CHAPTER I INTRODUCTION

1.1 Mitogen activated protein kinases (MAPK) in general

Cells communicate in multicellular organisms; by secreting and sensing signals, in order to adjust their behavior to the environment. Extracellular signals such as cytokines and growth factors bind to cell surface receptors and trigger the activation of multiple protein signal transduction cascades that mediate cellular responses such as proliferation, differentiation, apoptosis and motility. The Mitogen-activated protein kinase (MAPK) family is a group of homologous proteins forming several linear signal transduction pathways functioning in translating environmental cues into cellular behaviour **Fig1**. MAPKs exhibit their function in the form of sequential phophorylation events leading to two outcomes. The formation of functional signalling complexes in the nucleus to influence gene regulatory networks underlining cellular transformations induced by the environment. Alternatively, MAPKs could be retained in the cytoplasm and influence activity of cytoplasmic proteins [1,2].

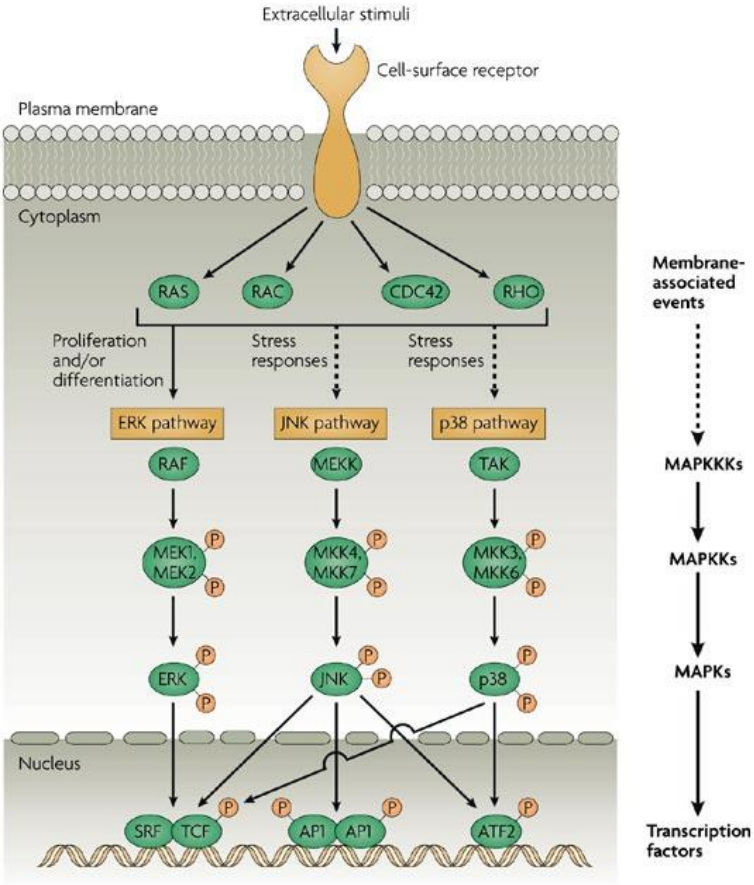

Fig1: ***MAPK pathways.*** The MAPK family consist of the ERK, JNK and P38 subfamilies. Most of the MAPK family members are functional within a MAPKKK-MAPKK-MAPK signaling cascade that is stimulated by members of the small GTPase family such as RAS,RAC,CDC42 and RHO. Small GTPases are kept in an GTP bound active state by Guanine nucleotide exchange factors (GEFs) which relay the signal from membrane bound activated receptors. Activated MAPK could translocate to the nucleus and influence gene expression networks. *Liu et al. 2007,Nature Reviews Immunology*

MAPK are classified into three subfamilies according to the middle amino-acid residue of the conserved Thr-X-Tyr dual-phosphorylation motif; the extracellular signal regulated kinases (Erk1,2,3,4,5,6,7) with a Thr-Glu-Tyr, the c-Jun amino-terminal kinases (Jnk1/2/3) have a Thr-Pro-Tyr motif and the P38 MAPK (α,β,γ, and

Δ) a Thr-Gly-Tyr. Phosphorylation of the Thr-X-Tyr motif activates the MAPK and occurs either by an upstream MAPK Kinase (MAPKK) or by autophosphorylation[2,3]. The MAPKK, (also called Mek) are in turn phosphorylated at Ser/Thr residues by upstream MAPKK Kinases (MAPKKK or Mekk) which could be activated by small GTP-binding proteins of the Ras/Rho family which relay signals from receptor tyrosine kinases (Rtk), integrins or G-protein coupled receptors[4-6].

MAPKs control the activity of numerous transcription factors and cytoplasmic proteins, including other kinases, by phosphorylation of Ser/Thr residues [7-9]. Transcription factor phosporylation occurs in the nucleus after translocation of MAPK as monomers [10]. MAPK could also form dimers and functional complexes with scaffold proteins to regulate signal transduction events in the cytoplasm. Scaffolding proteins are adaptor or anchoring proteins that bind multiple signalling proteins and create a functional complex at a particular subcellular location **Fig2**[11]. The cellular response is mediated by the duration of MAPK activity which depends on a dynamic exchange of phosphate groups on the dual phosphorylation

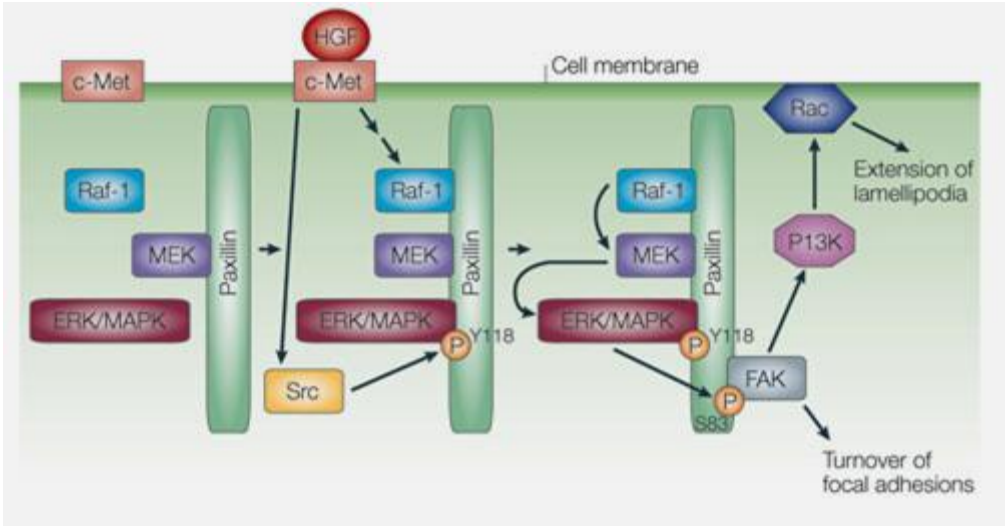

Fig2: Spatio-temporal regulation of the MAPK phosphorelay system by Scaffold proteins. MAPK signaling cascades can be organized into functional complexes at a specific subcellular location by interaction with scaffolding proteins. Paxillin is a scaffold protein recruiting the RAF-MEK-ERK cascade to focal adhesions which are large, dynamic protein complexes through which the cytoskeleton of a cell connects to the extracellular matrix. Following c-met activation, by the hepatocyte growth factor (HGF), paxillin binds and recruits the MAPK cascade to focal adhesion where activated ERK phosphorylates paxillin at S83 creating a binding site for focal adhesion kinase (FAK) at the scaffold and subsequent activation of phosphatidylinositol 3-kinase (PI3K) and Rac. This results in the extension of lamellipodia. *Walter Kolch 2005, Nature Reviews Molecular Cell Biology.*

motif by MAPK kinases and phosphatases[12]. MAPK phophatases (MKPs) inactivate the pathway by direct dephosphorylation of both the tyrosine and threonine residues of the dual phosphorylation motif[12]. Protein Tyrosine Phosphatases (PTPs) switch off the receptor signal through inactivation of upstream pathway members including RTK[13]. Finally, protein serine/threonine phosphatases (PSPs) act downstream of MAPK by removing phosphates of substrates. Interestingly, PP2A is a MEKK1 phosphatase which is activated by P38 during stress creating an inhibitory effect on Erk1/2 induced proliferation.

The MAPK family is conserved among eukaryotes and most vertebrates contain at least 14 MAPKs, 7 MAPKKs, and 20 MAPKKKs. Because of the characteristic conformation of both the MAPK activation loop containing the (Tyr-X-Thr) motif and the kinase pocket of MAPKKs this interaction is highly specific yet allows for some redundancy especially between closely identical isoforms from the same subfamily such as Erk1 and Erk2 or P38α and P38β. The 14 mammalian MAPKs can therefore be divided into four conventional MAPK signalling cascades and atypical MAPKs which are possibly constitutively active.
The first identified (canonical) cascade includes Erk1/2 which are specifically activated by Mek1/2 and initially discovered to be stimulated by growth factors (Egf, Pdgf, Igf and Fgf). The canonical MAPK pathway is stimulated after binding of Egf with its receptor. Ligand bound Egfr signals through adapter protein Grb2 and the Gef Sos to activate the small GTPase family member Ras. Ras transmits the signal to the MAPK cascade by recruiting Raf to the cell membrane for activation. Raf is a MAPKKK, the first kinase of the Erk1/2 cascade, which phosphorylates Mek1 and Mek2. Several other mitogen receptors, such as lpdgfr, Fgfr, Ngfr and Igfr, stimulate the pathway after ligand binding.
P38α,β,γ,Δ constitute the second cascade and are activated by Mek3 and Mek6 but also Mek4 which creates a crosstalk with the third cascade including Jnk1/2/3 and Mek7. Both P38α,β,γ,Δ and Jnk1/2/3 pathways are initially discovered to be stimulated by stress signals such as X-ray/UV irradiation, heat/osmotic shock, and oxidative/nitrosative stress as well as by proinflammatory cytokines. The fourth cascade with Erk5 and Mek5 is also activated by cellular stressors, cytokines and growth factors which are transmitted through Mekk2 and Mekk3. Erk5, also referred to as big MAPK kinase 1(Bmk1), has a larger C-terminal region containing an auto-inhibitory domain and nuclear localization signal[7,14-17]. **Fig1.**

Since the first discovery in 1990, research involving gene expression and functional analysis for identified members of the MAPK family is ongoing. Functional experiments with methods ranging from genetic engineering, pharmacological inhibition, siRNA or transgene expression are frequently used to outline specific and redundant functions of the various MAPK family members within certain contexts.

Some MAPK isoforms are widely expressed and functional in a great number of cell types ranging from embryonic to terminally differentiated lineages. In addition, MAPKs are discovered to be critically involved in severe disorders including cancer, Alzheimer, autoimmune/inflammatory diseases and also human genetic hereditary disorders [7,18-21]. How the existing closely related isoforms of a protein family, sharing sequence and functional domain similarities, take part in orchestrating cellular behaviours is in general a fundamental research topic with great scientific

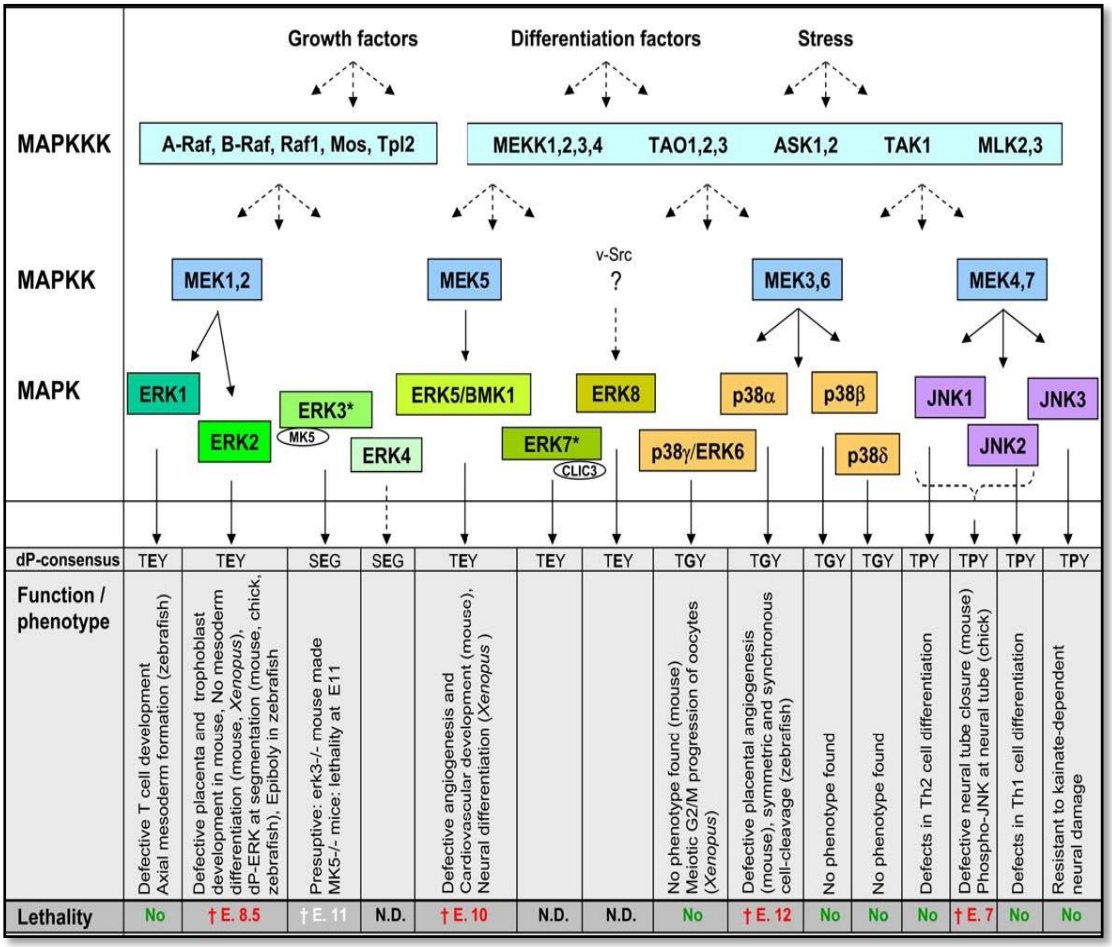

Fig3: The different MAPK cascades and their function in development. The identified MAPK pathways: ERK1/2, JNK1/2/3, p38α/β/γ/δ, ERK5, ERK3/4 and ERK7. The function, obtained by gene targeting or silencing studies are described for each MAPK. The lethality-index indicates if gene-disruption in mice resulted in a lethal developmental phenotype and at what day of development (†=lethal, No=not lethal, ND=not determined). erk3−/− mice neonates die within minutes after birth from acute respiratory failure. Recently (*Rousseau et al. 2010*) Erk4 is shown to be dispensable for mouse embryonic development. *Figure from Krens et al. 2006.*

value for molecular cell biology. Distinction of MAPK isoform signalling could also be beneficial in medical science related to MAPK associated diseases. Strategic invention of effective pharmaceuticals causing limited side effects requires facts of MAPK isoform specific signalling within relevant cellular and micro-environmental context.

1.2 MAPK in embryonic development

1.2.1 Lessons from *In vivo* and *In vitro* knock-out studies in mice.

In vivo functional distinction of MAPK family members has been achieved in particular with the gene-targeting technology in mice. Defects resulting from homologous recombination to disrupt the endogenous gene of interest are reported for most MAPK isoforms except Erk7 and Erk8 **Fig3**. Proteins indispensible for embryonic development could be functional in specific events such as patterning to establish the body plan or control pluripotent cell differentiation into lineages of cells and tissues. Functional studies could provide insight into the underlying mechanisms of human developmental genetic disorders.

Several studies have demonstrated Erk2, Erk5 and P38α to be essential for early vertebrate development and each fulfil a role that could not be compensated by other MAPK family members. Jnk1 and Jnk2 are fully functional redundant in regulation of early brain development. *Jnk*1-/- *Jnk*2-/- double knock-out mice die at E.11 due to defects in neural tube closure and deregulated neural apoptosis while embryos from single knock-outs showed no severe morphological abnormalities[22,23]. In contrast, a genetic study with embryonic stem cells (ESC) suggested that only the Jnk1 isoform was essential for neural differentiation induced by retinoic acid [24]. The expression of wnt-4 and wnt-6, inhibitors of ES cell neurogenesis, was found significantly elevated in JNK1(-/-) cultures relative to wild-type, JNK2(-/-), and JNK3(-/-) cultures. In addition, adult mice Jnk1 or Jnk2 single knock-out display defects of activated T lymphocyte differentiation into T-helper cells [25,26].

Erk2 plays a unique role during gastrulation, the differentiation of endoderm, mesoderm and ectoderm cell layers of embryonic blastocysts [27-29]. Gastrulation, the differentiation of endoderm, mesoderm and ectoderm cell layers of the blastocyst, is regulated by morphogens such as Fgf, Bmp, Wnt and Nodal which collectively orchestrate patterning and cell specification of the vertebrate and the invertebrate embryo. The Erk1/2 cascade functions downstream of Fgf and is involved in specification of the mesoderm, neural induction, anterior-posterior patterning and FGF formation during gastrulation[30]. Pluripotent mice ESC lacking either FGF4/ERK2 autocrine signalling resist mesodermal and neuronal induction

[31]. The Fgf-Erk pathway is an autoinductive stimulus for naive ES cells to exit the self-renewal programme and differentiate towards a state that is responsive to inductive cues for germ layer segregation[31,34,35]. The mesoderm defective phenotype was demonstrated *in vivo* with Erk2-deficient mice as well which also fail to induce the mesoderm cell fate[32]. Erk2 deficient embryos showed also increased apoptosis but proliferation was unaffected. In many somatic cell types, the Erk1/2 cascade is stimulated by mitogens and plays a prominent role in inducing proliferation. The mitogen stimulated ERK1/2 promotes proliferation by translocation to the nucleus for direct phosphorylation of transcription factors ets1, c-myc, c-jun, fos and indirect phosphorylation of CREB through p90RSK. Hyperactivation of the Erk1/2 cascade, as a result of upstream EGFR, RAS and RAF oncogene activity, is associated with uncontrolled proliferation of tumours. However undifferentiated mice ESC, sharing the same proliferative characteristics as transformed cells, do not require the Erk pathway for proliferation and expression of self-renewal markers Oct4, Nanog and Rex[33]. In fact, ESC and transformed cells can be propagated clonally, multiply in absence of serum and are not subject to contact inhibition or anchorage dependence. However attenuation of ERK signalling, by pharmacological inhibition of MEK activity or by ectopic expression of ERK phoshatases, facilitates self-renewal by reducing differentiation specifically in ESC.
Erk2 Knock-out in another *in vivo* background (C57BL/6 mice) leads also to embryonic lethality early in mouse development but after the implantation stage and reveals that Erk2 is essential for the formation of the placenta. Erk2-/- mice failed to form extra-embryonic ectoderm and the ectoplacental cone which differentiate from trophoblasts[36]. Erk2 is therefore involved in the first embryonic differentiation event where the blastocyst segregates into two cell lineages the trophoblast, which will specifically develop into extra-embryonic tissue forming the placenta, and the inner cell mass (ICM) which will predominantly give rise to the fetus.

Several studies using different background mice and knock-out approaches have shown that P38α knock-out is embryonic lethal within the first 12 days. Defects observed in P38α -/- mice include aberrant placental angiogenesis, increased apoptosis and massive reduction of the myocardium [27,37-39]. At the cellular level, P38α is abundantly present in ESC however knock-out did not affect endothelial, smooth muscle and neuronal cell differentiation. P38α-/- ESC have significantly increased cell adhesion to several extracelluar matrix proteins, correlating with elevated phosphorylation of focal adhesion kinase and paxillin. P38alpha -/- ESC also showed increased cell viability, correlating with increased expression of survivin and activation of AKT (protein kinase B)[40].

The Mek5/Erk5(Bmk1) cascade plays a role in embryonic development after the gastrulation stage. Erk5 deficient mice displays aberrant extra-embryonic (placental)

and embryonic blood vessel organization and maturation. Erk5 plays a role in controlling angiogenesis most likely by regulating endothelial cell survival. Histological and *In vitro* examination showed that endothelial cells lost their integrity, became round and eventually apoptotic. Also Cardiac development was largely retarded in Erk5-/- mice, and the heart failed to undergo normal looping [41].

1.2.2 MAPK in zebrafish development.

The disadvantage of the gene knock-out technology in mice is that it is time consuming and costly. The zebrafish is a much better alternative to screen for embryonic important genes. Synthetic antisense oligonucleotides known as morpholinos can be injected easily in batches of first stage zebrafish embryos for targeted knock-down of endogenous genes. A pair of adult zebrafish could lay 100 to 200 embryos according to a 10 hour light cyclus and batches of embryos of a particular stage can be obtained for experimentation. The ex-utero development and transparency of the embryo makes it possible to record phenotypes in wild type or transgenic fluorescent zebrafish lines. With the current available zebrafish tools molecular mechanisms could be identified and linked to cellular processes and morphological observations.

All 14 zebrafish MAPK genes are evolutionary conserved in zebrafish and analysis of phylogeny, based on zebrafish, mice, rat, xenopus, and human amino acid sequence similarity, shows that the different vertebrate MAPK cluster with their corresponding orthologs[28]. Most zebrafish MAPK are recently cloned and characterized by our laboratory using semi-quantitative RT-PCR expression analysis of different developmental stages and whole mount in situ hybridization for expression localization[42]. Further efforts for functional elucidation in zebrafish embryology have been performed for ERK1, ERK2, P38α and JNK1. The Current thesis described additional two MAPK members of the P38 subfamily: the P38β and P38δ isoforms.

FGF activation of the ERK1/2 cascade is also in the zebrafish embryo a critical molecular event during gastrulation. Zebrafish gastrulation occurs during epiboly a morphogenetic movement of the blastoderm over the interface of the yolk until fully covered. When the cells reach the equatorial region of the embryo the three cellular germ layers are formed depending on positional cues and distinct cell migratory properties provided by interplay of Bmp, Nodal, Wnt and Fgf morphogen gradients [43,44]. The Fgf protein family contains 23 members of which Fgf8, Fgf3, Fgf17b and Fgf24 are expressed at the margin of the migrating blastoderm during epiboly[45]. Erk1 and Erk2 are expressed throughout the blastoderm however activity is detected with Erk1/2 immuno-staining at the margin[46]. Morpholino knock-down of both MAPKs identifies Erk2 as a critical mediator of epiboly cell movements and differentiation of mesodermal cell fate[46]. Saturated Erk2 knock-

down prevents a large pool of embryos from initiating epiboly and the remaining from convergent extension movements that occur at 50% epiboly during the actual segregation of the germ layers. Transcriptional profiling of Erk2 morphants revealed downregulated mesoderm specific T-box transcription factors which are also known to be regulated by Fgf8[54]. Furthermore the Ap1 and Ets2 transcription factors are known Erk2 direct substrates and functional in Fgf induced mesoderm formation in xenopus[55,56]. Erk1 knock-down resulted only in a mild effect on epiboly progression without effect on mesoderm markers expression. P38α and its direct substrate Mapkapk2 are implicated in zebrafish epiboly as well [57,58]. However the molecular mechanism upstream and the downstream signaling during zebrafish gastrulation remain still unclear. Perhaps the Fgf pathway does also influence the P38α MAPK signaling. In somatic cells P38α can be activated by growth factors via the similar Grb2-Sos-Ras pathway which lead to the activation of the Raf-Mek1/2-Erk1/2 pathway from activated receptor tyrosine kinases. Growth factor stimulated Ras can also activate Cdc42 and Rac/Posh which will lead to activation of the Mixed Lineage Kinase Mlk2/3 which are MEKK upstream of P38 and also Jnk MAPKs[59]. The non-canonical Wnt pathway, also a major regulator of cell migration during gastrulation, could also recruit Rac to activate the Jnk module in Xenopus and mammalian cells [60]. Alterations in Rac or Jnk activity did disrupt Xenopus gastrulation and polarized cell movements. In zebrafish knock-down of Jnk1 did not result in severe defects in migration (unpublished data). Since functional redundancy of Jnk1 and Jnk2 was demonstrated previously in mice, the function in zebrafish epiboly might still be continued to be performed by Jnk2 despite reduced levels of Jnk1 in these embryos. Like in mice, a double Jnk1 and Jnk2 morpholino knock-down in zebrafish would reveal its possible function if this is the case. However this thesis focuses on the role of P38 and Erk2 MAPK in zebrafish early development.

Other early embryonic activities regulated by Fgf morphogens, and possibly the downstream Erk cascade, during gatrulation are dorso-ventral patterning, neural induction, endoderm formation and left-right assymmetry [30,47-49]. Over activation of Fgf8/Fgfr1/Ras/Mek signaling pathway leads to regional expansion of Bmp antagonists Chordin, Noggin and Follistatin expression which mark the dorsal side. Consequently the expression of Bmp ventral markers Bmp2b and Bmp4 is significantly reduced[49]. Dorsalized embryos appear extremely elongated which was the case after over activating a member of the Fgf8/Fgfr1/Ras/Raf/Mek cascade [47,50-52]. Neural induction is the process whereby naive ectodermal cells are instructed to adopt a neural fate. The ectoderm gives rise to both the neural tissue and the epidermis. Impeding FGF signaling results in a shift of posterior ectodermal markers from neural to epidermal. Dominant negative RAS blocks induction of posterior neural markers [53]. Conversely, over expression of Fgf3 expands posterior neural tissue. Anterior neural tissue, along with posterior

neuronal fate, fails to develop when inhibiting both FGF and Nodal signaling during zebrafish gastrulation. Specification of the neural plate requires repression of BMP signaling in the dorsal ectoderm. The FGF-MEK-ERK pathway induces expression of BMP antagonists and is able to block BMP binding to the receptor. However, an alternative pathway is also suggested since FGF signaling was able to repress BMP expression in absence of protein synthesis. The interaction of FGF/ERK pathway with BMP and Nodal signaling is also crucial for endoderm formation. While the ectoderm germ layer arises from the animal pole region of the embryo, the mesoderm and endoderm progenitors are located at the margin at the expression region of Nodal and Fgf. The endoderm progenitors arise from the first four rows of marginal cells, while the mesoderm develops from the entire marginal region. Endoderm specification is repressed in regions with both FGF/Erk and Bmp expression. Although FGF expression ovelaps the most vegetal marginal cells which develop into endoderm, BMP morphogens are absent in this region. Left-right assymmetry of the brain, heart and gut is regulated in the zebrafish by a ciliated epithelium inside a fluid-filled organ called Kupffer's vesicle which arise from dorsal forerunner cells during gastrulation. Multiple Kupffer's vesicles migrate ahead from the migrating margin establishing a directional fluid flow resulting in asymmetrical gene expression that leads to asymmetrical organogenesis. The fibroblast growth factor (FGF) signalling regulates cilia length and function in diverse epithelia during zebrafish and *Xenopus* development. Morpholino knockdown of FGF receptor 1 (Fgfr1) in zebrafish cell-autonomously reduces cilia length in Kupffer's vesicle and perturbs directional fluid flow required for left–right patterning of the embryo. Expression of a dominant-negative FGF receptor (DN-Fgfr1), treatment with SU5402 (a pharmacological inhibitor of FGF signalling) or genetic and morpholino reduction of redundant FGF ligands Fgf8 and Fgf24 reproduces this cilia length phenotype.

1.3 Structural comparison of P38 and ERK MAPK

The effect of MAPK signalling into different possible cellular outcomes depends on presence of kinases and phosphatases that regulate the on and off switching of the MAPK [61,62]. The mechanism of MAPK activation is much more understood since the crystal structures of both the low-activity, (unphosphorylated) and high-activity (phosphorylated) forms are resolved with X-ray crystallography **Fig4**. Especially research focused on Erk2 structural changes following phosphorylation provided insight into a common mechanism controlling activation and substrate specificity for protein kinases in general [61-65]. Folded protein kinases appear in two spherical lobes; the N-lobe and the C-lobe with the catalytic site in between **Fig4a**. The N- and C-lobes rotate apart to release substrates or together to align the catalytic site residues located at the interface of the two lobes [66]. The proper juxtaposition of active site residues on both lobes enables phosphoryltransfer from ATP to

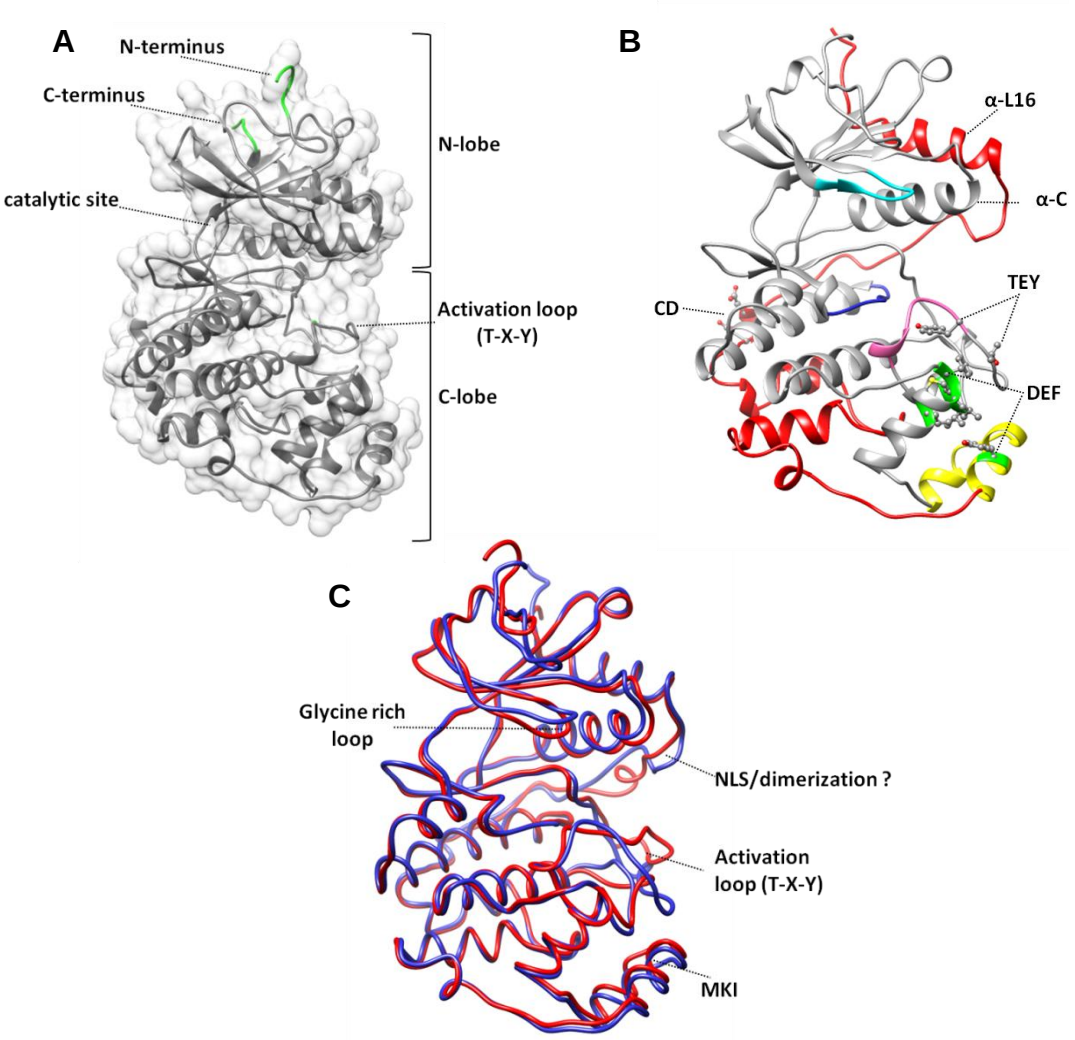

Fig4: Predicted three-dimensional structure of zebrafish Erk2. Homology models are obtained of unphosphorylated and phosphorylated zERK2 using 1ERKA and 2ERKA respectively (PDB). **A** Surface and ribbon figure showing protein features common to other protein kinases including cyclin-dependent kinase-2 (CDK2), cAMP-dependent protein kinase (PKA), phosphorylase kinase (PHK) and the Tyr kinase domain of the insulin receptor (IRK). Except the tyrosine residue of the dual phosphorylation site which is unique for MAPKs. **B** MAPK have two allosteric docking sites**.** The CD-site (ball and stick residues) is located on the C-terminal extension (red) and the groove for DEF domain docking is located between α-helix G, the MAPK insert (yellow) and activation loop. The P+1 site (pink) is also involved in substrate kinase interaction. Catalytic residues are located in the glycine rich loop (cyan), catalytic loop (blue) and α-helix C. **C** Superposition of unphosphorylated and phosphorylated zERK2. Phosphorylation of Thr-183 and Tyr-185 results in reorientation of the activation loop, glycine rich loop and MAPK insertion consequently leading to MAPK activation. Images made with UCSF Chimera http://www.cgl.ucsf.edu/chimera.

substrate. In the C-lobe the activation site of the kinase and allosteric docking sites for interacting proteins are situated. The C-terminal region folds back spans both domains and terminates in close approximation of the N-terminus **Fig4b (red)**. This C-terminal extension is longer in MAPK kinases and is important for stable protein folding and recognition of interacting proteins. The N-lobe of MAPKs consist of a several loops, a β-sheat and 2 helices α-helix C and α-helix L16. The latter being part of the C-terminal extension. Some residues of the catalytic site for ATP binding are located in α-Helix C. The C-lobe is mainly composed of α-helices but contains also several short β-strands and loops with catalytic residues that are clustered in the catalytic loop **Fig4b (blue)**. The catalytic loop is allosterically controlled by Loop 12 (activation loop) at the surface of the C-lobe.

Loop 12 contains the dual-phosphorylation site Thr-183 and Tyr-185 (TEY motif rat Erk2 numbering) for kinase activation. Upon phosphorylation the activation loop changes conformation making the MAPK kinetically active and accessible for substrates **Fig4c.** Thr-183 and Tyr-185 induce conformational changes differentially and contribute therefore separately to the activation of the protein. Tyr-185 is unique for MAPKs which is part of a highly conserved P+1 sequence (YVATRWYR) **Fig4b (pink)**. After phosphorylation the P+1 sequence forms an pocket for interaction with the phosphoacceptor site on substrates. MAPK phosphorylate substrates at Serine and Threonine residues next to a Proline residue (P+1). This interaction is inhibited by the side chain of unphosphorylated Tyr-185 which occupies the pocket. Phosphorylation of the Thr-X-Tyr motif induces rearrangements of hydrogen bonds and ionic contacts with other residues and cause the tyrosine side chain to face the surface of the molecule. This process opens the P+1 pocket for Proline and places the Ser/Thr residue of the substrate to the correct position for phospho-transfer[67]. Phospho-Thr-183 rearranges active site residues by forming direct ionic contacts with arginine residues in α-helix C (Arg-68), in the catalytic loop (Arg-146) and in the activation loop (Arg-170). These interactions create two extended networks of interacting amino acids that connect the activation loop with α-helix C and the C-terminal extension while changing the orientation of the N-terminal and C-terminal domains. Phosphorylated Tyr-185 increases the accessibility for substrates binding.

In addition the C-domain contains two allosteric docking sites that confer specificity for substrates referred to as the CD-site and a DEF/ED domain docking site. The CD-site is a docking site located on the C-terminal extension accessible for substrates containing a DEJL motif with consensus sequence Arg/Lys2-Xaa2–6-Faa-Xaa-Faa (where Faa are the hydrophobic residues Leu, Ile or Val). In the CD domain of MAPKs, two or three acidic amino acids (D330, D327 zERK2) forms an acidic cluster which is crucial for docking to a cluster of basic amino acids commonly present in MAPK-docking sites. MAPK substrates could also contain a

DEF/ED site (Phe-X-Phe) and dock in a hydrophobic groove **Fig4b (green)** between the loop the activation loop, α-helix G and the MAPK insertion **Fig4b (yellow)**[61,68-70] [71].

The existing structural differences between P38 and Erk influence the affinity for substrate binding and underlie therefore the specification of their biological function. The first obvious structural differences is the change in conformation of the activation loop which is 6 residues shorter in P38 than Erk2 causing a replacement of the Thr and Tyr phosphorylation sites. This could lead to differences in the binding affinity of activators (MEKs) and substrates. The length of the activation loop also plays an important role in controlling autophosphorylation. Other structural differences are observed when looking at the α-Helix C in the N-terminal domain which is rotated in P38 in respect to its position in Erk2. In addition the interactions between the C-terminal extension (α-Helix L16) and the other part of the N-terminal domain are weaker. These differences result in a relatively open ATP binding site in P38.

1.4 Scope of the thesis

The documented 30 years of research focus on the MAPK protein family is a rich source of information on molecular roles linked to cellular behaviour. Activation of MAPK cascades leads to precise cellular responses depending on intrinsic cell-type specific factors and the nature and abundance of the extracellular signal(s) in the cellular microenvironment. We are interested in the molecular mechanisms of MAPK signalling that facilitate proper development of the zebrafish embryo. The first stages of embryonic development involves divers, often in parallel occurring, cellular behaviours that contribute to the formation of the body plan such as proliferation, cell fate decisions, adjustment of cell motility and adhesiveness. Most MAPK family members are expressed at an early stage of development and might be part of the underlying mechanisms. Using four different approaches (**Chapter 2-5**) we aimed, with the present study, to contribute to the elucidation of Erk1/2 and P38 MAPK molecular mechanisms during zebrafish early development.

Description of the Erk/MAPK three-dimensional protein structure, identification of pathway components and biochemical studies describing signal amplitude, duration and localization in divers biological systems where of great value for explaining how a single MAPK cascade could be involved in different cellular behaviours. Models of distinguished MAPK transient, oscillatory, bistable and ultrasensitive signalling shed light into the multitasking capacity of Erk2. Further enrichment of this knowledge will add to our understanding of the imbalanced Erk2 signalling observed in tumorigenesis and provide a fundament for treatment design and prediction. To adjust Erk2 kinetic activity amino-acid substitutions were introduced and the effect

on downstream signalling and gene transcription were assayed based on well established molecular mechanisms. We constructed constitutive active zebrafish Erk2 mutants that will be used for identification of new signalling events in the context of embryology (**Chapter 2**). In future genomic incorporation of the mutants and engineering of zebrafish transgenic lines with tissue specific stable expression would be valuable for modelling Erk2 associated diseases.

Analysis of P38/MAPK isoform specific expression in organ tissues and during different time points of the developing zebrafish embryo is a first step for detailed functional elucidation of this cascade. With the second experimental chapter we completed expression analysis of all P38 isoforms by expression analysis of the P38β and P38δ isoforms. Like most MAPK, expression of both transcripts was detectable within the first 24 hours of zebrafish development and at 1dpf, when the body plan was completed, expression was most saturated in the brain compartments. The P38β transcript was maternally present and stably expressed during the first stages of development. P38δ expression started after zygotic transcription and varied in different stages (**Chapter 3**).

We performed also a comparative study between P38α and Erk2 MAPK since both are essential in a similar process during development. Morpholino knock-down of either P38α or Erk2 disrupts epiboly an embryonic process involving germ layer segregation which occurs in zebrafish from 5 to 10hpf. Transcriptome analysis of P38α and Erk2 morphants at high/oblong stage, approximately an hour before initiation of epiboly, revealed large, partly overlapping pools of genes functional in different aspects of early development such as morphogen pathway, cell adhesion and migration. Interestingly, some genes functional in later established tissues of the immune system or nervous system were affected by P38α or Erk2 knock-down. In addition, inhibition of endogenous P38α expression affects a significant pool of ribosomal proteins. We hypothesize that this molecular link can be attributed to the role of P38α in maintaining cellular integrity during stress (**Chapter 4**).

Mammalian P38α deficiency resulted in aberrant placental angiogenesis and many *in vitro* studies in endothelial cells mapped P38α downstream of Vegf a key growth factor for endothelial proliferation and vascular remodelling. We questioned whether this role was conserved in zebrafish by unsaturated morpholino knock-down in Tg:fli+eGFP zebrafish. We addressed the role of P38α in zebrafish angiogenesis and found that P38α depletion had no effect on vasculogenesis but disrupted outgrowth of intersegmental vessels (ISV) from the dorsal aorta in the trunk of the zebrafish embryos. Angiogenesis of intersegmental vessels was completely blocked after treatment with the P38 inhibitor Birb796. With transcriptional analysis we investigated whether P38α regulates expression of angiogenic factors (**Chapter 5**)

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