distinct functions for ERK1 and ERK2 in cell migration processes during zebrafish gastrulation

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submitted

Abstract

The MAPKs are key regulatory signaling molecules in many cellular processes. Here we define differential functions for ERK1 and ERK2 MAPKs in zebrafish embryogenesis. Morpholino knockdown of ERK1 and ERK2 resulted in cell migration defects during gastrulation, which could be rescued by co-injection of the corresponding mRNA. *Erk2* mRNA cross-rescued ERK1 knockdown, but *erk1* mRNA was unable to compensate for ERK2 knockdown. Cell-tracing experiments in knockdown embryos revealed a convergence defect for ERK1 morphants without a severe posterior-extension defect, whereas ERK2 morphants showed a more severe reduction in anterior-posterior extension. These defects were primary changes in gastrulation cell movements and not caused by altered cell fate specification. More stringent knockdown conditions showed that the absence of dual-phosphorylated ERK2 from the blastula margin blocked initiation of epiboly and arrested embryogenesis, whereas ERK1 knockdown had only a mild effect on epiboly progression. Together, our data show distinct roles for ERK1 and ERK2 in developmental cell migration processes during zebrafish embryogenesis.

Introduction

The mitogen-activated protein kinase (MAPK) cascade governs key signaling pathways that control cell proliferation, differentiation and survival responses in all eukaryotes. Altered MAPK signaling is associated with developmental defects and various pathologies, including cancer. The MAPK cascade involves sequential activation of a serine/threonine kinase (MAPKKK), followed by a dual-specific MAPK kinase (MAPKK) and a dual-phosphorylated MAPK target. The vertebrate family of MAPKs consists of three major subfamilies: ERK, JNK and p38 (Johnson and Lapadat, 2002). An important challenge is to understand the complexity of the different MAPK cascades. Although specificity of these cascades has been reported (Kolch, 2005), it is clear that cross-talk occurs and strong indications for redundancies exist (Johnson et al., 2005). Distinct cellular functions in cancer formation have recently been shown for ERK1 and ERK2, which are the most intensively studied MAPKs of the ERK subfamily (Lloyd, 2006). For example, tumorigenicity of transplanted NIH 3T3 cells stably expressing an oncogenic form of Ras in nude mice was largely inhibited by co-transfection of ERK1, but not by ERK2 or p38 (Vantaggiato et al., 2006).

The canonical pathways of ERK1/2 activation are well studied *in vitro* and different animal models have been used to address ERK1 and ERK2 functions

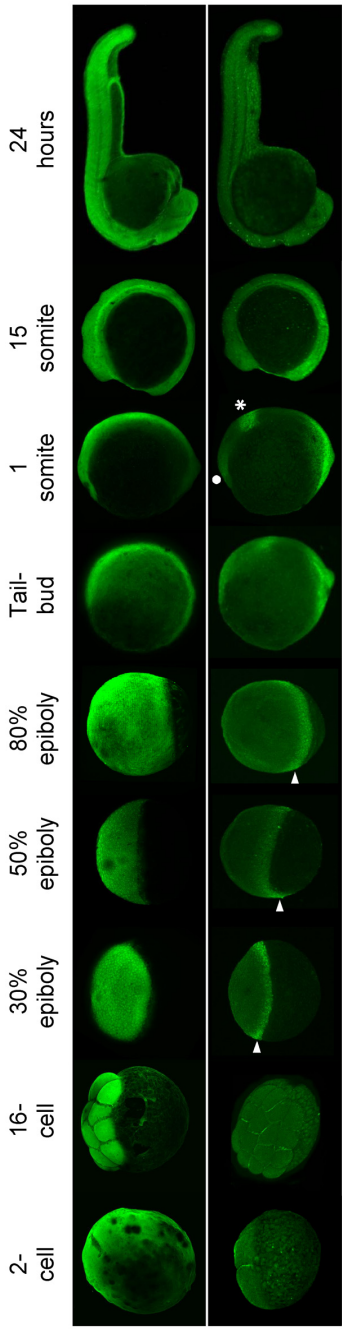

Figure 1. Spatio-temporal distribution of total ERK protein, compared to active dual-phosphorylated ERK (dpERK) protein by immuno-histochemistry. The dpERK signal was stronger on the cleavage-sites (2-cell and 16-cell stage). During gastrulation dpERK signal was enhanced in the margin (▲). After completion of gastrulation stronger signals were observed in the tailbud, MHB (*) and the anterior neural boundary (•). (5x objective, Biorad confocal laser scanning microscope)

in development. However, due to their crucial roles in early embryogenesis, the differential functions of ERK1 and ERK2 are not yet clearly defined. In *Drosophila*, FGF-dependent ERK activation was shown to be required for proper mesoderm dispersal (Gabay et al., 1997;Gryzik and Muller, 2004;Stathopoulos et al., 2004;Wilson et al., 2005) In chick, functions for activated ERK1/2 in axon growth were suggested (Kato et al., 2005). In *Xenopus*, ERK2 was shown to be required for mesoderm differentiation (Gotoh et al., 1995) and neural specification (Umbhauer et al., 1995;LaBonne et al., 1995;Uzgare et al., 1998). Studies using knockout mice clearly indicated that ERK1 and ERK2 have distinct functions. Gene disruption of *erk2* is lethal during early development, showing that ERK1 is not redundant to ERK2 (Yao et al., 2003). In contrast, *erk1*-/- mice are viable, fertile and of normal size. However, the proliferation and maturation of the thymocytes is affected, despite expression of ERK2 (Gilles Pagès et al., 1999) . Furthermore, ERK1 has a critical regulatory role in long-term adaptive changes of the brain, underlying striatum-dependent behavioral plasticity and drug addiction (Mazzucchelli et al., 2002). *Erk1*-/- mice showed improved rate of learning and better long term memory than wild type controls. Elevated ERK2 activation was observed in primary neurons isolated from *erk1* knockout mice, whereas no higher ERK2 levels were detected in the brains. 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. *Erk2*-deficient mice fail to form the ectoplacental cone and the extra-embryonic ectoderm, which gives rise to mature trophoblasts in the fetus (Saba-El-Leil et al., 2003). *Erk2*-/- embryos also fail to form mesoderm, based on histological criteria at E6.5 and E7.5 (Yao et al., 2003). Finally, ERK2 is critical for proliferation of the trophoblast stem cell-population present in the trophectoderm and proximal extra-embryonic ectoderm, which is essential for placenta formation (Ornitz and Itoh, 2001).

In this study we aimed to increase the understanding of the differential roles of ERK1 and ERK2 in early vertebrate embryogenesis and cell-migration processes, using the zebrafish model. Because of its *ex utero* development the zebrafish model is optimally suitable to study the link between the cellular and developmental functions of ERK1 and ERK2. Previously, immunohistochemical analysis of the spatio-temporal patterns of ERK1/2 phosphorylation showed that, like in mouse (Corson et al., 2003) and chick (Delfini et al., 2005), zebrafish ERK1/2 is activated locally during segmentation stages (Sawada et al., 2001). Furthermore, it was shown that insulin-like growth factors (IGFs) stimulated zebrafish cell proliferation by activating ERK-MAPK and PI3-kinase signaling pathways (Pozios et al., 2001). Subsequently, research on the ERK-MAPK pathway in zebrafish development has 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 (Tsang et al., 2004). Over-activation of the FGF/ERK-pathway led to dorsalized embryos by inhibiting expression of BMP genes (Furthauer et al., 2004). In contrast, over-expression of ERK-MAPK phosphatase MKP3 or injection of a high dose of mRNA of the inhibitor *Sef* resulted in an opposite ventralization (Furthauer et al., 2002;Tsang et al., 2004). More recently, endoderm formation was shown to be regulated by combinatorial Nodal, FGF and BMP signaling, where BMP and FGF signaling cooperate to endoderm formation in response to Nodal signaling (Poulain et al., 2006). Recently, we identified the zebrafish orthologs of the MAPK gene family and determined their specific spatial and temporal expression patterns during zebrafish embryogenesis (Krens et al., 2006). We showed that the zebrafish genome encodes for members of all MAPK-subfamilies; ERK, JNK and p38. Furthermore, we showed that *erk1* and *erk2* are differentially expressed compared to each other and to the other members of the zebrafish MAPK gene-family. However, no gene-targeting studies have been reported until now.

Here, we report on knockdown studies of ERK1 versus ERK2 and present evidence for differential effects on convergence extension cell movements. Cell tracing experiments showed that ERK1 morphants displayed reduced convergence cell movements, whereas ERK2 morphants showed a more severe reduction in anterior-posterior extension of the dorsal body axis, without significantly altering the early cell fate specification. Stronger knockdown conditions for ERK2 arrested embryogenesis at the onset of epiboly, thereby preventing the blastula to gastrula transition. Taken together, our data imply distinct functions for ERK1 and ERK2 MAPKs in gastrulation cell migration during zebrafish embryogenesis

Results and discussion

Immuno-histochemical analysis of the localization of active / phosphorylated ERK1 and ERK2 MAPK showed elevated levels of ERK phosphorylation in the cleavage furrows at the 16 cell stage, in the margin during epiboly and gastrulation, at locations of neural differentiation (anterior neural boundary and mid-hindbrain boundary, Fig.1) and segmentation (Pozios et al., 2001;Sawada et al., 2001). Immunohistochemistry cannot distinguish between ERK1 and ERK2. However, we recently showed that *erk1* and *erk2* display differential spatiotemporal expression patterns during zebrafish development (Krens et al., 2006), suggesting distinct functions for ERK1 and ERK2 during zebrafish development.

Morpholino knockdown of ERK1 and ERK2 results in specific phenotypes

To elucidate the functions of ERK1 and ERK2 in zebrafish development, gene-specific morpholinos (MO) directed to the 5' untranslated sequences were used to block the translation of *erk1* or *erk2* mRNAs. Injection of 0.2mM (1.7 ng) ERK1MO or ERK2MO induced severe developmental defects (Fig.2), while treatment with the same concentration of the standard control MO had no effect (data not shown). At 24 hpf approximately 10% of ERK1MO injected embryos were dead, and 85% (n=130) displayed phenotypes characterized by a shorter body axis and somites without the distinct v-shape (Fig.2A). The somitogenesis phenotype was confirmed by in situ hybridization with *myod* (supplemental data Fig.S1). In contrast, ERK2MO injected embryos (n=75) showed approximately 70% lethality at 24 hpf and 85% at 48 hpf (Fig.2E) and the surviving ERK2MO morphants showed more severe phenotypes than the ERK1 morphants. At 24 hpf, the head was still distinguishable, but the MHB was not properly formed as shown by lack of *pax2.1* gene expression (Brand et al., 1996). In addition, development of the tail structures was severely defected (Fig.2B, Fig.S1), resulting in shortened phenotypes at 48 hpf (Fig.2G). Somites structures were barely recognizable and the associated *myod* expression pattern was highly aberrant (Fig.S1). Approximately 50% of the embryos treated with ERK1MO and 15% treated with ERK2MO survived up to 48 hpf (Fig.2E). The surviving morphants were shortened at 24 and 48 hpf and had enlarged heart-cavities (Fig.2F,G). Protein analysis of the surviving embryos by western blot using a general ERK antibody showed that ERK1 and ERK2 protein levels were specifically and significantly reduced by the respective ERK1 and ERK2 MOs (Fig.2D).

To test the specificity of the obtained knockdown phenotypes, rescue experiments were performed using synthetic *erk1* and *erk2* mRNAs lacking the MO target site. Protein samples were made at the shield stage from embryos injected with 100 pg mRNA and western blot analyses showed that the synthetic mRNAs were correctly translated into proteins and were dual phosphorylated/activated in the embryo (Fig.S2B). For rescue experiments mRNA concentrations were used below those that induced over-expression phenotypes (Fig.S2). Co-injection of synthetic *erk1* mRNA (8 pg per embryo) with 0.2 mM ERK1MO rescued 67% of embryos with the shorter body axis at 24 hpf and 50% at 48 hpf (Fig.2E,F,H,J and L). Co-injection of *erk2* mRNA (1.5 pg per embryo) with 0.2 mM ERK2MO increased the percentage of survivors from ~20% to more than 70% at 24hpf and rescued the body axis defect in ~35% of the embryos (Fig.2E, G-M). The redundancy of the ERK1 and ERK2 was determined in cross-rescue experiments using the same mRNA concentrations as described above (Fig.2E and data not shown). ERK1 knockdowns were

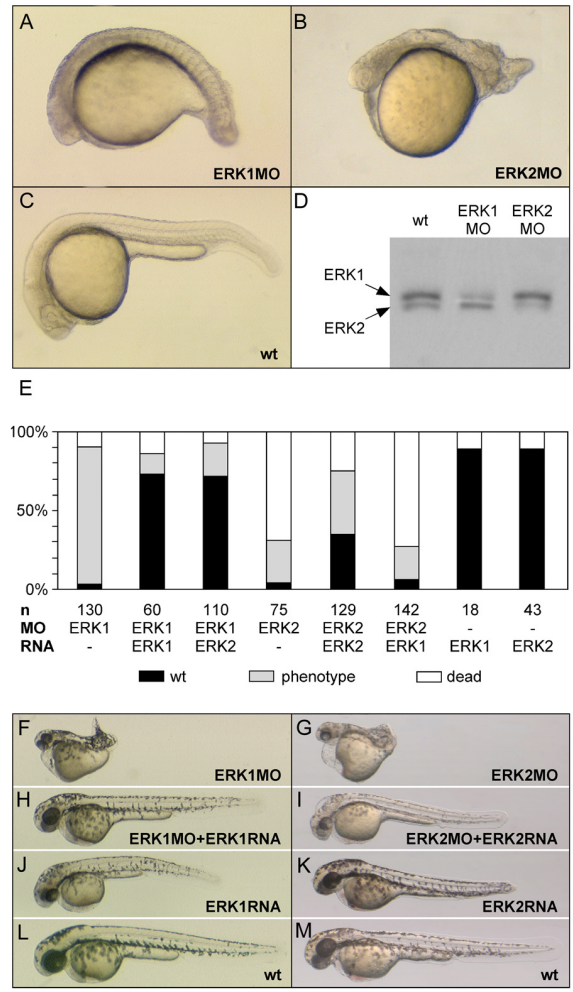

Figure 2. Specific ERK1 and ERK2 knockdown by morpholino injection. (A-C) Images show representative examples of the ERK1 (A) or ERK2 (B) knockdown phenotypes at 24 hpf, compared to wild type (wt) embryos (C). Specific knockdown was confirmed by Western blot analysis optimized to discriminate between the sizes of ERK1 (p44MAPK) and ERK2 (p42MAPK) protein and detected with global ERK antibody (D). Protein samples were isolated from 20 hpf embryos, injected with 0.2mM ERK1-MO or ERK2-MO. (E) Statistics of ERK knockdown and (cross-) rescue experiments determined by co-injection of ERK1 or ERK2 morpholino with synthetic *erk1* or *erk2* mRNA at 24 hpf. Black = wild type; gray = phenotype; white = dead.

(F-M) Images show phenotypes of surviving knockdown embryos at 48 hpf (F,G), embryos rescued by co-injection of corresponding mRNA (8pg *erk1* RNA, 1.5pg *erk2* RNA) (H,I), embryos injected with mRNA only (J,K) and wild type embryos at 48hpf (L,M)

rescued by co-injection of *erk2* mRNA with similar efficacy as by *erk1* mRNA. Co-injection of ERK2MO with *erk1* mRNA did not increase the number of surviving embryos nor rescued the body axis defects, indicating that the amount of *erk1* mRNA that rescued ERK1 knockdown cannot cross rescue the ERK2 knockdown phenotype.

Distinct functions for ERK1 and ERK2 in gastrulation cell movements

The low surviving rates and the severe phenotypes of ERK1 and ERK2 morphants at 24hpf indicate possible roles for these kinases in earlier developmental processes. To address this, embryos injected with ERK1MO or ERK2MO were closely monitored at yolk plug closure (YPC) until tailbud stages (Fig.3A-F). ERK1MO-injected embryos showed rather normal extension compared to control embryos (Fig.3A,B, distance between arrowheads), but showed a widening of the dorsal axial mesoderm structures (notochord, Fig.3D,E). Strikingly, ERK2MO-injected embryos showed a shortened anterior-posterior axis at the end of gastrulation (Fig.3C), but a far less severe effect on the widening of the notochord than ERK1MO-injected embryos (Fig.3F).

These results pointed towards a possible effect of ERK1 and ERK2 knockdown on dorsal-ventral patterning or convergence extension (CE) cell movements. We first addressed this question, using the marker genes *dlx3*, expressed in the neural plate, *sonic hedgehog* (*shh*), expressed in the notochord, and *hgg1*, expressed in the developing hatching gland, in 10 hpf embryos (Fig.3G-L) as previously described (Jopling and den Hertog, 2005). In ERK1 morphants, the *dlx3*-expression domain was much wider and the hatching gland was located more posterior (Fig.3H,K). Strikingly, ERK2 morphants did not show this widening of the neural plate (*dlx3*), suggesting that this defect was ERK1MO specific. In ERK2 morphants (Fig.3I,L) the *sonic hedgehog* (*shh*) expression domain in the midline was not fully extended in the anterior direction, resulting in a gap between the *dlx3* and shh expression domains. In addition, the expression domain of *hgg1* was located even more posterior than in ERK1 morphants, indicating affected extension in ERK2 morphants.

Depletion of ERK1 or ERK2 perturbs gastrulation cell movements differently, without significantly altering early cell fate specification

In order to address if the observed phenotypes in ERK1 and ERK2 knock down embryos were primary cell movement defects or secondary effects due to a patterning alteration we performed in situ hybridization with several different marker genes (Fig.4).

In ERK1MO injected embryos at late gastrula stages, in situ hybridization for markers of dorsal ventral patterning revealed a slight reduction of the ventral non neural ectoderm marker gene *gata2* (Fig.4B) and a concomitant expansion of the dorsal neural ectoderm marker *otx2*, suggesting mild dorsalization of ERK1MO embryos (Fig.4E). The expression domain of *cyclops* in the *axial* mesoderm was mediolaterally expanded (Fig.4H). This observation was confirmed with the non-axial mesoderm marker *snail1a* (Fig.4K) showing a bigger

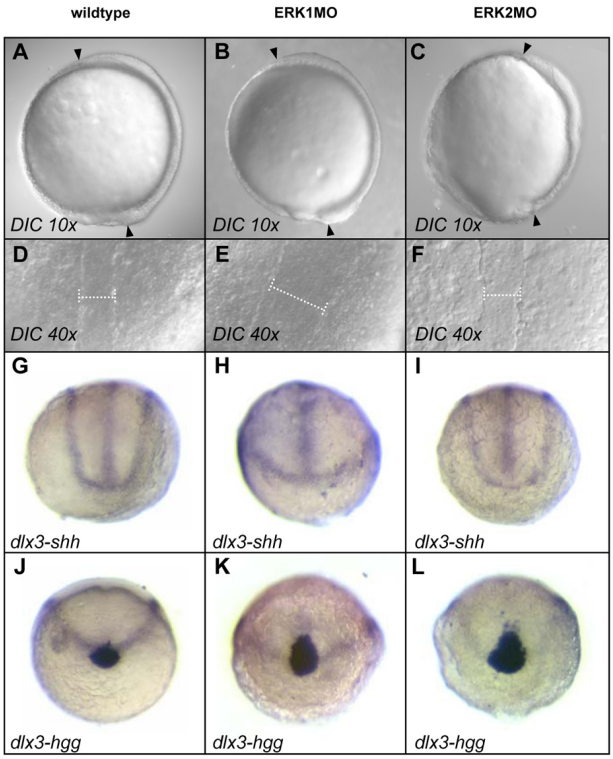

Figure 3. Phenotypic characterization of ERK1 and ERK2 morphants in late- gastrulation and early segmentation indicates affected convergence extension movements. (A,D,G,J); wild type control embryos (B,E,H,K); ERK1 knockdown embryos, injected with 1.7ng ERK1MO (C,F,I,L); ERK2 knockdown embryos, injected with 1.7ng ERK2MO. (A-C); Live embryos at yolk plug closure (YPC) to tailbud (TB) stages, animal pole up, dorsal to right. The distance between the arrowheads (►) resembles the length of the AP body axis (10x objective), or dorsal view (D-F), white spacer highlights the widening of the dorsal notochord (40x objective). (G-L); Combined in situ hybridization on 10 hpf old embryos with *dlx3* (edge neural plate), with *shh* (midline) or with *hgg1* (hatching gland) marker-genes (anterior view, dorsal to top).

gap in its expression at the dorsal axis and a broadening forerunner cells as detected by the endoderm marker gene *sox17* (Fig.4N). These changes were not observed in ERK2 knockdowns (Fig.4C,F,I,L and O)

In ERK2 morphants the expression domains of *gata2* and *otx2* were reduced (Fig.4R and U) as *gata2* and *otx2* expression did not expand as far ventrally and dorsally respectively compared to wild type and ERK1MO embryos (Fig.4P,S and Q,T). Analogous, the expression domain of *cyclops* and *notail* was shortened in the anterior-posterior axis in ERK2MO injected embryos (Fig.4X,A'), compared to wild type embryos (Fig.4V,Y) and ERK1 morphants (Fig.4W,Z). Consistent with the normal fate specification and regionalization in both ERK1MO and ERK2MO embryos at later gastrulation stages, normal expression of earlier patterning marker genes was observed. The dorsalizing factor *chordin* was expressed normally at 30% epiboly (Fig.4B'-D'), as was *goosecoid*, expressed at the dorsal margin (Fig.4E'-G'). Taken together, the expression patterns of different cell fate marker-genes indicated some slight morphological changes specific for either ERK1MO or ERK2MO, but the global cell patterning was not changed in these morphants. Therefore, these results suggest that ERK1 and ERK2 knockdown perturb gastrulation by affecting cell movements, without significantly altering the early cell fate specification.

In order to directly study the specific effect of ERK1 and ERK2 knockdown on cell movements during gastrulation, we performed cell-tracing experiments using DMNB-caged fluorescent dextran (Kozlowski et al., 1997;Sepich et al., 2000). To follow convergence (dorsal) cell migration, lateral mesoderm cells located 90° from the dorsal shield, were labeled by uncaging the fluorophore with a localized pulse of ultraviolet light at 6hpf and followed in time (Fig.5A-I and S). Extension cell migration processes were monitored by labeling cells in the dorsal shield (Fig.5J-R and T).

ERK1MO-injected embryos displayed a severely reduced movement of the labeled lateral cells towards the dorsal axis in time (Fig.5A-C), whereas only a slight reduction of dorsal migration was observed in ERK2MO-injected embryos (Fig.5D-F). In contrast, in ERK2MO injected embryos the extension movements were reduced to a greater extend, compared to ERK1MO injcted embryos that showed rather normal extension movements. Quantification of 10 embryos indicated that the reduction of dorsal migration for ERK1 (Fig.5S) and the reduced anterior extension migration for ERK2 (Fig.5T) were significant, and confirm the previous suggestions based on the in situ hybridization results (Fig.3 and 4). Together, these results indicate differential affected CE movements by ERK1 or ERK2 knockdown.

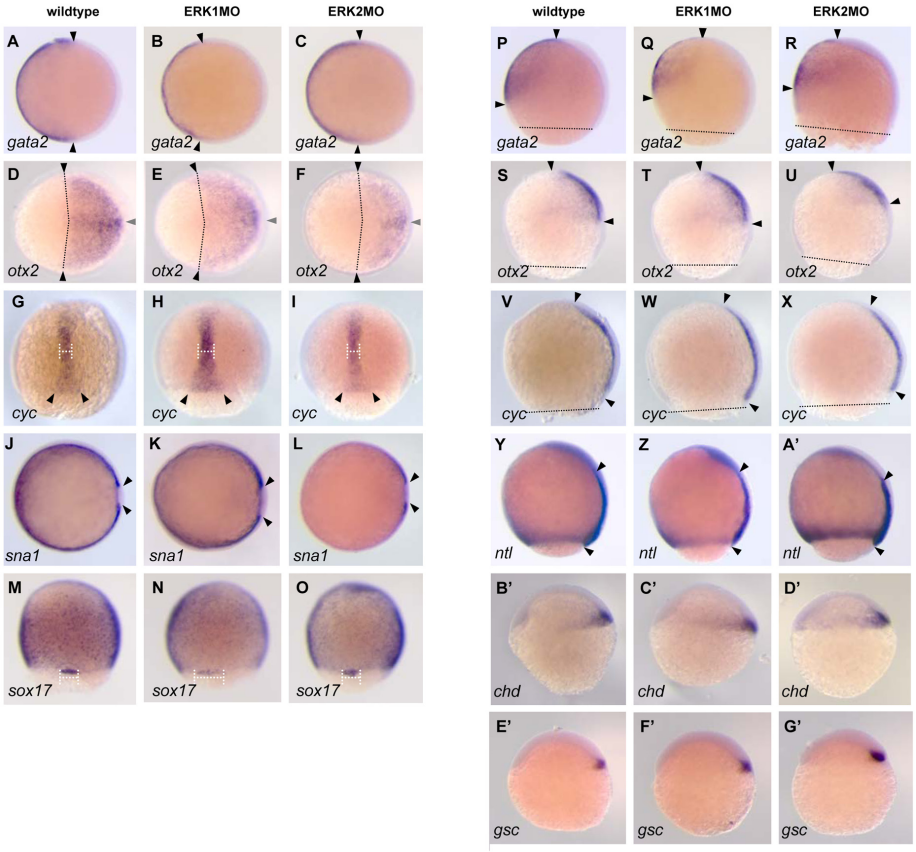

Figure 4. Expression of embryonic patterning marker genes in ERK1 and ERK2 morphants
Expression of mRNA was detected by whole mount in situ hybridization. (A-F, J-L) animal pole view, dorsal to right; (G-I,M-O) dorsal view, animal pole to top; (P-G') lateral view, dorsal to right, animal pole to top.
(A-C, P-R) *gata2*, expressed in non-neural ectoderm at 80% epiboly.
(D-F, S-U) *otx2*, expressed in ectoderm at 90% epiboly.
(G-I, V-X) *cyclops*/*ndr2* in axial mesoderm at 80-90% epiboly.
(J-L) *snail1a* in paraxial mesoderm at 90% epiboly.
(M-O) *sox17* in endoderm and forerunner cells at 80% epiboly.
(Y-A') *notail* in margin an axial mesoderm at 90% epiboly.
(B'-D') *chordin* in presumptive dorsal shield.
(E'-G') *goosecoid* in presumptive dorsal shield.

Arrowheads (►) mark the boundaries of expression domains.

ERK1 knockdown mildly affects epiboly progression, whereas ERK2 knockdown causes a developmental arrest at the onset of epiboly

Taken into account that the knockdown of ERK1 and ERK2 was not completely saturated (Fig.2D), we applied more stringent knockdown conditions by doubling the MO concentration and studied the effects on epiboly (Fig.6 and S3). Embryos injected with 0.2 mM ERK1MO (Fig.S3A) did not show any obvious phenotype until 80% epiboly, while doubling the concentration to 0.4 mM slightly delayed epiboly-migration compared to wild type embryos (Fig.S3B). Embryos injected with 0.2 mM ERK2MO were delayed to 65% epiboly at 8hpf (Fig.S3C), when wild type embryos reached 80%. Knockdown of ERK2 by injection of 0.4 mM ERK2MO prevented epiboly cell in 38% (Fig.6-arrest category and Fig.S3D) of the injected embryos and inhibited epiboly progression and further progression into gastrulation stages in 46% of the embryos (Fig.6 severe). This effect was rescued by co-injection of 20 pg synthetic *erk2* mRNA (Fig.S3E), which resulted in 82% (49 out of 60 embryos) of the co-injected embryos entering gastrulation. Injection of this amount of synthetic *erk2* mRNA did not induce any phenotype by itself at this stage.

Nomarski microscopy was performed to study the affected epiboly initiation at 4.5hpf (Fig.7A-F). The leading edge of the migrating cells in the margin and the enveloping layer (EVL) in wild type embryos (Fig.7A,D) and ERK1MO embryos (Fig.7B,E) showed a sharp front of migrating cells over the yolk. Knockdown of ERK2 induced a discontinuity in the margin and delayed crowding of the yolk syncytial nuclei (YSN) in the external yolk syncytial layer (YSL), resulting into a broadened YSL (Fig.7C, F).

Taking into account that active/phosphorylated ERKs were previously detected in the margin (Fig.1), we performed immuno-histochemistry against the dual phosphorylated ERK1/2 at 4.5hpf (dome-30% epiboly) and 8hpf (80% epiboly) (Fig.7G-I and J-L). In the control embryos (G,J) and the ERK1MO injected embryos (H,K) phosphorylated ERK was still detected in the margin in both stages. However, ERK phosphorylation was completely abolished in ERK2MO injected embryos (I,L) at both stages, indicating that ERK2 is the active ERK MAPK in the margin during gastrulation cell moments and that ERK2 plays a key-role in epiboly progression.

The migration of the YSN to the vegetal pole is considered as a driving force, next to radial intercalation (Myers et al., 2002; Montero and Heisenberg, 2004; Solnica-Krezel, 2006), through all stages of epiboly (Kane et al., 1996). At late blastula stages, the YSL forms a broad band and contains most of the YSN. At sphere/dome stage, the wide belt of external YSL narrows in the animal-vegetal direction and the YSN become increasingly crowded. When the blastoderm expands vegetally reaching 30% epiboly, the YSN of the exter-

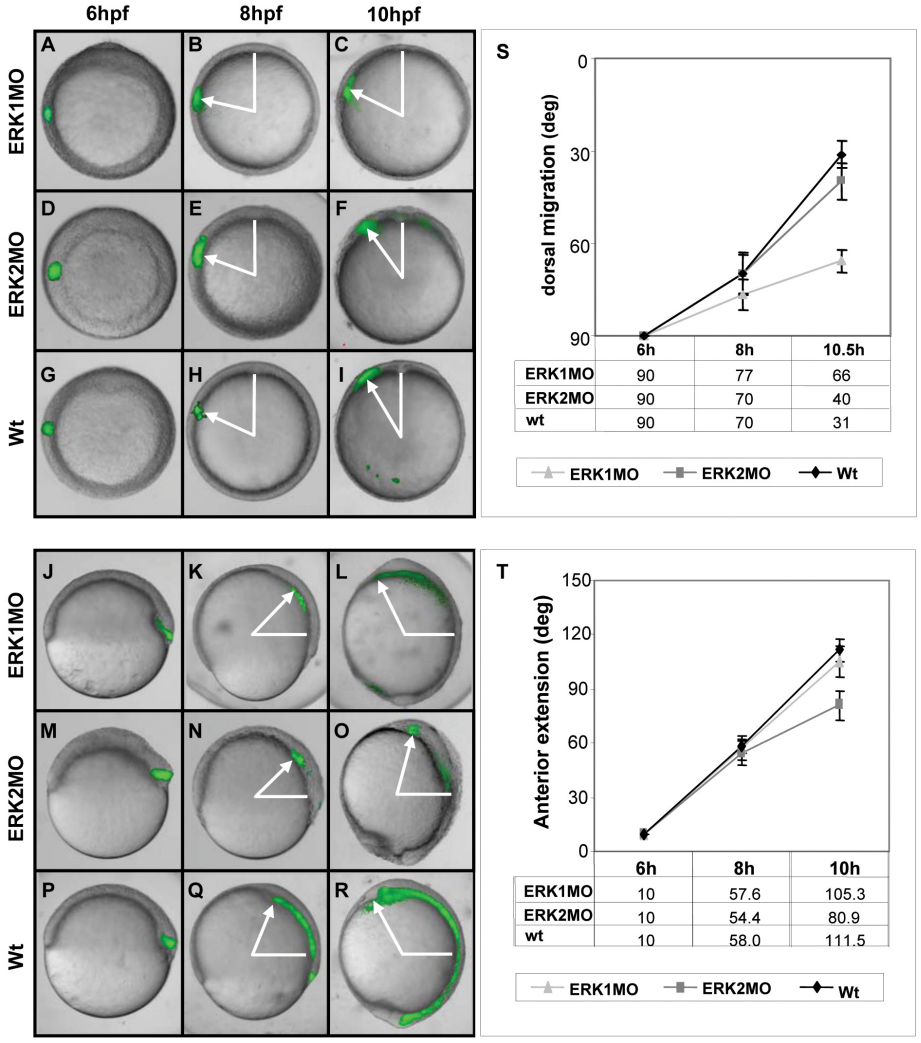

Figure 5. Knockdown of ERK1 and ERK2 affects CE cell movements differentially. Embryos were co-injected with MO and caged fluorescein dextran, which was activated at shield stage (6hpf) laterally to determine dorsal migration (A-I; animal pole view, dorsal to top) and dorsally to determine anterior extension (J-R; lateral view, dorsal to right). Images show uncaging cells directly after activation (A,D,G,J,M,P), at 8hpf (B,E,H,K,N,Q) and at 10hpf (C,F,I,L,O,R). Cell tracing experiments were performed in ERK1MO injected (A-C, J-L), ERK2MO injected (D-F, M-O) and wild type embryos (G-I, P-R). (S) Quantification of dorsal migration (n=10 embryos), measured as indicated with white arrows (A-I) as degrees from dorsal. (T) Quantification of anterior migration measured as indicated with white arrows (J-R) as degrees of anterior movement (n=10 embryos). ERK1MO: gray triangle, ERK2MO: dark gray square, wild type: black diamond.

nal YSL concentrate near the EVL at the margin (Solnica-Krezel and Driever, 1994). To follow migration of the YSN *in vivo,* 10 µM SYTOX-green nucleic acid stain was (co-)injected in wild type control embryos and morpholino-treated embryos (Fig.7M-O). At 8 hpf, ERK1 knockdown resulted in a delay of epiboly movements. Control embryos were at 80% epiboly (Fig.7M) when ERK1MO injected embryos were still at 50% epiboly (Fig.7N). In ERK2 morphants the YSN were still located at the top of the yolk at 8hpf and did not show any migration towards the vegetal pole (Fig.7O). Therefore we conclude that knockdown of ERK2, and subsequent depletion of activated ERK2 from the margin, also prevented the YSL from migrating to the vegetal pole of the embryo.

Together, these results provide evidence that knockdown of ERK1 and ERK2 affects convergence extension cell movements differentially during zebrafish gastrulation, without significantly altering early developmental patterning. ERK1 morphants showed a reduced convergence defect without a severe anterior posterior extension defect, whereas ERK2 morphants did show a severe anterior posterior extension defect without a significant convergence defect. Further analysis, using stronger knockdown conditions revealed that ERK2 is the active MAPK in the blastula margin. This indicates that absence of this active ERK2 from the margin at the blastula to gastrula transition prevents the initiation of epiboly. This subsequently blocks further progression of gastrulation cell migration processes, leading to an arrest in embryogenesis.

Discussion

The ERK1 and ERK2 MAPKs are among the most studied signaling molecules and numerous important functions for these proteins in cellular and developmental signaling processes have been observed. However, the specificity of MAPKs in developmental processes is not well understood. In this study, we show that ERK1 and ERK2 have distinct roles in epiboly and convergence extension (CE) movements. Specifically, we present data demonstrating that ERK1 knockdown affects convergence to a larger extend, whilst ERK2 knockdown predominantly affects anterior-posterior extension during gastrulation cell migration processes. In addition, complete depletion of active ERK2 from the blastula margin prohibited epiboly initiation and led to an arrest of embryogenesis.

	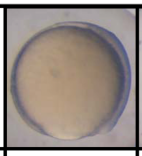	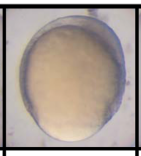	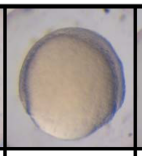	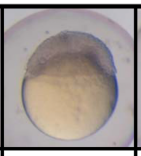	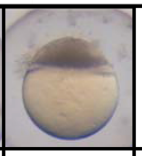	†
n	wt	light	mild	severe	arrested	dead
ERK2MO (0.2mM) 95	0%	20%	55%	15%	3%	7%
ERK2MO (0.4mM) 96	0%	0%	13%	46%	38%	4%
wt 44	100%	0%	0%	0%	0%	0%

Figure 6. Classification of the phenotypes of embryos injected with 0.2 mM or 0.4 mM ERK2MO, compared to wild type embryos at 8-9 hpf. Classification into wild type, light, mild, severe and arrested phenotype categories is indicated by percentages.

Different lethality of ERK1 versus ERK2 knockdown

Morpholino knockdown of ERK1 and ERK2 resulted in a shorter body axis and affected somite shape. Knockdown of ERK2 also disrupted the formation of the MHB and was more lethal than knockdown of ERK1 (Fig.2 and S1). Similar phenotypes were observed in previous studies after inhibition of the FGF pathway with the FGFR inhibitor SU5402, by over-expression of a dominant negative FGF receptor, and in embryos lacking FGF8 and FGF24 ligand activity (Griffin et al., 1995;Draper et al., 2003;Griffin and Kimelman, 2003;Mathieu et al., 2004), suggesting that the functions of ERK1 and ERK2 are linked to FGF signaling in zebrafish.

The observation that ERK2 knockdown is more lethal than ERK1 knockdown is in agreement with gene targeting results in mice showing that *erk1*-/- mice are viable and without embryonic phenotypes, whereas *erk2*-/- mice are lethal (Gilles Pagès et al., 1999). In zebrafish, the same amount of *erk2* mRNA that rescued morpholino knockdown of ERK2 was also able to cross-rescue knockdown of ERK1. In contrast, the *erk1* mRNA could not cross-rescue ERK2 morphants. Our results support the suggestion of Yao et al. that ERK2 can compensate for the loss of ERK1 (Yao et al., 2003;Saba-El-Leil et al., 2003). The difference between the observed developmental defects and lethality in zebrafish ERK1 morphants and the lack of a severe phenotype in *erk1*-/- mice (Mazzucchelli et al., 2002) is remarkable and suggests different redundancy and adaptation mechanisms in these organisms.

Distinct roles for ERK1 and ERK2 in convergence and extension cell movements during gastrulation

Phenotypes of surviving ERK1 and ERK2 morphants indicated possible effects on the dorsal-ventral patterning and convergence extension cell movements. In situ hybridization using combinations of markers for these processes supported this hypothesis (Fig.3), while in situ hybridization experiments with patterning marker genes did not show a significant altering of the early cell fate specification (Fig.4). The expression domain of *dlx3*, as a marker for the edge of the neural plate, was widened in ERK1 morphants and in ERK2 morphants a gap was observed between the expression-domains of *dlx3* and *shh* (notochord). This showed a distinct loss-of-function defect of ERK1 versus ERK2 during gastrulation cell migration processes. In order to directly define the specific functions of ERK1 and ERK2 in convergence extension (CE) cell movements, cell tracing experiment were performed in ERK1 and ERK2 morphants (Fig.5). We found distinct defects upon ERK1 and ERK2 knockdown during CE movements, as ERK1 morphants showed a convergence defect without a severe posterior-extension defect, whereas ERK2 morphants showed a more severe reduction in anterior-posterior extension. Importantly, defects underlying CE movements were primary effects, and not secondary to patterning defects, since in situ hybridization analyses with marker genes revealed no significant patterning changes in ERK1 and ERK2 morphants (Fig.4).

The CE movements during zebrafish gastrulation involve different cellular events, depending on the position of the cells along the dorsoventral axis (Myers et al., 2002). Dorsal convergence appears to require migration of cells on the extracellular matrix, while extension movements might be more dependent on cell-cell adhesion, involving neighboring cells crawling over the surfaces of the surrounding cells. Importantly, these findings are consistent with recent zebrafish work demonstrating that convergence and extension cell movements may be considered as separate morphogenetic movements of gastrulation cell migration in zebrafish (Glickman et al., 2003;Bakkers et al., 2004;Daggett et al., 2004).

In other model systems interaction between ERK signaling and cell migration during gastrulation were also observed. In *Drosophila*, *Xenopus*, mouse and zebrafish, the functions of ERK MAPKs are related to FGF signaling. Throughout zebrafish development, activated ERK protein is localized in overlapping expression regions with various FGF ligands and other components of the FGF-pathway (Fig.1) (Pozios et al., 2001;Sawada et al., 2001;Furthauer et al., 2002;Tsang et al., 2002;Poulain et al., 2006). In the early mouse gastrula, FGFs are required for the migration of the epiblast cells out of the primitive streak, which consists of the endoderm and mesoderm progenitor cells (Sun

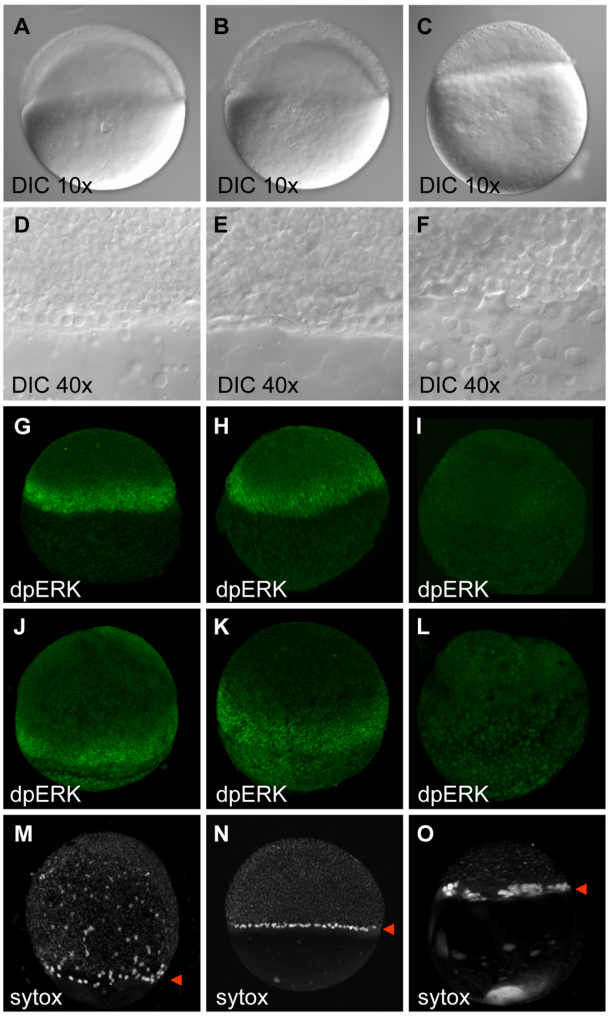

Figure 7. Stronger knockdown of ERK2 prohibits epiboly initiation and revealed ERK2 to be the active MAPK in the margin. Embryos were injected with 0.4mM (3.4 ng) ERK1MO or ERK2MO and compared to wild type embryos at the indicated stages. Differential interference contrast (DIC) microscopy using a 10x objective (A,B,C,) or a 40x objective (D,E,F) was used to monitor the onset of gastrulation (4.5hpf). Localization of dpERK was detected by immuno-localization in wild type, ERK1MO and ERK2MO injected embryos at 4.5 hpf (G-H) and 8hpf (J-L) by phospho-specific ERK antibody. YSL-migration was followed by co-injection of 10µM sytox green, red arrowhead, 8hpf (M,N,O). Images in G-O were taken by confocal microscopy.

et al., 1999). In chicken, FGFs signal chemotactic cues during gastrulation, and coordinate cell movements during ingression of the epiblast cells through the primitive streak (Yang et al., 2002). During early mesoderm migration in *Drosophila*, the local MAPK activation pattern suggests that the FGF receptor (Htl) is specifically activated at the leading-edge of the migrating mesoderm cells (Gabay et al., 1997), with FGF8-like1 and FGF8- like2 being required as ligands for Htl (Gryzik et al., 2004). Here we showed that disruption of these signals by knockdown of either of the key FGF downstream targets ERK1 or ERK2 results in differential effects on the regulation of convergence extension movements. This also points towards different downstream targets of ERK1 and ERK2 and divergence of ERK-signaling at this level.

Epiboly initiation requires active/phosphorylated ERK2 in the blastula margin

More stringent knockdown conditions were applied to address the possible functions of ERK1 and ERK2 as phosphorylated ERK1 and ERK2 were previously detected in the blastula margin (Fig.1). The increased knockdown of ERK1 only slightly delayed epiboly, whereas 40% of the ERK2MO injected embryos did not initiate epiboly at all. Immuno-histochemical studies on ERK1 and ERK2 morphants using a phospho-ERK antibody showed that ERK2 is specifically activated in the blastula margin (Fig.7I and L) and crucial for epiboly progression. Our results indicate that depletion of this active ERK2 from the margin prevents cells from the animal pole to migrate over the yolk-cell at the onset of epiboly (Fig.7). In addition, migration of the YSL to the vegetal pole was prevented, thereby removing one of the motors for gastrulation movements (Solnica-Krezel et al., 1994; D'Amico and Cooper, 2001; Solnica-Krezel, 2006).

Integration of ERK MAPKs in other developmental signaling networks

Our results suggest that activation of ERK2 plays a major role in early epiboly initiation. The question remains which signaling cascade. that triggers the onset of epiboly, is blocked by ERK2 knockdown. At the upstream signaling level multiple interconnected signaling pathways, such as Nodal, Wnt, BMP and FGF, act together to coordinate epiboly and gastrulation processes (Schier and Talbot, 2005) and are therefore possibly connected with ERK2 activation.

Similarities were observed between the severe phenotypes of ERK morphants and embryos in which the FGF pathway was inhibited. Mesoderm initiation was previously shown not to be affected in the absence of FGF signaling (Raible and Brand, 2001; Draper et al., 2003; Furthauer et al., 2004; Tsang

et al., 2004) as in severe ERK2 morphants that still expressed *ntl* and *papc* (data not shown). However, *erk2*-/- mice fail to form mesoderm (Yao et al., 2003). Attempts to block FGF signaling by triple knockdown of FGF8,17b and 24 did also not inhibit epiboly and endoderm/ectoderm differentiation as in ERK2 morphants, but resulted in a large increase of endoderm precursor cells (Poulain et al., 2006). This indicates that multiple pathways may be involved in the regulation of ERK2 in the margin at the onset of epiboly.

A number of mutants described for epiboly arrest show delayed or arrested epiboly at later stages, but not a block at blastula to gastrula transition (Kane et al., 1996). For example, different *hab* alleles, containing mutations in the cell adhesion molecule *E-cadherin* (*cdh1*), arrest epiboly of deep cells at mid-gastrulation, whereas epiboly of EVL and YSL proceeds further (Kane et al., 2005;Shimizu et al., 2005). Reduction of E-cadherin protein levels by morpholino knockdown also delayed epiboly and affected intercalation and possibly extension cell migration (Kane et al., 2005). Stronger loss of E-cadherin function impaired epiboly at earlier stages (Babb and Marrs, 2004), similar as observed in our study of ERK2 morphants. An interesting link is starting to emerge between activation of ERK MAPKs via hepatocyte growth factors and the regulation of *E-cadherin* expression via *snail* in cancer processes (Medici et al., 2006;Grotegut et al., 2006). In future studies we would like to address the link of ERK MAPK with FGF, E-cadherin and other signaling components using microarray analyses in the different ERK knockdown backgrounds.

Experimental procedures

Cloning

Total RNA was isolated from adult Tuebingen zebrafish, using TRIZOL® Reagent protocol (GIBCO BRL, Life technologies ™). cDNA for zebrafish *erk1* and *erk2* genes were cloned into pCR®4Blunt-TOPO® (Invitrogen) using primers flanking the coding region, based on EST AB030902 and AB030903, and subcloned in the pCS2+ vector using the EcoR1-site. Constructs were checked by sequence analysis.

Micro-injection of morpholinos and mRNAs

One-cell stage embryos were injected with 1 nl of the solubilized compounds in 1× Danieau's buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES; pH 7.6] containing 1% Phenol red solution (Sigma). Definition of stages was according to Kimmel et al. At the 1K-stage (3hpf), embryos with a red animal pole were selected as positive-injected embryos. To block translation of the *erk1* or *erk2* mRNA, 1.7 ng or 3.4 ng of MOs were injected per embryo. MOs were targeted against the 5'- UTR of the respective mRNAs (GeneTools Philomath, OR, USA): ERK1MO, 5'-TCTGTCCGCAAATCGTCGCCTTCGC; ERK2MO, 5'-CACCCAAAAGCACCAGGAAAAGCTC.

The pCS2+ constructs containing the zebrafish ERK1 and ERK2 genes were linearized and synthetic mRNAs were transcribed with SP6 RNA polymerase using the mMessage mMachine Kit (Ambion). Per embryo 1 to 150 pg mRNA was injected.

Cell tracing

Embryos were (co-)injected at the one cell stage with 0.3% 4,5-dimethoxy-2-nitrobynzyl (DMND)-caged fluorescein dextran (molecular mass 10,000; Molecular probes, kind gift from the Hammerschmidt lab.). Uncaging was performed as previously described (Bakkers et al., 2004) with UV light at shield stage (6hpf) using a Zeiss axioplan microscope with 40x objective and adjustable pinhole. Embryos were imaged at 6, 8 and 10.5 hpf. The dorsal convergence and anterior extension angles were measured using Image J (NIH imaging software).

Whole-mount immuno-staining

Embryos were fixed overnight in 4% paraformaldehyde at 4°C. Embryos were washed three times and incubated for 2 hours in blocking buffer (PBS, 0.1% BSAc, 1% Triton X100) followed by overnight incubation at 4°C in 1:100 dilution of primary antibody (Polyclonal phospho-p44/42 MAP kinase antibody from Cell Signaling; ERK antibody K-23, Santa Cruz Biotechnology) in blocking buffer. Embryos were washed three times with blocking buffer, followed by 1 hour, incubation in 1:100 diluted secondary antibody (Goat anti-rabbit Alexa 488 conjugated). Embryos were washed three times in blocking buffer, and three times in PBS containing 0.1 % Tween.

Whole-mount in situ hybridization

Embryos were fixed overnight in 4% paraformaldehyde in PBS at 4 °C and in situ hybridization was performed as described previously (Thisse et al., 1993) using described probes for *chd*, *cyc*, *gsc*, *gata2 myod*, *ntl*, *otx2*, *pax2*.1, *snai1a*, *shh1*, *sox17*. The *dlx3* and *hgg1* probes were kindly provided by Jeroen den Hertog.

Protein isolation and western blot analysis

Embryos were dechorionated and de-yolked in Ca^{2+}/Mg^{2+} free solution. Cells were pelleted and washed with PBS followed by a passive lysis in buffer (0.125% NP40, 25mM Tris-HCl, 2 mM EDTA, 1 mM Na_3VO_4, 25 mM NaF and 1 complete mini EDTA-free protease inhibitor cocktail tablet (Roche) per 10 ml lysis-buffer).

Protein extract were separated by SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by and proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Den Bosch, The Netherlands) by western blotting. The membranes were blocked in 5% w/v non-fat dry milk in Tris-buffered saline-Tween 20 (TBST). The blots were incubated with a 1:1000 dilution of the indicated antibody in TBST with 3% BSA (Sigma) overnight at 4°C. Signal was detected using a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies and the enhanced chemiluminescence method (Amersham).

Acknowledgements

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Supplementary data

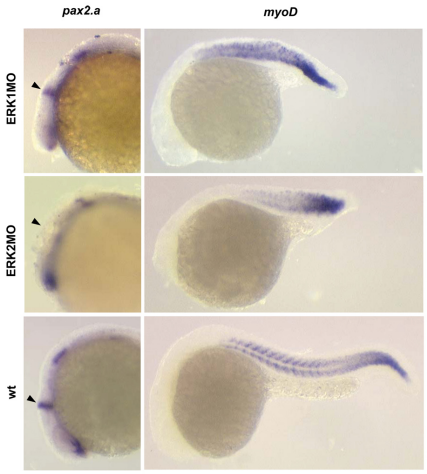

Figure S1. In situ hybridization with *pax2.1* or *myod* on ERK1MO or ERK2MO injected embryos compared to wild type embryos at 24hpf. Surviving embryos after knockdown of ERK1 or ERK2 show disturbed somite formation. Knockdown of ERK2 affects MHB formation.

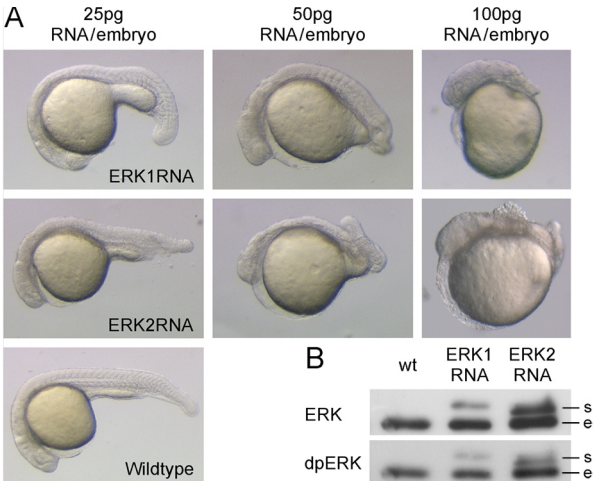

Figure S2. Over-expression of *erk1* and *erk2* led to concentration-dependent phenotypes. (A) Embryos were injected with 25, 50 or 100pg *erk1* RNA or *erk2* RNA per embryo and developmental phenotypes were recorded at 24hpf. (B) Western blot analysis was performed with global ERK and dpERK antibody on protein samples of 100pg RNA injected embryos in shield stage. The proteins derived from synthetic mRNAs (s) are slightly larger than the endogenous proteins (e) due to the introduction of a small linker, and can therefore be distinguished by size.

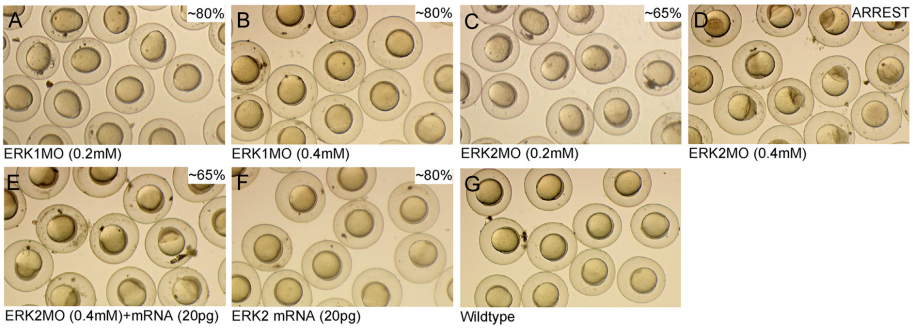

Figure S3. Specific functions of ERK1 and ERK2 revealed by concentration dependent knock-down during early embryogenesis. Embryos were injected with 0.2 mM (1.7 ng) and 0.4 mM (3.4 ng) ERK1MO (A,B) or ERK2MO (C,D) respectively. Rescue was performed by co-injection of 3.4ng ERK2MO with 20pg *erk2* mRNA (E) and compared to phenotypes of 20 pg *erk2* mRNA injected (F) and wild type embryos at 8hpf (H).

V

ERK1 and ERK2 MAPK are key regulators of distinct target gene signature sets in zebrafish embryogenesis

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submitted

Abstract

The MAPK signaling proteins are involved in all eukaryotic cellular processes and in signaling networks. However, specific function of most of these proteins in vertebrate development remain elusive because of potential redundancies. For instance, the upstream activation pathways for ERK1 and ERK2 are highly similar, and also many of their known downstream targets are common. In contrast, mice and zebrafish studies indicate distinct roles for both ERKs in cellular proliferation, oncogenic transformation and development. A major bottleneck for further studies is that relatively few i*n vivo* downstream targets of these kinases have been identified conclusively.

Microarray based gene expression profiling of ERK1 and ERK2 knockdown zebrafish embryos resulted in specific gene expression signature sets that showed pronounced differences in gene ontology analyses. Signaling pathways were analyzed for the BMP, FGF, Nodal and Wnt signaling pathways that are important for embryogenesis. Specific effects of ERK1 and ERK2 knockdown treatments were confirmed by whole mount in situ hybridization experiments.

The gene ontology analyses showed that ERK1 and ERK2 have specific roles in embryogenesis and target distinct gene sets involved in vertebrate development, confirming the embryonic knockdown phenotypes. For ERK1 we identified a connection with genes involved in dorsal-ventral patterning and subsequent embryonic cell migration. For ERK2 we identified a connection with genes involved in cell-migration, mesendoderm differentiation and patterning. The outcome of the predictions for ERK2 knockdown on developmental signaling were confirmed by the observed effects on mesoderm and endoderm patterning and subsequent whole mount in situ hybridization experiments.

Introduction

ERK1 and ERK2 (Extra-cellular signal Regulated protein Kinases) are most likely the best studied members of the mitogen activated protein kinase (MAPK) proteins. Despite efforts and their biological - medical importance, still relatively few *in vivo* downstream targets of these kinases have been identified conclusively, especially when considering the numerous cellular events and signaling networks they are involved in (Johnson et al., 2005). Most of the target proteins and downstream genes have been identified by *in vitro* studies using cell culture systems.

Specific roles for both ERKs are described for cellular proliferation, as mouse embryos fibroblasts (MEF) isolated from *erk1*-/- mice grew faster

than wild type cells. The tumorigenicity of transplanted NIH 3T3 cells stably expressing an oncogenic form of Ras in nude mice was largely inhibited by co-transfection of ERK1, but not by ERK2 or p38 (Vantaggiato et al., 2006). In diseases ERK1 and ERK2 can display distinct cellular functions, as have been shown for the formation of cancer (Lloyd et al., 2006). In addition, divergent roles for ERK1 and ERK2 were already shown by the different effect of the knockout studies performed in mice as *erk1*-/- mice are viable and fertile (Pagès et al.1999), while *erk2*-/- mice die *in utero* before embryonic day (E) 8.5 (Saba-El-Lei et al., 2003).

To study and compare the developmental roles of ERK1 and ERK2 we used specific morpholino antisense oligonucleotides (MO), to block translation of ERK1 and ERK2. We previously showed that mild knockdown of ERK1 or ERK2 differentially affected convergence extension movements, but did not disturb patterning of the embryo. Stronger knockdown conditions of ERK2 led to a more severe phenotype, as ERK2MO morphants did not go into epiboly, whereas ERK1MO morphants still developed further and entered gastrulation stages. In addition, immuno-histochemical studies showed that ERK phosphorylation was completely abolished in the blastula margin of ERK2 morphants, indicating that ERK2 is the active ERK MAPK in the margin and essential for epiboly initiation and further progression of the developmental program (chapter 4, this thesis). Possibly ERK2 also functions in mesendodermal differentiation processes in the blastula margin, as FGF is known to activate the canonical MAPK pathway in a Ras dependent manner (Gotoh and Nishida, 1996; Bottcher and Niehrs, 2005). The severe phenotype that we observed upon ERK2 knockdown in zebrafish embryos indicate that ERK2 has a more dominant role during early developmental processes, as also suggested by the mice knockout phenotypes.

Here we aim to further determine specific downstream gene targets of ERK1 and ERK2 during vertebrate development, by performing expression profiling analysis using a microarray approach. We compared the expression profiles of ERK1 and ERK2 knockdown embryos, using specific morpholino antisense oligonucleotides (MO), which specifically block the translation of a gene of interest into a functional protein (Nasevicius and Ekker, 2000).

Microarray technology enables gene characterization based on systematic and comprehensive expression studies of large sets of genes. In addition, the availability of the zebrafish genome sequence and the annotation of all predicted genes help us to understand the link between genes and their functions. Newly developed software programs and web-based analysis tools, e.g. Rosetta Resolver, GenMAPP and GeneTOOLS eGOn, are helpful for the processing and comparisons of large expression datasets and biological in-

terpretation of the data. For instance, these tools facilitate the prediction of interconnection between developmental signaling pathways that can be tested by biological assays.

Analysis of the obtained data revealed that ERK1MO and ERK2MO knockdown affect signature sets of common target genes, as well as signatures sets of specific genes. Surprisingly, we also identified gene sets in which the expression patterns were anti-correlated. Several signature marker genes identified in this study were confirmed by quantitative real time PCR and in situ hybridization. We performed signaling pathway analysis on the obtained ERK1 and ERK2 transcriptome signatures, using the GenMAPP software program (Dahlquist et al., 2002) for the analysis of important signaling cascades during early vertebrate development. These include BMP, FGF, Nodal and Wnt signaling pathways (Schier and Talbot, 2005). For ERK1 knockdown we identified a connection with genes involved in dorsal-ventral patterning and subsequent embryonic cell migration. For ERK2 knockdown we identified a connection with genes involved in mesoderm and endoderm initiation, differentiation and patterning. Many of these genes also play a role in morphogenic cell migration processes during later stages of development. The outcomes of the predictions for ERK2 knockdown on developmental signaling were confirmed by the observed effects on mesoderm and endoderm induction and subsequent whole mount in situ hybridization experiments.

Results and discussion

Distinct gene expression signature sets of ERK1 and ERK2 knockdown embryos

A morpholino knockdown approach was used to block translation of either ERK1 or ERK2 by injection of 0.4mM (=3.4 ng/embryo) morpholinos (MO) targeting ERK1 (ERK1MO) or ERK2 (ERK2MO). The knockdown embryos, also referred to as morphants, showed severe phenotypes after depletion of ERK2. These embryos did not enter epiboly at 4.5 hpf and the blastula cells remained on top of the yolk, preventing further development of the embryo (Fig.1C). Wild type embryos reached 30% epiboly at this time (Fig.1A) (Kimmel et al., 1995). In contrast, ERK1 morphants did not show any obvious phenotypes at this point, and had entered epiboly (Fig.1B). The severe phenotypes of ERK2 morphants indicate defects in crucial early developmental processes and it is likely that the expression of a significant number of genes has changed.

To identify specific gene pools affected by the knockdown of ERK1 or ERK2,

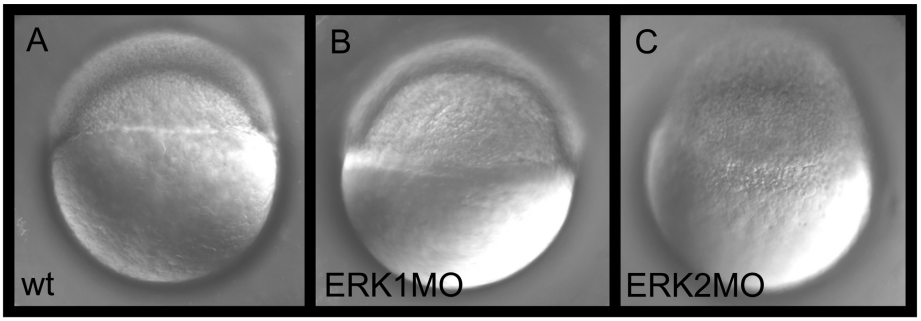


Figure 1. Differential Interference Contrast (DIC) microscopy images of 4.5h old embryos. Wildtype (wt) and ERK1MO morphants are at approximately 30% epiboly stage and undergo epiboly, whereas ERK2 morphants do not initiate epiboly.

and to identify possible downstream targets, microarray based transcriptome analysis was performed using Agilent zebrafish microarrays. Total RNA was isolated from the morpholino injected embryos at 30% epiboly time point. As a control for general morpholino effects, a standard control morpholino (GeneTools Philomath, OR, USA) was injected in the same concentration. This did not result in any phenotypes during zebrafish development. Still, injection of standard control MO could have specifically affected the transcriptome of the developing embryos. Therefore, we also performed microarray analysis comparing standard control MO to embryos injected only with MO injection-buffer (1× Danieau's buffer, containing 1% Phenol red solution). The obtained standard control MO signature set consists of 574 probes that showed a significant (combined p-value smaller than 10^{-5}) change in their expression by injection of the standard control MO at 30% epiboly. Of this signature set only 26 genes were common with both the ERK1 and ERK2 knockdown expression signature sets (Table S7; online supplementary data at http://biology.leidenuniv.nl/~krens). Since in these comparisons use was made of ratios against the standard control MO, we can conclude that the expression of this overlap gene set is specifically affected by the standard control MO. Therefore, this set of 26 genes was flagged as putative false positives in further microarray analyses. The remainder of the 574 set of probes, most likely influenced by aspecific effects of morpholino injections, will be compensated for by using ratios against standard control MO in all ERK1 and ER2 analyses.

The RNA from standard control MO injected embryos was used as a reference also in dye swaps to compare the transcriptomes of both ERK1MO and ERK2MO injected embryos. Comparison of the gene expression profiles of ERK1 and ERK2 morphants at 30% epiboly showed a larger number of probes with significant changes ($p<10^{-5}$) in ERK2 than in ERK1 morphants,

as illustrated in a Venn-diagram (Fig.2A). Furthermore, the number of probes with an altered expression was larger and with a higher fold of change in expression for ERK2 then for ERK1 morphants (Fig.3). This is in agreement with the phenotype of ERK2 knockdown embryos that indicates a more prominent role for ERK2 in early development. (Fig.1). The probes that were found also to be effected by the standard control MO (less than 1% of the total number affected genes), were marked with an asterisk (*) in the annotated gene-tables for ERK1 and ERK2 knockdown (Table S1-S6; online supplementary data at http://biology.leidenuniv.nl/~krens). Several genes are represented by multiple

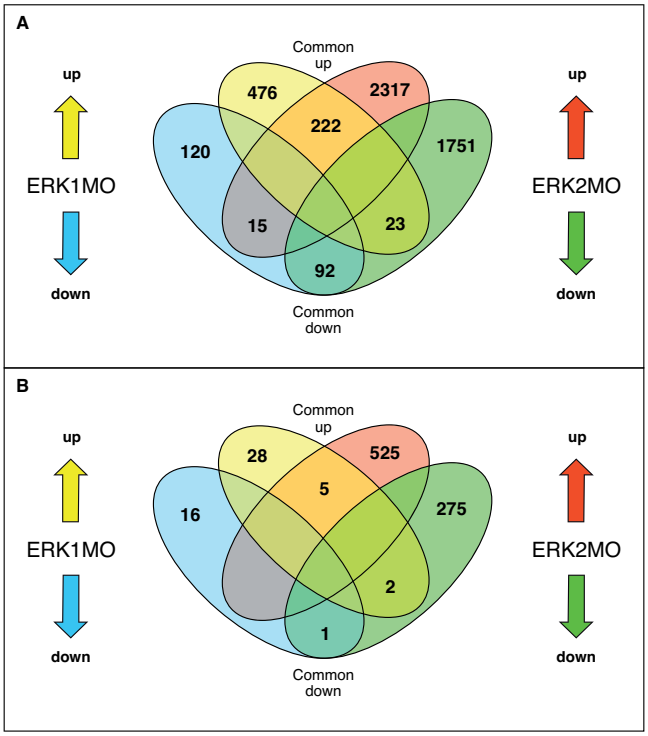


Figure 2. Venn-diagram comparison of the ERK1 and ERK2 knockdown expression profiles at 30% epiboly stage. The Venn diagram was constructed twice, with the complete signatures (A) and with the stringent signatures (B). The signatures of ERK1 and ERK2 morphants are split in up and down regulated, therefore the Venn diagram shows the numbers of specifically up and down regulated genes, common up and down regulated genes, and two anti-correlated gene pools (up regulated in ERK1MO – down regulated in ERK2MO and down regulated in ERK1MO – up regulated in ERK2MO); yellow = up-regulated by ERK1MO (ratio > 1), blue = down-regulated by ERK1MO (ratio <1), red = up-regulated by ERK2MO (ratio > 1), green = down-regulated by ERK2MO (ratio <1).

probes on the microarray slides and therefore the same gene occurs several times in the annotated tables. Observations such as this give additional support for the obtained gene expression signatures and confirm that the expression of these genes is affected by ERK1 or ERK2 knockdown.

The result show that different probes were specifically regulated by either knockdown of ERK1 (476 probes up-regulated, 120 probes down-regulated) or knockdown of ERK2 (2317 probes up-regulated, 1751 probes down-regulated). Furthermore, a number of 222 probes were commonly up-regulated, 92 probes are commonly down-regulated. Maybe even more interesting are the probes which were regulated in an anti-correlated manner: 23 probes were up-regulated by knockdown of ERK1 whereas they were down-regulated by knockdown of ERK2 (anti-correlated gene-pool 1) and 15 probes were down-regulated by knockdown of ERK1 whereas they were up-regulated by knockdown of ERK2 (anti-correlated gene-pool 2). The commonly and anti-correlated regulated

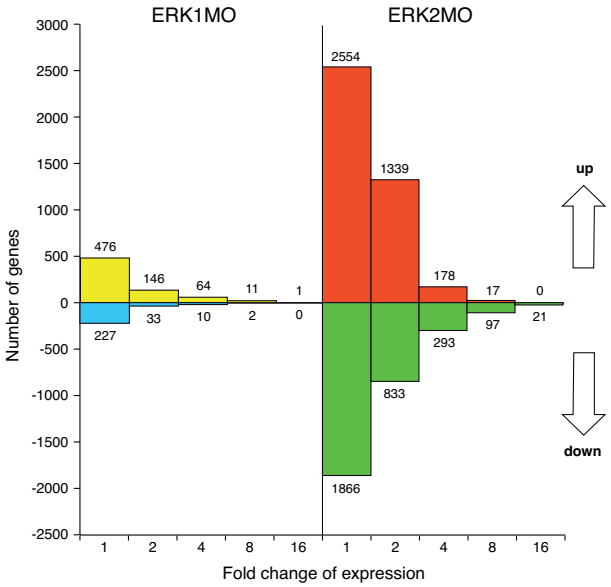


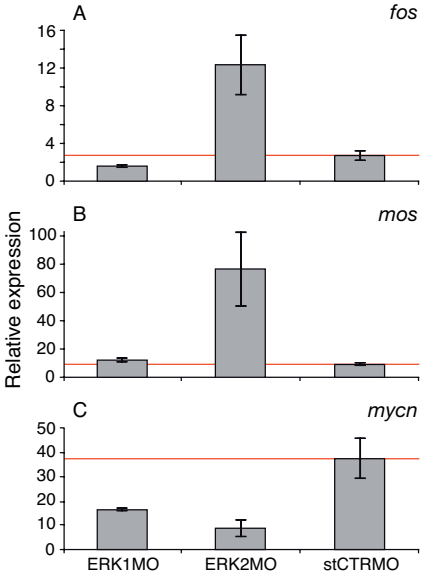
Figure 3. Knockdown of ERK2 affects the expression of more genes, and with a higher fold of changes than knockdown of ERK1. The graph represents the total number of genes that showed changes in expression, as well as their fold of change (greater than 1, 2, 4, 8, and 16 fold changes) upon knockdown of ERK1 or ERK2 at 30% epiboly ($p<10^{-5}$). The number of genes with changed expression levels and the fold of change after knockdown of ERK1 were lower than upon ERK2 knockdown.

probes have been annotated and are listed in tables S1 to S4 and assigned gene designations. To identify the ERK1MO and ERK2MO specific genes, we focused on the probes that were most significantly affected. Therefore we used the following criteria: the absolute fold change must be at least 1.5 in each independent replicate and the common p-value provided by the error-model taking into account all hybridizations must be smaller than 10^{-5} to compensate for multiple testing false positives. These probes are also depicted in a Venn-diagram (Fig.2B). The stringent selected probes that were only found in either ERK1MO or ERK2MO gene-pools were manually annotated and assigned gene designations as listed in table S5 and S6.

Quantitative real-time PCR analyses confirm the different ERK1MO and ERK2MO gene expression profiles

To confirm the obtained gene expression profiling by the microarrays experiments, quantitative real-time PCR (qPCR) analysis was performed on three differentially regulated genes. The expression level were tested on the same RNA samples as used for the microarray analysis for *mycn* (v-myc, myelocytomatosis viral related oncogene, neuroblastoma derived, NM_212614), *fos* (FBJ murine osteosarcoma viral oncogene homolog, NM_205569) and *mos* (moloney murine sarcoma viral oncogene homolog, NM_205580) (Fig.4). β-actin was taken as reference to compare the expression levels of the selected genes in ERK1MO, ERK2MO and standard control MO injected embryos. The qPCR expression levels for *fos*, *mos* and *mycn* in ERK1MO or ERK2MO were compared to standard control MO injected embryos, in the same way as was performed for the microarray analysis. The obtained qPCR data for the expression of *fos* showed an anti-correlated regulation comparing ERK1 and ERK2 knockdown to standard control MO conditions. *Fos* is down-regulated by knockdown of ERK1 and up-regulated by knockdown of ERK2, compared to the expression-level of *fos* in standard control MO (Fig.4A). The expression level of *mos* is up-regulated, whereas *mycn* is down-regulated in both ERK1MO and ERK2MO conditions, compared to the standard control MO (Fig. 4B and C respectively).

The data obtained by qPCR and microarray experiments for the expression-levels of *fos*, *mos* and *mycn* in ERK1MO and ERK2MO were compared to the expression levels of these genes in the standard control MO injected embryos (Fig.4D). The qPCR data confirmed the change in expression levels of the selected genes as observed by microarray analysis, and thus the unique gene expression profiles for ERK1MO and ERK2MO mediated knockdown in early zebrafish development (30% epiboly).



D

Gene	morpholino	qPCR	Agilent 22K	
fos	ERK1MO	-1.92	-1.46	⇩
	ERK2MO	4.50	2.31	⇧
mos	ERK1MO	1.37	3.44	⇧
	ERK2MO	8.87	10.35	⇧
mycn	ERK1MO	-2.22	-1.71	⇩
	ERK2MO	-4.35	-5.37	⇩

Figure 4. Quantitative real-time PCR confirmation of the microarray results. (A-C) qPCR was performed on three genes that showed differential regulation of expression in response to knockdown to either ERK1, ERK2 and the standard control MO control: *fos* (NM_205569, down in ERK1MO, up in ERK2MO), *mos* (NM_205580, up in both ERK1MO and ERK2MO), *mycn* (NM_212614 down in both ERK1MO and ERK2MO), correlated to the β-actin housekeeping gene. The fold of changes of these genes, detected by qPCR assay and microarray, are listed in a table (D). ⇧ = induction of expression, ⇩ = repression of expression, compared to the standard control MO.

Gene Ontology (GO) analysis

The gene expression signatures of the ERK1 and ERK2 morphants were used to perform gene ontology analysis. Gene ontology consists of three structured controlled vocabularies (ontologies) that describe genes and gene-function (http://www.geneontology.org) and each gene ontology has a unique nu-

merical identifier. The GO-clusters are structured in a tree, which can be queried at different levels. This allows us to assign properties from genes at different levels, depending on the depth of knowledge or interest concerning the genes of interest. Gene ontologies describe gene products in terms of their associated 'biological processes' (GO:0008150), 'cellular components' (GO:0005575) and 'molecular functions' (GO:0003674) in a species-independent manner. To do so, we first annotated the complete Agilent 22K-zebrafish microarray chip by BLAST searches with all oligonucleotide sequences in the zebrafish genome. From the complete number of 21506 oligonucleotides from the Agilent 22K zebrafish chip, 21485 oligonucleotides were assigned an Unigene ID according to the highest similarity. Next, the Unigene ID-linked signature sets for ERK1 and ERK2 knockdown, were uploaded into the GeneTools eGOn V2.0 web-based gene ontology analysis software (explore Gene Ontology, database build #97) (Beisvag et al., 2006). These signature sets comprised 575 Unigene IDs in the case of ERK1 morphants and 2987 Unigene IDs in the case of ERK2 morphants were compared to the complete set of 21485 Unigene IDs from the Agilent 22K zebrafish microarray chip ('biological process'; 6036 Unigene IDs, 'molecular function'; 6322 Unigene IDs and 'cellular component'; 5606 Unigene IDs). As a result we were able to determine which gene ontology clusters were significantly over- or under represented in the ERK1MO and ERK2MO Unigene ID linked signature sets (Fig.5; p-values smaller than 0.05 are indicated with asterisk, p-values smaller than 0.02 are indicated with double asterisk). Several GO-clusters shared high similarity and were identified by similar groups of genes. We reduced the number of terms by excluding these overlapping GO-clusters. To ensure statistical relevance, also the GO-clusters that contained less than 10 Unigene IDs were removed. For the selected GO-terms, we calculated the relative fold of enrichment within the ERK1- and ERK2-morphant signature sets (Fig.5). The results showed a significant relative over- or under-representation of the number of Unigene IDs in ERK1 versus ERK2 morphants within the GO categories. For ERK1 versus ERK2 knockdown signature sets we obtained 5 or 14 GO-terms associated with 'biological processes' (Fig.5A), 3 or 16 associated with 'molecular functions' (Fig.5B) and 3 or 8 associated with 'cellular components' (Fig.5C), respectively.

Comparing the ERK1 and ERK2 knockdown signature sets some of these GO-terms show striking over- or under-representations in the number of representing Unigene IDs. For example, both the GO-terms 'cell cycle' (GO:0007049) and 'apoptosis' (GO:0006915) are significantly enriched upon ERK2 knockdown. When looking at the gene-lists in more detail (also using GenMAPP analysis, data not shown) inhibitory factors of apoptosis are down-regulated, whereas positive regulators of cell cycle were up-regulated. 'Cell

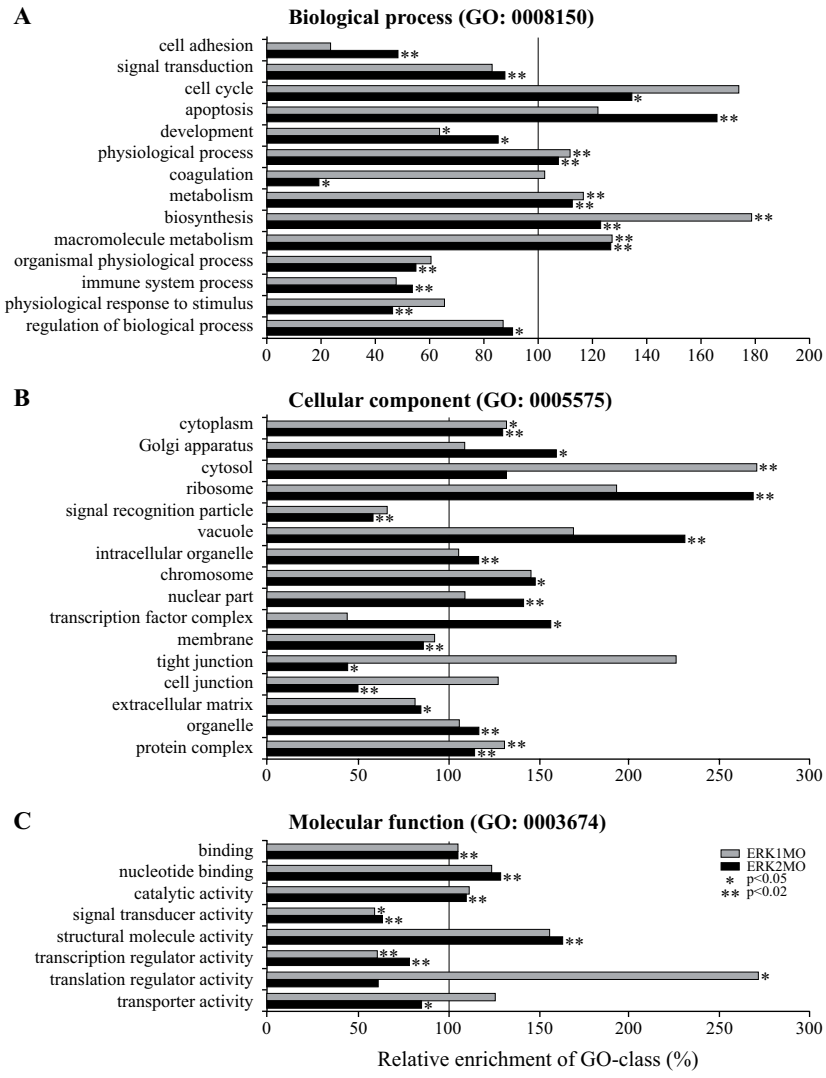


Figure 5. Statistical comparison of the Gene-Ontology distribution within the gene expression profiles, in ERK1 versus ERK2 knockdown embryos. (A) Biological process (GO:0008150), (B) Cellular component (GO:0005575) and (C) Molecular function (GO:0003674). ERK1MO and ERK2MO gene expression profiles were split in up- (red) and down- (green) regulated gene expression profiles and compared to the whole 22K Agilent chip, based on the Unigene-ID identifiers. The graph depicts the relative fold of enrichment (x-axis) of the statistically selected GO-clusters (y-axis), within one of the gene-pools: ERK1MO-up, ERK1MO-down, ERK2MO-up and ERK2MO-down (* = P<0.05, ** = P<0.01). Values greater than 1 were considered over-represented, values less than1 are considered as under-represented.

adhesion' (GO:0007155) and the cellular component GO-terms 'tight junction' and 'cell junctions' are significantly under-represented in the signature set of ERK2 morphants. Regulation of cell adhesion and the organization of tight- and cell-junctions are crucial for cell migration processes. Therefore these findings corroborate the crucial function of ERK2 in developmental cell migrations processes, as previously described in chapter 4.

Specifically for ERK1 knockdown a significantly enrichment of the 'translator regulator activity' (GO:0030528) GO-cluster was found. In contrast, the relative enrichment of this GO term in ERK2 morphants showed an underrepresentation. A significant overrepresentation of the GO term biosynthesis in ERK1 morphants correlates with these observations.

Interestingly, the GO-enrichment analysis showed that the number of genes within the GO-cluster 'development' (GO:0007275) were significantly underrepresented for both ERK1 (19 genes) and ERK2 (136 genes) morphants. This gives further support for the notion that both ERK1 and ERK2 have an important function during embryogenesis. From the 19 development-related genes whose expression was affected by ERK1 knockdown, 12 genes (63%) were not found in the ERK2 knockdown signature set. This shows that ERK1 and ERK2 have specific roles in embryogenesis and target distinct genes during vertebrate development, confirming the embryonic knockdown phenotypes.

Pathway Analysis of ERK1MO and ERK2MO mediated knockdown expression profiles

To further analyze putative down stream targets of ERK1 and ERK2 involved in early development, we focused on essential signaling pathways that are involved in early embryonic differentiation and patterning; Nodal, FGF, WNT and BMP- signaling pathways (Fig.8). For our study, we used the signaling pathway analyzing software program, GenMAPP (Gene Microarray Pathway Profiler, www.GenMAPP.org) (Dahlquist et al., 2002). This program is designed for viewing and analyzing gene expression data in the context of biological pathways and allows microarray-mediated gene expression signature sets to be displayed on biological (signaling) pathways. To do so, we first constructed *in silico* GenMAPP pathways for the zebrafish Nodal, FGF, (canonical) WNT and BMP signaling pathways (Fig.6). The construction of these GenMAPP signaling pathways is based on what is described in literature for zebrafish development, supported by the described knowledge for other vertebrate signaling processes and canonical signaling models, found on the Science's STKE Connections Map Database (http://stke.sciencemag.

org/cm/). Although it is clear that the Nodal, FGF, Wnt and BMP pathways are all interconnected, resulting in a complex signaling network, we performed a pathway-based analysis focusing on separate signaling pathways since the ways these signaling pathways exactly interconnect on a molecular scale is hardly understood yet.

The Unigene ID linked ERK1MO and ERK2MO signature sets that were used for GenMAPP analysis were not limited by fold change but instead we used all genes that had a combined p-value smaller than 10^{-5}. As previously mentioned, the number of genes that showed a changed expression in ERK2MO compared to ERK1MO injected embryos was far larger. Therefore, as expected, more genes with changed expression levels were found in the *in silico* GenMAPPs signaling pathways for ERK2MO, than for ERK1MO.

Knockdown of ERK1 did show only one gene (*smurf1*) with a significantly changed expression level within our BMP signaling GenMAPP. However, more genes were affected in FGF signaling: *fgf17b* (-1.4 fold) the MAPKKK *mos*, (+3.5 fold), transcription factor *cmyc* (-1.7 fold) and *srf* (serum response factor, -1.4 fold) showed significant changes in expression. In the Nodal pathway, the Nodal antagonist *lft1*/*antivin1* (+2.6 fold) and the EGF-CFC co-receptor *oep* (*one eyed pinhead*, -1.5 fold) were the only components found to be affected in ERK1 morphants. Furthermore, the ventrally expressed Wnt8-mediated organizer inhibitory gene *vent* (Melby et al., 2000) was down-regulated (-1.5 fold, Fig.6). Other genes involved in Wnt-signaling affected by ERK1 knockdown were *dab2* (disabled homolog 2, +1.5 fold), *ck2b* (casein kinase II beta subunit, -1.2 fold) and *ppp2r5e1* (Protein phosphatase 2A, regulatory B subunit, B56, +1.3 fold). These genes are also considered to be involved in early embryonic pattering pathways. Two genes involved in regulating gastrulation cell migration, *oep* and *quattro* (Warga and Kane, 2003; Dagget et al., 2004), were altered in expression.

The effect of depletion of ERK2 was far more severe in most of the analyzed signaling processes (Fig.6A-C). Key components of the FGF-pathway (*fgf8*, *fgfr4, frs2*, *bRaf*, *aRaf* and *mek1l*) and downstream target genes (*erm*, *eve1*, *pea*, *mkp3*, *spry2*, *ntl*, *spt*/*tbx16* and *tbx6*) were down-regulated, indicating already a block of the FGF-ERK pathway by ERK2 knockdown. Expression of some of these (mesoderm) target genes is initiated by Nodal. The Nodal-genes like *boz*/*dharma*, *squint*/*ndr1* and *smad2* are up-regulated, whereas inhibiting genes *lefty1* (*lft1*, -6 fold) and the ventral genes *vox* (-2 fold) and *ved* (-4 fold) are down-regulated in ERK2 morphants. Other nodal signaling mediator genes that are down-regulated are *oep* (-4 fold), *p300* (-2 fold), *foxh1*/*sur* (*schmalspur*, -2 fold) and the negative regulator of TGFβ signaling *TGIF* (-2 fold). The nodal-mediated endoderm gene *sox32*/*casanova*, expressed in the

margin, was down-regulated (-6 fold), and also the downstream target-gene *axial*/*foxA2* (-2 and -4 fold). Interestingly, *squint*/*ndr1* also functions as a positive regulation of fibroblast growth factor receptor signaling pathway (Maegawa et al.,2006).

The Wnt ligand *Wnt11* and receptors (*frz7a*, *7b*, *8a*, *9* and *10*) and the central mediator β-*catenin1* were down-regulated in ERK2 morphants, suggesting a severe inhibitory effect or even complete block of these pathways at this level. This inhibition of the Wnt pathway is also supported by the up-regulation of *axin2*/*conductin*, a scaffold protein from the β-catenin destruction complex, responsible for the degradation of β-catenin (Behrens et al., 1998). Down-regulation of the putative Wnt-target genes *vox*, *vent*, but also *otx2*, *sp5*, and *lim1* further support impaired Wnt-signaling. However, ERK2 knockdown also led to the down-regulation of the inhibitors *dkk1* and *sfrp1*, and up-regulation of the intracellular Wnt-signaling components *fxd8c*, *dab2*, β-*catenin2* and *tcf1*.

The effect of ERK2 knockdown on BMP signaling is also complex, as *bmp4* is up-regulated whereas *bmp1a*/*tolloid* and *bmp6* are down-regulated. This opposing effect is also found in the BMP antagonists, as *chordin* (*chd*) and the ventrally expressed membrane bound bmp-inhibitor *bambi* were down-regulated, whereas a different BMP antagonist *gremlin* is up-regulated. Adding to this complexity is the fact that the agonist *twisted gastrulation* (*twsg1a*) is up-regulated. The results clearly show that that dorsal-ventral patterning and also mesoderm patterning is severely affected but it is difficult to speculate about the downstream effects of all these changes of expression in the BMP pathway.

To confirm predicted effects of the Genmapp pathway analysis experimentally, we performed whole mount in situ hybridization on ERK1 and ERK2 morphants at 30% epiboly with marker genes regulated by Nodal, BMP, Wnt and FGF (Fig.7, Fig.8). This technique not only indicates the level of expression of a certain gene, but importantly also shows the localization of its expression. Different components of the Wnt / β−catenin pathway showed lower expression levels in ERK2 morphants. We showed that *goosecoid* (*gsc*) (Stachel et al., 1993), a downstream marker gene for the Wnt pathway at early developmental stages (Fig.7A-C) is not expressed in the ERK2 morphants. Knockdown of ERK1 did lead to a significant effect on the expression of *gsc*, but after knockdown of ERK2 no expression of *gsc* was detected by whole mount in situ hybridization. This confirms that canonical Wnt signaling was severely affected in ERK2 morphants, preventing subsequent expression of the Wnt-target gene *gsc*.

The *lefty 1* (*lft1* / *antivin1*) gene is a member of the TGF-beta superfamily that regulates left-right axis formation during embryogenesis via antagonistic activ-

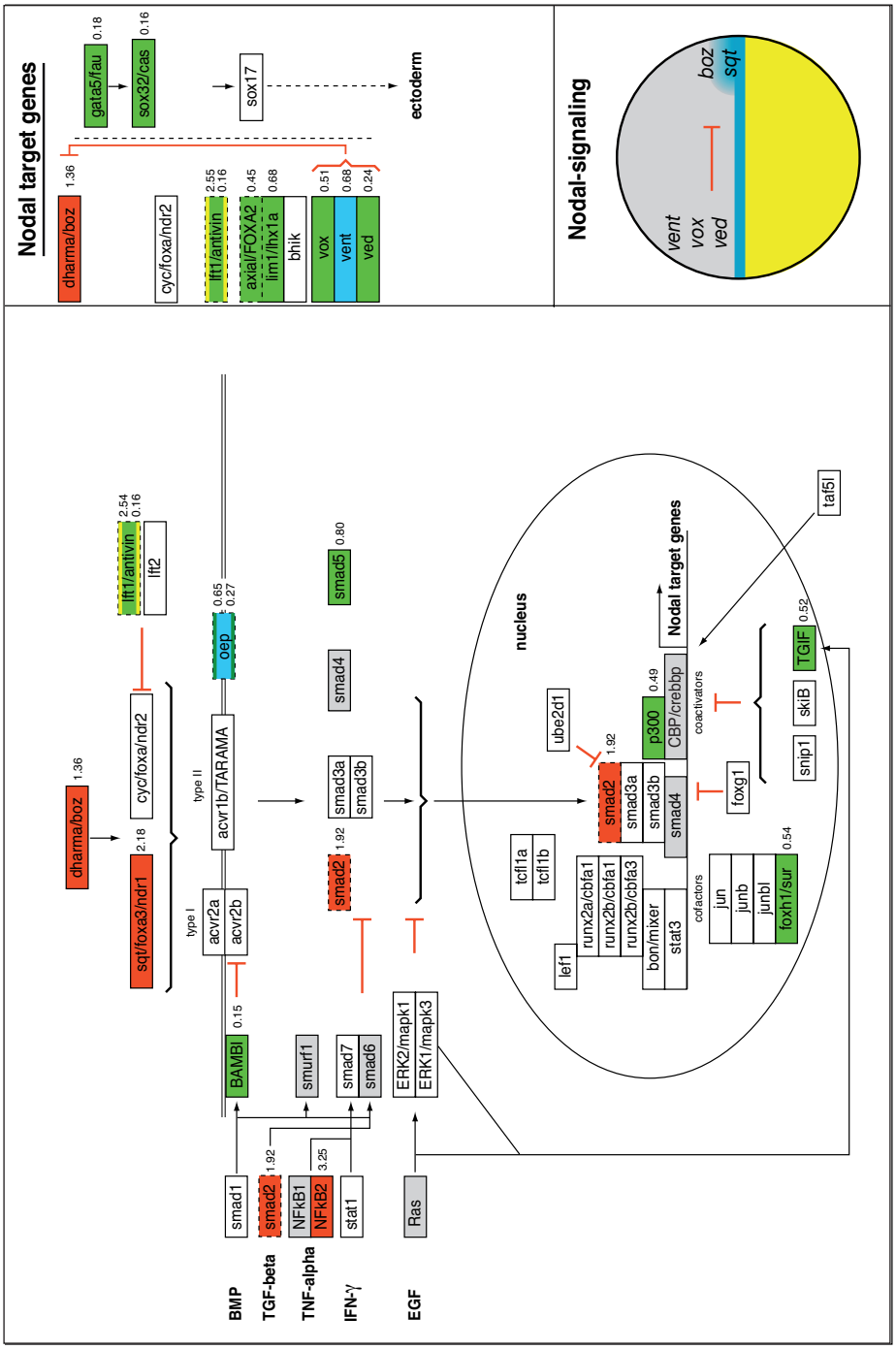

Nodal target genes
dharma/boz
1.36
gata5/fau
0.18
sox32/cas
0.16
cyc/foxa/ndr2
lft1/antivin
2.55
0.16
axial/FOXA2
lim1/hx1a
bhik
0.45
0.68
sox17
ectoderm
vox
vent
ved
0.51
0.68
0.24
Nodal-signaling
vent
vox
ved
boz
sqt
BMP
TGF-beta
TNF-alpha
IFN-γ
EGF
smad1
smad2
1.92
NFkB1
NFkB2
3.25
stat1
Ras
BAMBI
0.15
smurf1
smad7
smad6
ERK2/mapk1
ERK1/mapk3
type I
acvr2a
acvr2b
sqt/foxa3/ndr1
2.18
dharma/boz
1.36
cyc/foxa/ndr2
type II
acvr1b/TARAMA
lft1/antivin
2.54
0.16
lft2
oep
0.65
0.27
smad2
1.92
smad3a
smad3b
smad4
smad5
0.80
nucleus
tcfl1a
tcfl1b
lef1
runx2a/cbfa1
runx2b/cbfa1
runx2b/cbfa3
bon/mixer
stat3
cofactors
ube2d1
smad2
1.92
smad3a
smad3b
smad4
p300
0.49
CBP/crebbp
coactivators
Nodal target genes
foxg1
jun
junb
junbl
foxh1/sur
0.54
snip1
skiB
TGIF
0.52
taf5l

Figure 6A

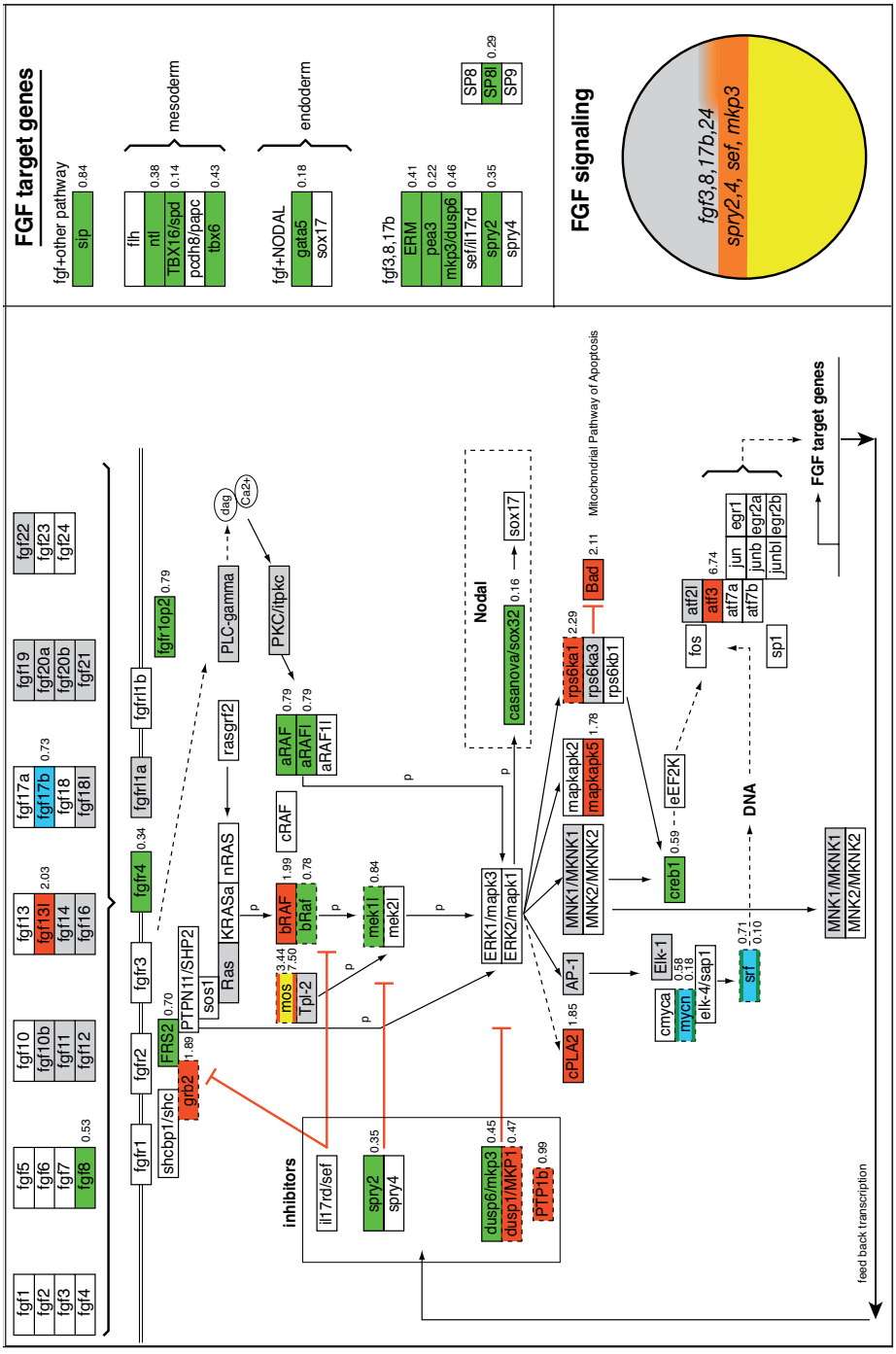
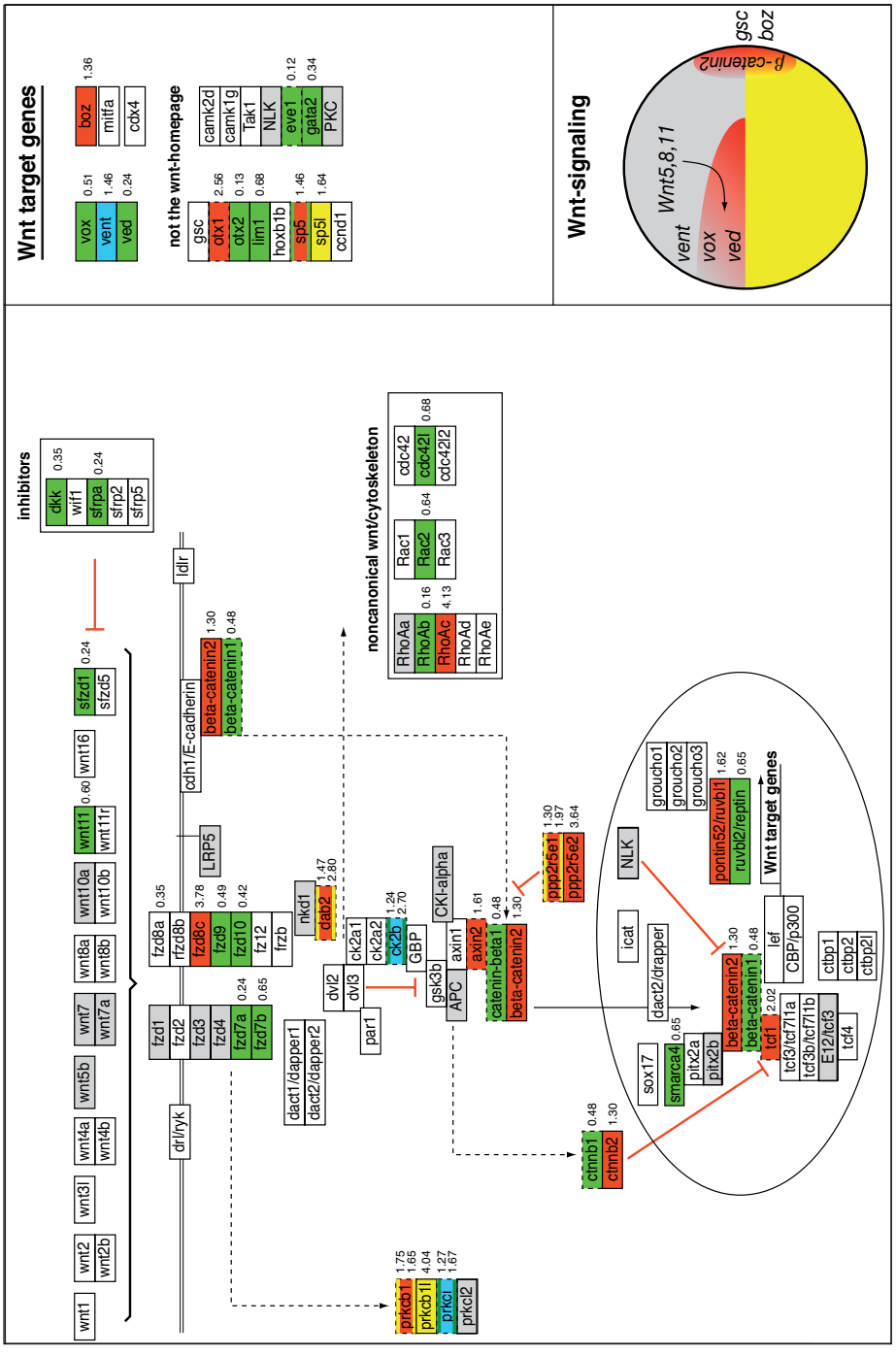

FGF target genes
fgf+other pathway
sip
0.84
flh
ntl
TBX16/spd
pcdh8/papc
tbx6
0.38
0.14
0.43
mesoderm
fgf+NODAL
gata5
sox17
0.18
endoderm
fgf3,8,17b
ERM
pea3
mkp3/dusp6
sef/il17rd
spry2
spry4
0.41
0.22
0.46
0.35
SP8
SP9
SP8
0.29
FGF signaling
fgf3,8,17b,24
spry2,4, sef, mkp3
fgf1
fgf2
fgf3
fgf4
fgf5
fgf6
fgf7
fgf8
0.53
fgf10
fgf10b
fgf11
fgf12
fgf13
fgf13l
fgf14
fgf16
2.03
fgf17a
fgf17b
fgf18
fgf18l
0.73
fgf19
fgf20a
fgf20b
fgf21
fgf22
fgf23
fgf24
fgfr1
fgfr2
fgfr3
fgfr4
0.34
fgfr1a
fgfr1b
fgfr1op2
0.79
shcbp1/shc
grb2
1.89
FRS2
0.70
PTPN11/SHP2
sos1
Ras
KRASa
nRAS
rasgrf2
PLC-gamma
dag
Ca2+
PKC/tpkc
mos
3.44
7.50
Tpl-2
bRAF
1.99
bRaf
0.78
cRAF
aRAF
0.79
aRAF1
aRAF1l
0.79
mek1l
0.84
mek2l
ERK1/mapk3
ERK2/mapk1
Nodal
casanova/sox32
0.16
sox17
inhibitors
il17rd/sef
spry2
0.35
spry4
dusp6/mkp3
0.45
dusp1/MKP1
0.47
PTP1b
0.99
cPLA2
1.85
AP-1
MNK1/MKNK1
MNK2/MKNK2
mapkapk2
mapkapk5
1.78
rps6ka1
2.29
rps6ka3
rps6kb1
Bad
2.11
Mitochondrial Pathway of Apoptosis
cmyca
mycn
0.58
0.18
elk-4/sap1
Elk-1
creb1
0.59
eEF2K
fos
atf2l
atf3
6.74
atf7a
atf7b
jun
junb
egr1
egr2a
egr2b
junbl
sp1
srf
0.71
0.10
DNA
FGF target genes
MNK1/MKNK1
MNK2/MKNK2
feed back transcription

Figure 6B


Wnt target genes
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vent 1.46
ved 0.24
boz 1.36
mitfa
cdx4
not the wnt-homepage
gsc
otx1 2.56
otx2 0.13
lim1 0.68
hoxb1b
sp6 1.46
sp5l 1.64
ccnd1
camk2d
camk1g
Tak1
NLK
eve1 0.12
gata2 0.34
PKC
Wnt-signaling
gsc
boz
β-catenin2
Wnt5,8,11
vent
vox
ved
inhibitors
dkk 0.35
wif1
sfrpa 0.24
sfrp2
sfrp5
wnt1
wnt2
wnt2b
wnt3l
wnt4a
wnt4b
wnt5b
wnt7
wnt7a
wnt8a
wnt8b
wnt10a
wnt10b
wnt11 0.60
wnt11r
wnt16
szd1 0.24
szd5
dkr/ryk
fzd1
fzd2
fzd3
fzd4
fzd7a 0.24
fzd7b 0.65
fzd8a 0.35
fzd8b
fzd8c 3.78
fzd9 0.49
fzd10 0.42
fz12
frzb
dact1/dapper1
dact2/dapper2
nkd1
dab2 1.47 2.80
dvl2
dvl3
par1
ck2a1
ck2a2 1.24 2.70
ck2b
GBP
gsk3b
APC
CKI-alpha
axin1
axin2 1.61 0.48
catenin-beta1
beta-catenin2
1.30
cdh1/E-cadherin
beta-catenin2 1.30
beta-catenin1 0.48
LRP5
ldlr
noncanonical wnt/cytoskeleton
RhoAa
RhoAb 0.16
RhoAc 4.13
RhoAd
RhoAe
Rac1
Rac2 0.64
Rac3
cdc42
cdc42l 0.68
cdc42l2
ppp2r5e1 1.30 1.97
ppp2r5e2 3.64
prkcb1 1.75 1.65
prkcb1l 4.04
prkci 1.27 1.67
prkcl2
ctnnb1 0.48
ctnnb2 1.30
NLK
icat
dact2/drapper
sox17
smarca4 0.65
pitx2a
pitx2b
groucho1
groucho2
groucho3
pontin52/ruvbl1 1.62
ruvbl2/reptin 0.65
Wnt target genes
beta-catenin2 1.30
beta-catenin1 0.48
tcf1 2.02
lef
CBP/p300
ctbp1
ctbp2
ctbp2l
tcf3/tcf7/7l1a
tcf3b/tcf7l1b
E12/tcf9
tcf4

Figure 6C

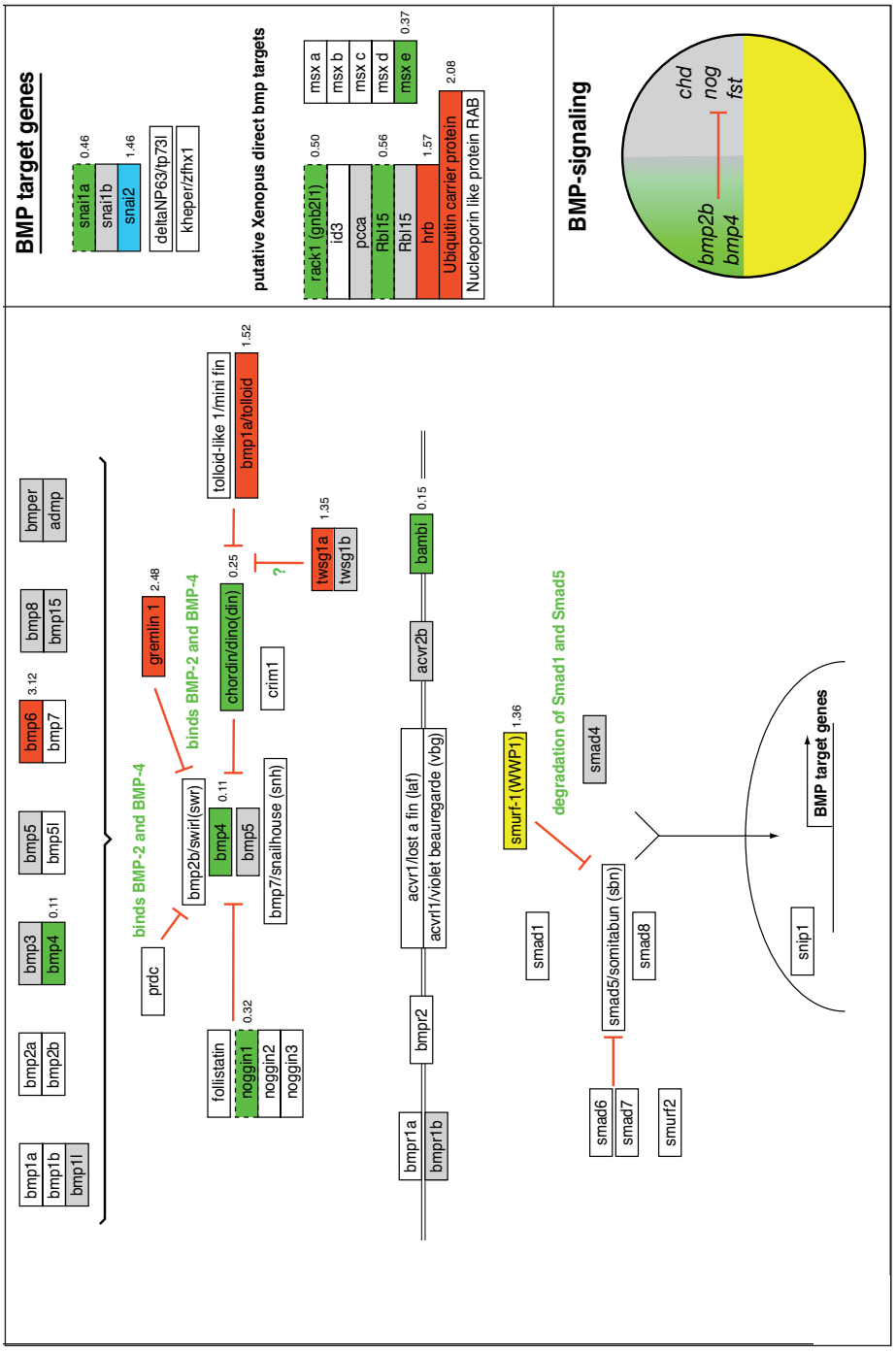
BMP target genes
snai1a
0.46
snai1b
1.46
snai2
deltaNP63/tp73l
kheper/zfhx1
putative Xenopus direct bmp targets
rack1 (gnb2l1)
0.50
id3
pcca
Rbl15
0.56
Rbl15
1.57
hrb
Ubiquitin carrier protein
2.08
Nucleoporin like protein RAB
msx a
msx b
msx c
msx d
msx e
0.37
BMP-signaling
chd
nog
fst
bmp2b
bmp4
bmp1a
bmp1b
bmp1l
bmp2a
bmp2b
bmp3
bmp4
0.11
bmp5
bmp5l
bmp6
3.12
bmp7
bmp8
bmp15
bmper
admp
binds BMP-2 and BMP-4
prdc
bmp2b/swirl(swr)
follistatin
noggin1
0.32
noggin2
noggin3
bmp4
0.11
bmp5
bmp7/snailhouse (snh)
gremlin 1
2.48
binds BMP-2 and BMP-4
chordin/dino(din)
0.25
crim1
toloid-like 1/mini fin
bmp1a/tolloid
1.52
?
twsg1a
twsg1b
1.35
bmpr1a
bmpr1b
bmpr2
acvr1/lost a fin (laf)
acvrl1/violet beauregarde (vbg)
acvr2b
bambi
0.15
smurf-1(WWP1)
1.36
degradation of Smad1 and Smad5
smad1
smad4
smad6
smad7
smurf2
smad5/somitabun (sbn)
smad8
snip1
BMP target genes

Figure 6D

ity against Nodal, another member of the TGF-beta super-family. Expression starts at blastula stage, immediately after initiation of zygotic transcription, and is localized in the whole blastula margin at late blastula – 30% epiboly stage (Thisse et al., 1999). In ERK1 knockdown embryos, *lft1* expression was up-regulated (+2.54 fold) and down-regulated in ERK2 morphants (-3.27 fold), as also shown in Fig.6A. Whole mount in situ hybridization with *lefty1* probe (Fig.7D-F) at 30% epiboly shows a possible increase of *lefty1* expression in ERK1 morphants (Fig.7E), but the decrease of expression in ERK2 morphants (Fig.7F) was clearly visible.

The T-box gene *notail* (*ntl*) is involved in mesoderm development, as described in the legend to figure 8. At 30% epiboly *ntl* is expressed in the blastula margin (Schulte-Merker S., et al., 1992). This expression is synergistically regulated by FGF and Nodal signaling pathways (Draper et al., 2003; Griffin and Kimelman, 2003). Both of these pathways show a negative regulation in the ERK2 morphants, as shown by the GenMAPP analysis (Fig.6). The negative effect on these pathways and the array-data itself suggested a down-regulation of the *ntl*-gene upon ERK2 knockdown. The expression signatures from the ERK1 and ERK2 morphants revealed that *ntl* expression was not significantly changed in ERK1 morphants (p-value > 10^{-5}), but was down-regulated (-3 fold) in ERK2 morphants. This decrease in expression of *ntl* was also confirmed by whole mount in situ experiments (Fig.7G-I). The *ntl* gene expression in ERK1 morphants was comparable to expression in wild type embryos, but *ntl* expression was decreased in ERK2 morphants. Strikingly, expression of *ntl*

Figure 6. Analysis of developmental signaling processes ERK1MO and ERK2MO gene expression profiles indicate defects in early developmental signaling processes.

(A) Nodal, (B) FGF, (C) canonical Wnt and (D) BMP signaling pathways are overlaid with gene-expression color criterion and ratios of gene-expression from the program GenMAPP: yellow = up-regulated by ERK1MO (ratio > 1), blue = down-regulated by ERK1MO (ratio <1), red = up-regulated by ERK2MO (ratio > 1), green = down-regulated by ERK2MO (ratio <1), gray = gene is not present on the Agilent zebrafish 22k microarrays or in the GenMAPP database, white = not significantly changed.

The genes that were affected in their expression in both ERK1 and ERK2 morphants show multicolored gene-boxes with the expression ratios for both conditions depicted on the right of the gene; the ration for ERK1 knockdown at the top and ERK2 knockdown below. At the right side of the subfigure a list of responsive target-genes is listed for each signaling pathway. The bottom right of each subfigure shows a small representation of the predicted signaling activity in the wild type embryos, based on the potential range of signals and the expression patterns and range of antagonists adopted from Schier and Talbot (2005), late blastula stage, lateral view, dorsal to right and animal pole to top.

was not constant in the marginal ring, as stronger expression was detected in the putative dorsal side of the ERK2 morphants.

The obtained results by whole mount in situ hybridization using *gsc*, *lft1* and *ntl*, confirm the predictions made by the GenMAPP analysis, as the changes in their expression levels are conform the predictions obtained by the signaling pathway analysis of the microarray data.

Discussion

In this study we have performed an expression profiling analysis that gives new mechanistic insights in how ERK signaling is functioning and how it integrates with other known effectors of vertebrate embryogenesis. This expression profiling study compared ERK1 and ERK2 knockdown at late blastula stage revealing different gene expression signature sets and downstream targets for these proteins. The gene expression signatures of ERK1 and ERK2 knockdown were used to obtain new insights in the embryonic processes that were perturbed and to indicate potential downstream target genes that were specifically affected by the knockdown of either ERK1 or ERK2.

Comparison of the effects of ERK1 and ERK2 knockdown

Comparison of the gene expression profiles of the ERK1 and ERK2 morphants with standard control MO injected embryos as a shared reference showed specific gene expression profiles, as different number of genes and different gene pools were found. The gene expression signature sets included genes involved in cell cycle, proliferation, cell differentiation, metabolism, cytoskeleton dynamics, signal transduction, migration and transcription. The gene expression profiles of ERK1 and ERK2 and knockdown embryos showed a set of genes that were commonly regulated (Fig.2B). Surprisingly, we also found a set of genes that was regulated in an anti-correlated manner. This observation is in line with the notion that ERK1 and ERK2 have specific down stream targets.

The higher number of genes affected by the knockdown of ERK2 is in agreement with the severe phenotype of ERK2 knockdown embryos. In order to understand the severe effects of ERK1/2 knockdown, we have to consider the results in the context of the known signaling pathways that govern developmental programs as proliferation, cell migration and differentiation processes. Therefore we performed signaling pathway analysis on the ERK1 and ERK2 transcriptome signatures using the GenMAPP software program for analysis of

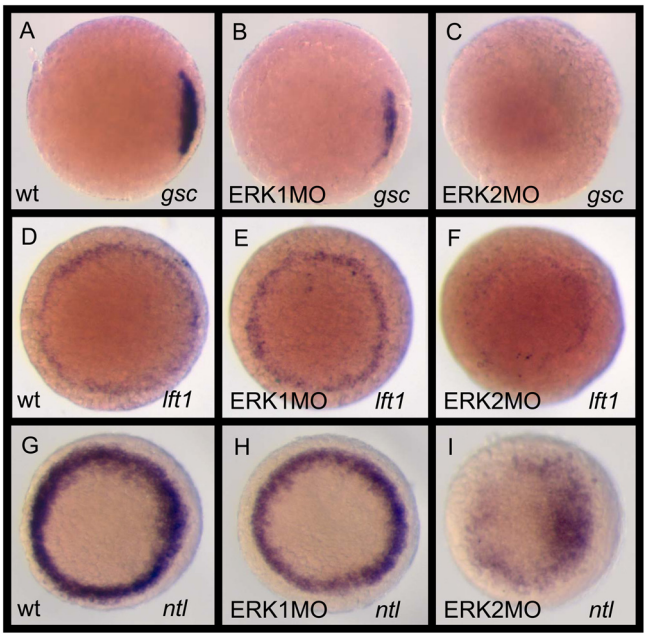

Figure 7. Effects of ERK1 and ERK2 knockdown are confirmed by whole mount in situ hybridization. The zebrafish embryos were injected with 3.4ng ERK1MO (B,E,H) or ERK2MO (C,F,I) and in situ expression patterns were compared to wild type embryos (A,D,G). The embryos were fixed at 4.5hpf, processed for whole mount in situ hybridization, and imaged (animal pole view, dorsal to right). A,B,C: *goosecoid* (*gsc*, presumptive shield/dorsal organizer); D,E,F: *lft1*/*antivin* (blastula margin); G,H,I: *notail* (*ntl*, blastula margin)

important signaling pathways involved in early vertebrate development. These include the Nodal, FGF, Wnt and BMP signaling pathways.

ERK1 and ERK2 are involved in different developmental processes

For biological interpretation of the obtained expression profiles, analysis of gene ontology (GO) can be used as a tool to indicate processes that are likely to be affected. Different gene ontology clusters showed a relative enrichment in ERK1 versus ERK2 knockdown gene expression signatures. Since the annotation of the zebrafish genome is the limiting factor in assigning biological functions we have focused on gene onthologies that are relatively well known and have further supported the analyses by manual annotation of our signa-

ture sets. For instance, this leads to the conclusion that the biological GO-clusters 'development' was significant under-represented for both ERK1 and ERK2 knockdown. More detailed analysis was performed using the signaling-pathway based GenMAPP gene map annotator and pathway profiler program. This led to a model for the distinct effects of ERK1 and ERK2 knockdown in developmental signaling processes as summarized in figure 8. Early embryo developmental processes include mesoderm formation, endoderm formation dorsal-ventral pattering, anterior-posterior patterning and gastrulation movements. To establish a mesodermal zone, next to the dorsal-ventral patterning, also induction processes occur at the animal-vegetal axis. Complex signaling processes are used by the embryo to induce mesoderm, as nicely reviewed by Kimelman (Nature reviews 2006). Based on literature data it is possible to interpret the observed expression patterns and knockdown effects in the context of known signaling pathways underlying these processes as described in the legend to figure 8.

Under the milder knockdown conditions used in chapter 4, reduced expression of ventral markers and concomitant expansion of dorsal marker-genes was found. However, these changes in patterning were not considered sufficient evidence to conclude an altered cell specification. The use of more stringent knockdown conditions as applied in this array-based study showed that in ERK1 morphants the ventrally expressed patterning gene *vent* was down-regulated, but also the BMP inhibitory gene *smurf1* was up-regulated, possibly responsible for inhibition of BMP signaling on the ventral side (Fig. 8, panel D and B). This may lead to a dorsalization of ERK1 knockdown embryos. Surviving ERK1 morphants showed a tailless phenotype (data not shown / chapter 4). This supports a block of BMP-signaling, as tail formation is combinatory regulated by BMP and FGF signaling since mutant embryos for *bmp2b* fail to form tails (Agathon et al., 2003) and embryos with impaired FGF-signaling show tailless phenotypes. However, it is important to note that also genes involved in regulating gastrulation cell migration were altered in expression (*oep* and *quattro*) (Hammerschmidt et al., 2003; Dagget et al., 2004). This makes it difficult to determine whether ERK1 morphants show affected convergence cell migration, due to altered patterning and cell fate specification or due to cell migratory events as described in chapter 4 using less stringent knockdown conditions.

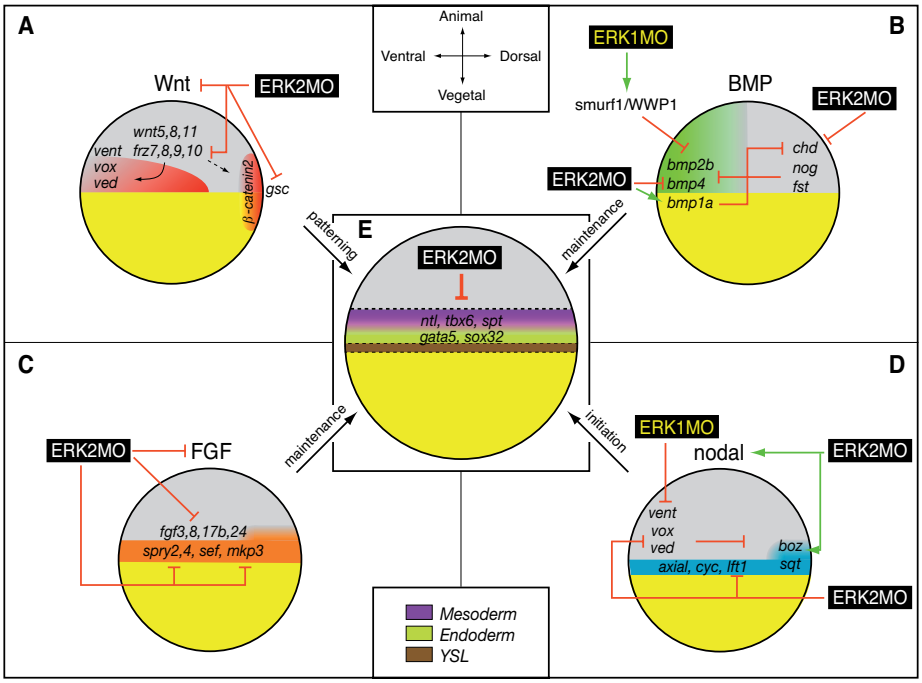

Figure 8. ERK1 and ERK2 knockdown differently affect signals involved in patterning of the early embryo. (A-D): Schematic representation of the effects of ERK1 and ERK2 knockdown on the activities of Nodal, FGF, Wnt and BMP signaling pathways in late blastula embryos. (E): effect of ERK2 knockdown (ERK2MO) on early embryonic mesendoderm differentiation. The representation of predicted signaling activity in the wild type embryos is based on the potential range of signals, the expression patterns and range of antagonists, adopted from Schier and Talbot (2005). The combined signaling activities from these pathways are responsible for the differentiation and fate-map of the late blastula/early gastrula stage of the zebrafish embryo (E, late blastula stage, lateral view, dorsal to right, animal pole to top). In the zebrafish embryo, dorsal ventral patterning starts as early as the 128-cell stages by accumulation of β-catenenin at the nuclei of the dorsal cells, rapidly followed by the expression of *goosecoid* (A). Soon after mid-blastula transition, β-catenin also activates the expression of a number of zygotic genes, including *chordin*, *bozozok* and *squint* (A and D), and FGF signals (C). These genes act to inhibit the action of ventralizing factors or induce mesoderm and endoderm cell fates at the dorsal side. Subsequently, the expression of these genes quickly spreads over the complete margin (panel E). To establish a mesodermal zone induction processes occur at the animal-vegetal axis. Complex signaling processes are used by the embryo to induce mesoderm. In a over-simplified manner, it can be said that Nodal (D) signaling is involved in initiation of mesoderm formation, FGFs (C) and Wnt (A) are involved in maintaining the mesoderm state and BMPs (B) are involved in further patterning of the mesoderm (Kimelman, 2006).

Figure 8. continued: Knockdown of ERK1 (ERK1MO) resulted in an increased expression of the BMP-inhibiting protein *smurf1*/*wwp1* and the ventrally expresses gene *vent* (A). Combined, this indicates a reduction of ventral signaling, possibly leading to a dorsalization of the embryo. ERK2 knockdown (ERK2MO) promotes Nodal signaling by repressing the expression of Nodal inhibitors (*vox*, *ved*, *lft1*) (D). Furthermore, it perturbs FGF signaling (repression of *fgf8* and components of the RAS-ERK pathway and down regulation of its target genes) and Wnt signaling (repressed expression of frizzled receptors and key components of the Wnt pathway). In addition, perturbed BMP signaling results in incorrect patterning of the mesoderm (B). In summary, this shows that mesendoderm differentiation is still initiated by Nodal signaling (D), but mesendoderm maintenance by FGF and Wnt signaling is defected. This results in reduced expression of mesoderm (*ntl*, *tbx6* and *spt*) and endoderm (*gata5*, *sox32*) marker genes (B, C and E), showing that ERK2 is essential for mesendoderm differentiation (E).

ERK2 signaling is essential for the maintenance of the mesendodermal cell fates

In ERK2 morphants no active MAPK was detected at the margin at 4.5hpf (chapter 4) suggesting that Ras-Raf-MEK-ERK dependent FGF signaling and subsequent downstream signaling was blocked. FGF signaling acts as a competence factor for cells to respond to Nodal mediated mesoderm induction. As our data shows that ERK2 morphants are severely impaired in both FGF and Wnt signaling it is likely that mesoderm progenitor cells in the margin are affected in the maintenance of the mesodermal cell fates (Fig.8E). However, it has been reported that Nodal and FGF pathways interact through a positive regulatory loop and synergize to maintain mesodermal cell populations (Mathieu et al., 2004), in addition FGF signaling negatively regulates Nodal-dependent endoderm induction in zebrafish (Mizoguchi et al., 2006). This would suggest that Nodal-mediated initation of mesoderm differentiation is still present, but the maintenance of the mesoderm, mediated by FGF and Wnt, is affected.

In *Drosophila*, FGF-dependent ERK activation was shown to be required for proper mesoderm dispersal (Gabay et al.,1997; Gryzik and Muller, 2004; Stathopoulos et al., 2004). In *Xenopus*, ERK2 was shown to be required for mesoderm differentiation (Gotoh et al., 1995). Mouse *erk2*-/- embryos also fail to form mesoderm at E6.5 and E7.5 based on histological criteria, but *erk2*-/- embryonic stem cells were still capable of forming mesoderm. However, treatment of these ES cells with the MAPK inhibitor PD184352 decreased total ERK activity in these cells and expression of the mesoderm marker *brachyury*/*ntl* (essential for posterior mesoderm and axis formation) (Yao et al., 2003). Our gene expression profiling shows that ERK2 plays a role in mesoderm development based on additional mesoderm markers (e.g. *spt*/*tbx16*, *tbx6*), but

importantly also by addressing the upstream signaling mechanisms involved in mesoderm initiation and maintenance. It should be noted that ERK-activation is not only mediated by FGF signaling, but also influenced by other growth factors (PDGF, VEGF), G-protein coupled receptor signaling and hormone- and Ca^{2+} signaling pathways. A nice example that shows the complexity of interconnections, redundancy and crosstalk between the different pathways is the work of Poulain et al. (2006), showing that combinatorial Nodal, FGF and BMP signaling regulates endoderm formation in zebrafish. The authors also show that activation of FGF-signaling or injection of constitutive active (rat) ERK2 lead to phosphorylation of Sox32 and repression of the endoderm marker *sox17*. However, in our study, ERK2 morphants showed a reduced expression of the upstream Nodal responsive genes *gata5*, *sox32* and *sox17*. These genes are normally expressed in presumptive endoderm progenitor cells in the margin at 4.5 hpf (Aoki et al.,2002). This suggests that depletion of ERK2 also affects endoderm differentiation (Fig.8). Follow-up experiments, using different times of development in combination with chromatin immunoprecipitation (chIP-chip) methodology will be needed to further understand the crucial function of ERK2 in mesendoderm development and determine specific target genes.

Conclusions

Our analysis of the gene expression microarray data revealed that ERK1 and ERK2 knockdown affected a set of common, as well as specific downstream genes. Surprisingly we also discovered a set of genes with anti-correlated expression. The gene ontology analyses show that ERK1 and ERK2 have specific roles in embryogenesis and target distinct gene sets involved in vertebrate development, confirming the embryonic knockdown phenotypes. The signaling pathway analysis on the ERK1 and ERK2 transcriptome signatures using the GenMAPP software program for analysis of BMP, FGF, Nodal and Wnt signaling pathways showed distinct roles for these MAP kinases. For ERK1 knockdown we identified a connection with genes involved in dorsal-ventral patterning and subsequent embryonic cell migration. For ERK2 knockdown we identified a connection with genes involved in mesoderm and endoderm initiation, differentiation and patterning. The outcome of the predictions for ERK2 knockdown on developmental signaling were confirmed by the observed effects on mesoderm and endoderm patterning and subsequent whole mount in situ hybridization experiments. Our results show the strength of gene expression profiling of morpholino knockdown embryos in combination with versatile bioinformatics tools.

Experimental procedures

Zebrafish Morpholino knockdown experiments

Zebrafish embryos were injected at the one-cell stage 1 nl of the solubilized compounds in 1× Danieau's buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES; pH 7.6] containing 1% Phenol red solution (Sigma). Definition of stages was according to Kimmel et al. at 1K-stage (3hpf), embryos with a red animal pole were selected as positive-injected embryos.

To block translation of the ERK1 or ERK2 protein, 0.4mM (3.4ng) morpholinos (MOs) were injected per embryo. MOs were targeted against the 5'-UTR of the respective mRNAs (GeneTools Philomath, OR, USA): ERK1-MO, 5'-TCTGTCCGCAAATCGTCGCCTTCGC; ERK2-MO, 5'-CACCCAAAAGCACCAGGAAAAGCTC. As a control, the standard control MO 5'-CCTCTTACCTCAGTTACAATTTATA was used at the same concentration. Injected embryos were kept at 28°C until desired stages, until sacrifice.

RNA isolation from zebrafish embryos

The zebrafish embryos were homogenized in liquid nitrogen and total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. To remove genomic DNA, RNA samples were incubated at 37°C for 15 min with 10 units of DNaseI (Roche). The RNA samples were purified using the RNeasy kit (Qiagen) according to the RNA Cleanup protocol. Total RNA concentrations were determined spectrophotometrically using a Nanodrop ND-1000 (Isogen Life science). Optical density A260/A280 ratios of all samples ranged from 1.8-1.9, indicating high purity.

Experimental design, Labeling and Hybridization of Agilent 22K-microarrays

A total of 7 Agilent 22K-microarray hybridizations were performed for this experiment. 2 independent biological replicates were analyzed for each gene and in the case of ERK2, at least an additional technical replicate were hybridized for each biological replicate, including 2 dye swaps. For each biological sample, a number of 70-100 morpholino injected embryos were collected at 30% epiboly stage. The RNA from standard control MO injected embryos was labeled with Cy3 and those of ERK1MO and ERK2MO injected embryos were labeled with Cy5, using the Agilent Low RNA Input Linear Amplification kit. Hybridization and scanning were performed by Service XS (Leiden, the Netherlands).

Data analysis of Agilent 22K-microarrays

Feature extraction also performed by Service XS using Agilent FE 8.5 software; all subsequent analysis were performed using the default settings implemented in Rosetta Resolver v 6.0 for an error modeling-based normalization.

For the analysis and detailed annotation shown in the Venn diagrams and tables, we focused on the genes that were most significantly affected. For that selection we used the following criteria: the absolute fold change should be at least 1.5 in each independent replicate; and the p-value provided by the error-model taking into account all hybridizations combined must be smaller than 10^{-5} to compensate for multiple testing false positives. For the tables used for GenMAPP (www.genmapp.org) analysis we took a less stringent approach not limiting the number of genes by fold change, therefore using all genes that had a combined p-value smaller than 10^{-5}.

cDNA synthesis and Quantitative PCR

cDNA synthesis was performed using a TGradient Thermocycler 96 (Whatman Biometra) according to the manufacturer's instructions. RNA samples were identical to those used for microarray hybridization. Reactions were performed in a 20 µl mixture of 150 ng RNA, 4 µl of 5x iScript Reaction mix (Bio-Rad) and 1 µl of iScript Reverse Transcriptase (Bio-Rad). The reaction mixtures were incubated at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

Quantitative real-time PCR was performed using the Chromo4 Four-color Real-time PCR detection system (Bio-Rad laboratories, Hercules, CA) according to the manufacturers' instructions. Gene-specific primers for quantitative real-time PCR were designed, using Beacon Designer software, to generate single gene-specific amplicons of 75-150 nucleotides. Reactions were performed in a 25 µl volume comprised of 1 µl cDNA, 12.5 µl of 2x iQ SYBR Green Supermix (Bio-Rad) and 10 pmol of each primer. Cycling parameters were 94 °C for 3 min to activate the polymerase, followed by 40 cycles of 94 °C for 15 sec and 59 °C for 45 sec. Fluorescence measurements were taken at the end of each cycle. Melting curve analysis was performed to verify that no primer dimers were amplified. All reactions were done in duplicate or triplicate and the threshold cycle C_T values were plotted against the base 10 log of the amount of cDNA by using Opticon Monitor 3.1 (Bio-Rad) according to the manufacturer's instructions. For evaluation of PCR efficiencies of all primers sets standard curves were generated using serial diluted cDNA samples (dilution factors of 1, 5, 25, 125 and 625) and strong linear correlations between the C_T values and the log of input cDNA amount were obtained, indicating correla-

tion coefficiencies ranging from 98% to 101%. Data were normalized using the Genex macro provided by Bio-Rad.

The expression level were tested for *mycn (*v-myc, myelocytomatosis viral related oncogene, neuroblastoma derived; NM_212614), *fos* (*v-fos,* FBJ murine osteosarcoma viral oncogene homolog; NM_205569) and mos (v-mos, Moloney murine sarcoma viral oncogene homolog; NM_205580). β-actin was taken as reference and it showed unchanged expression level between standard control MO injected and ERK1MO or ERK2MO injected embryos. Sequences of forward and reverse primers were 5'- CGAGCAGGAGATGGGAACC and 5'- CAACGGAAACGCTCATTGC for β-actin (AF057040); *mycn* (NM_212614) qP1fw GAGGATGATGAGGAAGATGATGAAG, qP2rv CCTGCCTGAGAGTTGGAGAC; *fos* (NM_205569) qP1Fw TGACCTGGAGCCGCTTTGC, and qP2rv GGTAGGTGAACATGAAGGAAGACG; *mos* (NM_205580) qP1fw CCCTCACCAATCCCCGTCAC , and qP2rv GAGCCTGTGTGCGACTTTACC
.

Whole mount in situ hybridization

Embryos were fixed overnight in 4% paraformaldehyde in PBS at 4 °C and in situ hybridization was performed as described previously (Thisse et al., 1993) using described probes for *gsc*, *ntl* and *lft1*/*antivin*.

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Supplementary data

The following supplemental data files can be found online at;
http://biology.leidenuniv.nl/~krens

Table S1. *Anti-correlated genes1: ERK1MO up-regulated, ERK2MO down-regulated*
The genes with an unigene-IDs flagged with an asterisk (*) = were also found in one of the microarray experiment where standard control MO injected embryos were tested versus control-injection (phenol red), with an absolute fold change of 1.5 and a p-value smaller than 10^{-5}

Table S2. *Anti-correlated genes2: ERK1MO down-regulated, ERK2MO up-regulated*
The genes with an unigene-IDs flagged with an asterisk (*) = were also found in one of the microarray experiment where standard control MO injected embryos were tested versus control-injection (phenol red), with an absolute fold change of 1.5 and a p-value smaller than 10^{-5}.

Table S3. *Common down-regulated genes by ERK1MO and ERK2MO*

Table S4. *Common up-regulated genes by ERK1MO and ERK2MO*

Table S5. *ERK1MO specific genes at 4hpf (30% epiboly)*
The ERK1MO specific genes were selected according to the following stringent filter: the fold change must have been 1.5 fold in each individual experiment and the combined p-value must be 10^{-5}.

Table S6. *ERK2MO specific genes at 4hpf (arrested)*
(1.5 fold in each individual experiment, combined p-value = 10^{-5})

Table S7. *Common effected genes by ERK1MO, ERK2MO and CTRMO*
(combined p-value = 10^{-5})

VI

Summary and General Discussion

Summary and General Discussion

Mitogen activated protein kinase (MAPK) are enzymes that are conserved in plants, fungi and animals. All eukaryotic cells use multiple MAPK modules for signal transduction. MAPK signaling cascades are critical signaling pathways that mediate intracellular responses induced by extracellular signals. Pharmacological and genetic studies using *in vitro* cell culture systems have revealed that these pathways control numerous cellular processes, such as tissue morphogenesis, cell proliferation and differentiation, cell survival, immune responses and adaptation. As a result of these broad functions, perturbed MAPK signaling can lead to diseases or affect the developmental program during embryogenesis. In mammals, 14 *mapk* genes have been identified, of which the archetype MAPK pathway is the extracellular signal-regulated kinase 1 (ERK1)/ERK2 module. The main aim of the study described in this thesis was to investigate activation and function of ERK1 and ERK2 signaling in developmental processes like cell migration and differentiation. To investigate these issues we used both embryonic zebrafish cells and whole zebrafish embryos.

First we addressed the question whether Chitin Oligosaccharides (COs) are able to activate the canonical ERK pathway in embryonic zebrafish cell cultures. The MAPK/ERK pathway was chosen as biological tool because of their major role in the integration and control of multiple cellular and developmental processes. The large extracellular polysaccharide hyaluronan (HA) and its synthesizing enzymes (HAS) have been implicated in regulating the migratory potential of metastatic cancer cells. Members of the HAS family have been shown to be involved in the biosynthesis of both hyaluronan and COs. We found that chitin tetrasaccharides transiently induced phosphorylation of ERK1/2 with a maximum after 15 minutes stimulation. We also examined the signaling pathway to ERK activation by COs and concluded that a high-affinity oligosaccharide receptor system exists that transduces the signal to Raf, MEK, and ERK. Once activated, ERK phosphorylates the downstream target p90RSK kinase (chapter 2). Injection of the chitin tetrasaccharides into zebrafish embryos also elevated the level of ERK activation during gastrulation stages, detected with antibodies recognizing dual-phosphorylated ERK1/2. This result suggests that CO signaling mechanisms are functional during early developmental processes.

Initially, we planned to find specific CO targets using a morpholino antisense oligonucleotides knockdown of ERK1 and ERK2. However, these knockdown studies resulted in severe phenotypes which changed the direction of the initial research outline. Phenotypic characterization of the obtained ERK1 and ERK2 knockdown embryos indicated distinct functions of ERK1 and ERK2

during vertebrate development. To study the role of the different mapk genes in zebrafish, we first identified, cloned and characterized the zebrafish *mapk* gene family (chapter 3). The zebrafish genome encodes for members of all *mapk* subfamilies, the ERK, JNK and p38. We determined their specific spatial and temporal expression patterns during zebrafish development. All *mapk* are expressed during zebrafish development and their expression was predominantly localized in the brain structures at 24 and 48 hpf, except for *p38b*. *P38b* expression was only detected at blastula stage. The dynamic expression patterns of the mapk genes led us to conclude that their expression is tightly regulated and the spatiotemporal differences in expression indicate towards distinct functions of the different MAPKs during embryogenesis. Subsequently, we focused on specific functions for ERK1 and ERK2 during early embryogenesis using morpholino knockdown approach and micro-array based gene expression profiling of ERK1 and ERK2 knockdowns.

Our study showed distinct effects of morpholino knockdown of ERK1 and ERK2 on gastrulation cell movements. The observed cell migration effects were considered to be primary changes in gastrulation cell movements and not caused by altered cell fate specification since the expression of patterning genes was not significantly altered in the knockdown embryos. However, the question of which upstream signals coordinate ERK1 and ERK2 activity and divergent downstream responses still remains elusive.

The upstream mechanisms of activation of ERK1 and ERK2 are mostly considered as highly similar, or even identical. Activation of ERK signaling is often the result of a response to growth factors or hormones, but can also be the result of a response to other compounds like neurotransmitters. Fibroblast growth factors (FGFs) are the best described activators of ERK signaling during developmental processes. Also throughout zebrafish development, activated ERK protein is localized in overlapping expression regions with various FGF ligands and other components of the FGF-pathway (chapter 4). Studies using other model systems, such as *Drosophila*, *Xenopus*, chicken, mouse and zebrafish, showed a link between FGF signaling and developmental cell migration processes. However, as previously mentioned, ERK activation is not only mediated by FGFs. Next to other growth factors (e.g. IGF, MDKB, NGF and TGF), also different compounds such as COs, that are known to activate ERK1 and ERK2, are produced during early zebrafish development. Interestingly, a previous study by Bakkers et al. showed a possible function for COs as signaling molecules in cell growth, differentiation and development of zebrafish embryo (Bakkers et al., 1997). In a follow-up study, Bakkers et al.. showed that MO-mediated knockdown of zebrafish HAS2 leads to the loss of hyaluronan, and severe migratory defects during gastrulation, somite mor-

phogenesis and primordial germ cell migration. In addition, their data provided evidence that convergence and extension are separate morphogenetic movements of gastrulation. This supports the data observed in our cell tracing experiments in ERK1 and ERK2 morphants embryos. In chapter 2 we showed that ERK1/2 can be activated *in vitro* by stimulation of embryonic zebrafish cells with COs. HAS2, the CO and hyaluronan synthesizing enzyme, has a role in convergence extension cell migration. In combination, this may suggest that HAS mediated CO signaling is involved in the direction of cell migration processes by affecting ERK signaling. Secondly, the data showed that convergence extension (CE) are separate morphogenetic movements during gastrulation. This observation is in line with the distinct effect on CE movements that we observed by comparing ERK1 with ERK2 knockdown embryos. ERK1 morphants revealed a convergence defect without a severe posterior-extension defect, whereas ERK2 morphants showed a more severe reduction in anterior-posterior extension.

A more stringent knockdown condition showed that the absence of activated ERK2 from the blastula margin blocked initiation of epiboly, a process driven by radial internalization of these cells. ERK2 morphants were arrested in embryogenesis, as the blastula stayed on top of the yolk, whereas ERK1 knockdown had only a mild delayed epiboly progression (chapter 4).

In order to identify specific distinct target genes for ERK1 and ERK2 and address which processes were affected by the depletion of ERK1 or ERK2, we compared the gene expression profiles from ERK1 and ERK2 stringent knockdown embryos using microarray technology (chapter 5). The obtained gene expression profiles of ERK1 and ERK2 morphants were analyzed using software programs, and web-based analysis tools, e.g. Rosetta, GenMAPP and eGOn (GeneTOOLS). Analysis of the obtained data revealed that ERK1MO and ERK2MO knockdown affect signature sets of common target genes, as well as signatures sets of specific genes. Surprisingly, we also identified gene sets in which the expression patterns were anti-correlated. The gene ontology (GO) analysis revealed that ERK1 and ERK2 knockdown signature sets showed significant differences in over- and under representation of the number of genes in distinct and common gene ontology clusters. For example, the commonly under-represented gene ontology cluster ‘development’ was represented by different genes. Further analysis of the obtained knockdown transcriptomes was therefore focused on the main early developmental pattering and differentiation signaling pathways Nodal, FGF, Wnt and BMP. The obtained results corroborate distinct developmental functions for ERK1 versus ERK2.

ERK1 morphants showed a possible disturbed dorsal-ventral patterning, as genes involved in the development of the ventral side of the embryo were

altered in their expression, possibly leading to a dorsalization of the embryos. However, only a small number of ventral genes were affected in ERK1 morphants and a concomitant up-regulation of dorsal genes was not observed. Therefore we can not conclude that ERK1 knockdown dorsalized the embryos. In addition, genes involved in gastrulation cell migration processes were found to be affected, in line with the previously described function for ERK1 during gastrulation cell migration processes. Still, it is important to note that the array analysis was performed before gastrula-stages. Therefore we can only hypothesize that the genes whose expression is affected by ERK1 knockdown are involved in the later gastrulation cell migration processes and are in fact responsible for the observed phenotypes.

The analysis of the gene expression profiles of ERK2 morphants showed that key pathways involved in mesoderm and endoderm differentiation were affected. This resulted in the reduced expression of mesoderm and endoderm marker genes. Analysis of signaling pathway, using the software program GenMAPP, showed that Nodal signaling (mesoderm initiation) was positively affected, whereas FGF and WNT signaling (mesoderm maintenance) were negatively affected. Combined, this indicates that mesendoderm was still initiated in ERK2 morphants, but its maintenance was perturbed. These predictions based on pathway analysis were confirmed by in situ hybridization with mesoderm and endoderm marker genes. In addition, also dorsal-ventral patterning was possibly affected, since ventrally expressed genes as well as dorsal (organizer) genes were differentially regulated, indicating that ERK2 is also of importance for the patterning of the mesoderm.

Many early mesoderm genes, which are crucial for developmental cell migration processes at later stages, were affected by ERK2 knockdown. Again here it is important to note that the ERK2 knockdown gene expression profile was obtained at blastula stages, before the gastrulation cell migration processes take place. However, stringent ERK2 knockdown depleted the active ERK signal from the margin and subsequently prevented the initiation of epiboly. In addition, the main cell movement to drive epiboly is radial intercalation. In a simplified way, it can be said that blastula cells from deeper layers start outward and intercalate with cells already at the surface, pushing the blastula cells over the yolk. To undergo these cell migration processes, the blastula cells need to recognize a signal or chemo-attractant gradient, polarize, organize their adhesiveness and rearrange their cytoskeleton. The ERK2 knockdown gene expression signature set showed changes in expression of genes involved in all these processes. Still little is known about the molecular basis of the complex movements occurring during epiboly. Follow-up experiments, using different knockdown conditions, timepoints during development

and active forms of both ERKs will help us to further understand the differential roles of ERK1 and ERK2 during theses processes. Importantly, this will also help to determine specific roles and target genes for ERK1 and ERK2 during vertebrate embryogenesis and link towards different functions of ERK1 and ERK2 in oncogenesis.

Future perspective

The work in this thesis provides evidence for distinct functions for ERK1 and ERK2 in cell migration processes and development. Morpholino knockdown technology was used to determine specific functions and downstream target genes for ERK1 and ERK2. To elucidate their individual roles at a detailed molecular level, cellular analysis and transcriptomal profiling after constitutive activation of ERKs by upstream signals or the introduction of constitutively active mutant ERK isoforms will be necessary tools.

Constitutive activation of ERK-signaling is found in several human malignancies and is likely to be involved in the development of tumors. The multiple roles for the ERK-pathway in the acquisitioning of a complex malignant phenotype of potential oncogenic cells suggests that specific blocking of the ERK pathway will result in anti-proliferative, anti-metastatic and anti-angiogenic effects in tumor cells. To further study the distinct roles of ERK1 and ERK2 as potential oncogenes in the formation and initiation of cancer and metastasis the zebrafish can be used as a novel model organism. The generation of transgenic fish that can be induced to express oncogenic forms of proteins involved in MAPK signaling will be useful to address this question. The transplantation of inducible oncogenic zebrafish cells into a transgenic zebrafish background provides an *in vivo* system to monitor progression of cancer and to conduct large scale anti-cancer drugs screenings.

In addition to the work concerning ERK1 and ERK2, this thesis also opens the possibilities to study the developmental role of other MAPKs for which no developmental studies have been reported yet. Improvement of gene annotation and ontology, and tools for proteomics and kinomics, will help to further identify molecular targets and identify new responsive downstream target genes.

Nederlandse Samenvatting

Nederlandse samenvatting

De ‘Mitogen Activated Protein Kinases (MAPK’s) vormen een enzym-familie die een belangrijke rol speelt bij het doorgeven van signalen binnen de cel. Deze enzymfamilie is evolutionair geconserveerd in planten en dieren. De MAPK-modules zijn essentiële signaaltransductieroutes die intracellulaire reacties beïnvloeden welke door extracellulaire signalen worden veroorzaakt. Door farmacologische en genetische studies en door het gebruik van *in vitro* celcultuursystemen, hebben we inzicht gekregen in talrijke cellulaire processen die beïnvloed worden door de MAPK-eiwitfamilie, zoals weefselmorfogenese, celproliferatie, celdifferentiatie, celoverleving en immuunreacties. Een consequentie van deze brede rol van MAPK’s is dat een verstoorde MAPK-signaaltransductieroute grote gevolgen kan hebben, zoals het ontstaan van ziekten of de verstoring van de embryonale ontwikkeling.

In zoogdieren zijn tot op heden 14 *mapk*-genen geïdentificeerd. Eén van de best gekarakteriseerde MAPK-signaaltransductiemodules is die van de Extracellular signal-Regulated Kinases 1 en 2 (ERK1/ERK2). De belangrijkste doelstelling van de studie die in dit proefschrift wordt beschreven, is het begrijpen van het activeringsmechanisme en de functie van ERK1 en ERK2 tijdens ontwikkelingsprocessen, zoals celmigratie en celdifferentiatie. Om de activering en functie van ERK1 en ERK2 te onderzoeken hebben wij gebruik gemaakt van embryonale zebraviscelculturen en zebravisembryo’s.

In eerste instantie heb ik mij gericht op de vraag of Chitine Oligosaccharides (CO’s) de ERK-MAPK’s kunnen activeren in gekweekte embryonale zebraviscellen. Deze vraagstelling was gebaseerd op eerder onderzoek waaruit bleek dat deze molekulen een belangrijke rol vervullen tijdens ontwikkelingsprocessen van gewervelde dieren. Tevens zijn hyaluronan-oligosacchariden (HA’s), die erg lijken op CO’s, betrokken bij het reguleren van de migratie-eigenschappen van metastaserende kankercellen. Bij de biosynthese van zowel HA’s als CO’s zijn leden van de hyaloronan synthase (HAS) eiwitfamilie betrokken. In een studie van Bakkers et al. is aangetoond dat uitschakeling van HAS2 in zebravisembryo’s, celmigratieprocessen tijdens de embryogenese van de zebravis verstoort (Development, 2004).

Uit onze resultaten bleek dat stimulatie van gekweekte zebraviscellen met CO’s leidt tot een transiente activatie van ERK1 en ERK2, met een maximum dat bereikt werd 15 minuten na toediening. Daarnaast hebben wij ook de signaaltransductieroute die leidt tot activatie van ERK1 en ERK2 door CO-stimulatie bestudeerd. Wij concluderen dat er een receptorsysteem met een hoge affiniteit voor dit oligosaccharide moet bestaan, dat na binding een signaal doorgeeft via de ERK-signaaltransductieroute, bestaande uit RAF, MEK en ERK. Na activatie fosforyleert ERK het target-eiwit p90RSK (RSK1, zie

hoofdstuk 2). Daarnaast werd er na injectie van chitine tetrasachariden in zebravisembryo's een verhoogde activering van ERK-MAPK gedetecteerd tijdens vroege ontwikkelingsstadia (gastrulatie, niet getoonde gegevens). Dit resultaat suggereert dat de signaleringsmechanismen van CO's tijdens vroege ontwikkelingsprocessen al functioneel zijn.

Vervolgens hebben we ons geconcentreerd op het bepalen van de functie van de downstream signaal componenten ERK1 en ERK2 tijdens de zebravis ontwikkling, door gebruik te maken van morpholino antisense oligonucleotiden (morpholinos; MO's). Met behulp van MO's kan het vertalingsproces van een mRNA naar een bepaald eiwit (translatie) specifiek geblokkeerd worden, resulterend in een verlaagde aanwezigheid ('knockdown') van het eiwit van interesse (ERK1 of ERK2). Deze aanpak resulteerde in zebravisembryo's met ernstige ontwikkelingsdefecten ten gevolge van het blokkeren van de translatie van ERK1 en ERK2. Aangezien er nog weinig bekend is over de specificiteit van ERK-MAPK's is het interessant om het zebravismodel te gebruiken om meer inzicht in de specifieke functies van ERK1 en ERK2 te verkrijgen.

Om de rol van de verschillende *mapk*-genen verder te kunnen bestuderen, gebruik makend van de zebravis als modelorganisme, hebben we eerst de leden van de *mapk*-genfamilie in het zebravis genoom geïdentificeerd, gekloneerd en gekarakteriseerd (hoofdstuk 3). Het zebravisgenoom bevat genen die coderen voor de leden van alle verschillende MAPK-subfamilies: ERK, JNK en p38. De expressie van deze genen bleek een grote dynamiek te vertonen gedurende verschillende ontwikkelingsstadia en in verschillende weefsels. Dit wijst erop dat de expressie van deze genen strak gereguleerd is. Tevens suggereren de verschillen in expressie verschillende functies voor de individuele MAPK's tijdens embryogenese.

Vervolgens heb ik me geconcentreerd op het ontrafelen van de mogelijk specifieke functies van ERK1 en ERK2 tijdens embryonale ontwikkeling. Hiervoor werd gebruik gemaakt van de al eerder genoemde morpholino 'knockdown' methode (gen-specifieke inhibitie van eiwittranslatie). Uit onze studie bleek dat gen-specifieke "knockdown"van ERK1 en ERK2 verschillende effecten had op de celmigratieprocessen die plaatsvinden tijdens de gastrulatiestadia, namelijk de convergentie- en extensie- celmigratieprocessen. Convergentie-celmigratie is het proces waarbij cellen van de ventrale zijde naar de dorsale zijde van het embryo migreren, terwijl door het extensie-celmigratieproces de (anterior-posterior) lichaamsas wordt verlengd.

Embryo's met een gen-specifieke 'knockdown' van ERK1 vertoonde een vertraagde convergerende celmigratie, terwijl de extensie van de lichaamsas niet significant veranderd was. Dit in tegenstelling tot ERK2 knockdown embryo's, die een vermindering in de extensie van de lichaamsas ver-

toonden, maar geen significante verandering van de convergentie-celmigratie. Een stringentere toepassing van de morpholino-'knockdown' techniek (het gebruik van een hogere MO-concentratie) toonde aan dat ERK2 de actieve MAPK is in de blastula-margin en dat de afwezigheid van geactiveerd ERK2 in de blastula-margin zelfs de initiatie van epibolie (de migratie van blastulacellen over de vegetatieve dooiercel) blokkeert. Deze ERK2 'knockdown' embryo's werden geblokkeerd in hun verdere embryonale ontwikkeling, terwijl ERK1 'knockdown' embryo's slechts een geringe vertraging in epibolie lieten zien (hoofdstuk 4).

Om meer inzicht te krijgen in de verschillende functies van ERK1 en ERK2 bij de beschreven ontwikkelingsprocessen en om de specifieke verschillende 'target'-genen van ERK1 en ERK2 te identificeren, hebben wij de genexpressie-profielen van ERK1- en ERK2 knockdown embryo's bepaald met behulp van de microarray-technologie (hoofdstuk 5). De vergelijking van de verkregen genexpressieprofielen van ERK1- en ERK2- knockdown embryo's liet zien dat ERK1 en ERK2 zowel gemeenschappelijke als specifieke genen beïnvloeden. Tevens werden er genen gevonden die anti-gecorreleerd waren in hun expressie in ERK1- versus ERK2 knockdown embryo's. Deze resultaten bevestigen de conclusie uit onze morpholino-studies dat ERK1 en ERK2 verschillende functies hebben tijdens embryonale ontwikkelingsprocessen.

Het genexpressieprofiel van ERK1 knockdown embryo's wees op een mogelijk verstoorde 'dorsal-ventral patterning', aangezien een aantal genen die betrokken zijn bij de ontwikkeling van de ventrale zijde van het embryo verminderd tot expressie kwamen. Dit leidt mogelijk tot een dorsalisatie van het embryo. Daarentegen werd slechts een klein aantal ventrale genen beïnvloed in ERK1-morphants en werd geen verhoogde expressie van dorsale genen waargenomen. Hierdoor kunnen wij niet definitief concluderen dat ERK1 knockdown de embryo's dorsaliseert. Ook genen waarvan bekend is dat zij een rol spelen bij gastrulatie-celmigratieprocessen vertoonden een veranderde expressie in ERK1 knockdown embryo's. Dit is in overeenstemming met de hiervoor beschreven ontwikkelingsdefecten van ERK1 knockdown embryos die wezen op een rol voor ERK1 voor gastrulatie-celmigratie-processen.

Uit de analyse van het genexpressieprofiel van ERK2 knockdown embryo's bleek dat belangrijke signaaltransductieroutes voor mesoderm en endoderm ontwikkeling verstoord waren. Dit resulteerde in een verlaagde expressie van mesoderm- en endoderm-specifieke genen. Genen die de initiatie van mesodermaal weefsel stimuleren (Nodal-signalen) kwamen verhoogd tot expressie, terwijl signalen die betrokken zijn bij het behoud en onderhoud van mesodermale weefsels (FGF- en WNT-signalen) negatief werden beïnvloed.

Gecombineerd wijst dit erop dat mesendoderm-differentiatie in ERK2 knockdown embryo's nog wel geïnitieerd werd, maar het behoud van de mesendoderm-identiteit verstoord was. Dit resulteert in een verstoorde mesoderm ontwikkeling en blokkering van verdere embryonale ontwikkeling. Wij concluderen uit onze data dat ERK2 van belang is voor het vormen van mesoderm. Veel vroege mesoderm specifieke genen zijn tijdens latere ontwikkelingsstadia ook betrokken bij celmigratieprocessen. Dit feit ondersteunt onze eerdere aanwijzingen voor een rol voor ERK2 tijdens embryonale celmigratieprocessen.

Conclusies

Uit dit onderzoek is gebleken dat het zebravisgenoom codeert voor leden van alle subklassen van MAPK's. Hierdoor is het mogelijk om de functie van deze eiwitten te bestuderen in de zebravis. Chitine Oligosaccharides (CO's) activeren ERK1 en ERK2 in culturen van embryonale zebraviscellen via een receptorsysteem met een hoge affiniteit voor oligosachariden. Verdere studie naar de functie van ERK1 en ERK2 tijdens embryonale ontwikkelingsprocessen laat zien dat ERK1 en ERK2 verschillende rollen hebben tijdens embryonale celmigratieprocessen en differentiatie van verschillende weefsel- en celtypen. Tenslotte reguleren ERK1 en ERK2 de expressie van zowel gezamenlijke als specifieke 'target'-genen tijdens de embryogenese.

Curriculum vitae
&
Publications

Curriculum vitae

Gabby Krens werd geboren op 7 juli 1977 in Roosendaal (gemeente Roosendaal en Nispen). In 1995 behaalde hij zijn HAVO diploma aan de Professor Zeemanschool te Zierikzee. Vervolgens begon hij de opleiding Biochemie aan de Hogere Laboratorium Opleiding; Hogeschool Rotterdam. Na deze opleiding start hij in het jaar 2000 met de studie Biologie aan de Universiteit Leiden, met als specialisatie moleculaire celbiologie en behaalde in 2002 zijn doctoraal diploma (cum laude).

In september 2002 begon hij als promovendus aan het in dit proefschrift beschreven onderzoek, in dienst van het IBL (Instituut Biologie Leiden) en LION (Leiden Institute of Physics) aan de Universiteit Leiden. Dit promotieonderzoek, gesubsidieerd door het BioScience Initiative (BSI), was een samenwerkingsverband tussen de groepen van prof. dr. Herman Spaink (moleculare celbiologie) en prof. dr. Thomas Schmidt (biophysica) en werd uitgevoerd onder de directe begeleiding van dr. Ewa Snaar-Jagalska. Vanaf januari 2007 werkt hij als post-doc in de groep van prof. dr. Herman Spaink, IBL, Universiteit Leiden.

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