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Identification of novel roles and new modes of regulation for the atypical MAP kinases ERK3 and ERK4

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Rania Al-Mahdi

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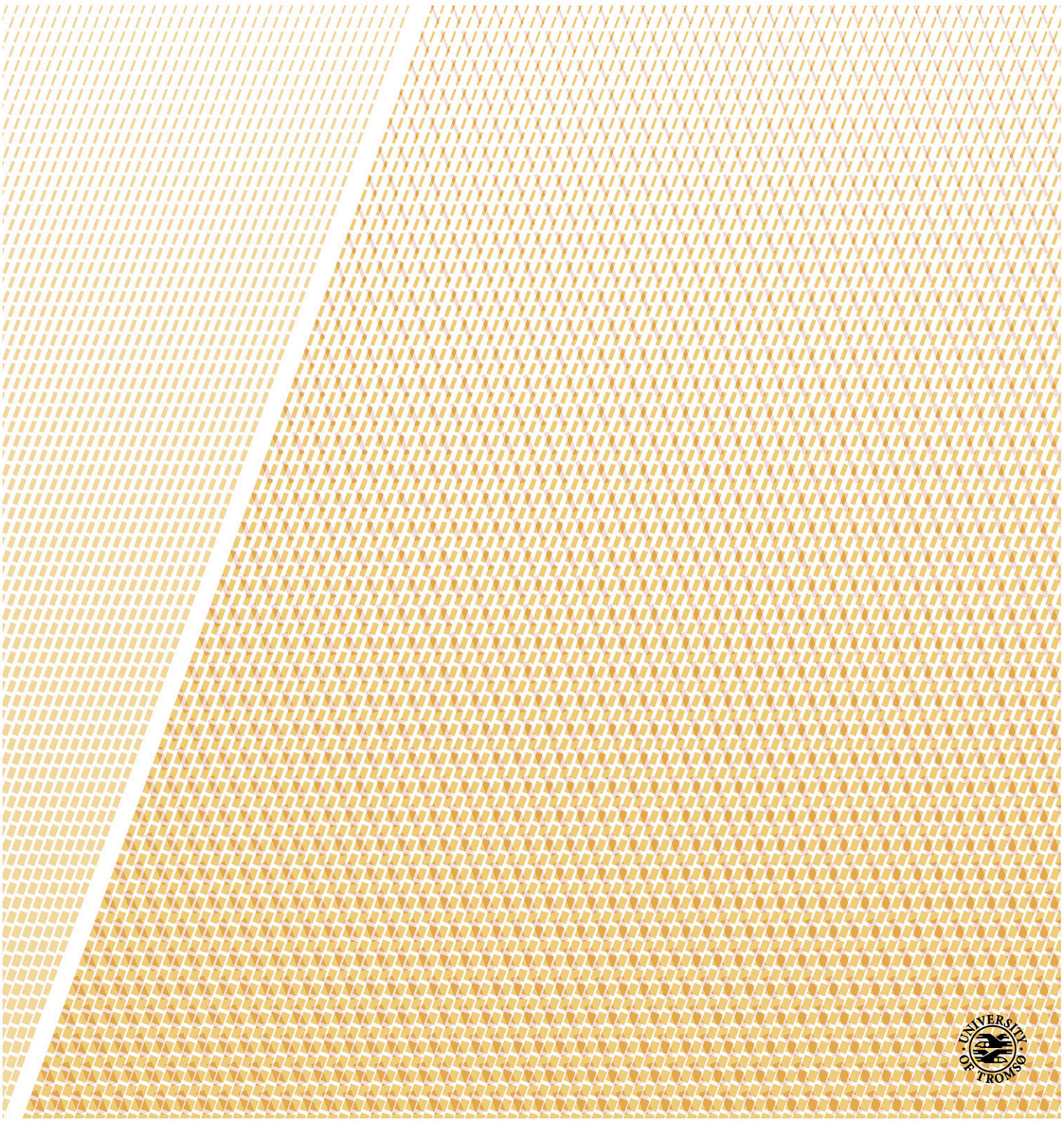

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NORWEGIAN **CANCER** SOCIETY

Rania Al-Mahdi
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Acknowledgements

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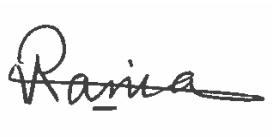

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A novel role for atypical MAPK kinase ERK3 in regulating breast cancer cell morphology and migration

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Accepted Manuscript for publication in Cell Adhesion & Migration Journal, Volume 9, Issue 6.

Paper III

Regulation of the atypical MAP kinases ERK4 by Hsp90

Rania Al-Mahdi, Bjarne Johansen, Hanne Kildalsen and Ole-Morten Seternes

Manuscript

Abbreviations

17-AAG	17-Allylamino-17-demethoxygeldanamycin
Ala	Alanine
aPK	atypical protein kinase
Arg	Arginine
ATP	Adenosine triphosphate
CD	Common docking
DUSP	Dual-specificity phosphatase
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ePK	eukaryotic protein kinase
ERK	Extracellular signal-regulated kinase
FA	Focal adhesion
Glu	Glutamic acid
Gly	Glycine
Hsp90	Heat shock protein 90
kDa	kilodaltons
KIM	Kinase-interacting motif
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MK5	MAPK-activated protein kinase-5
MKP	Mitogen activated protein kinase phosphatase
MMP	Matrix metalloproteinase
NSCLC	Non-small cell lung cancer
PAK	p-21 activated kinase
Pro	Proline
RTK	Receptor tyrosine kinases
Ser	Serine
SRC-3	Steroid receptor co-activator 3
TCR	T-cell receptor
Thr	Threonine
Tyr	Tyrosine
UV	Ultraviolet

I. Introduction

Since 2010, cancer is a leading cause of death worldwide, according to the *World Cancer Report* from the International Agency for Research on Cancer and United Nation report. In Norway, with a population of almost 5 million people, around 30,401 new cases were reported in 2013 were reported. Cancer does not discriminate between developed and developing countries. High cancer incidence and death rates are seen all over the world but to a lesser extent in developed countries according to the World Health Organization (WHO).
Cancer is a complex disease in which many of the characteristics of normal cell behavior are highly deregulated (1). It is a disease of the genome, where each tumor has its own set of genetic alterations. The progression of the cancer is associated with a complex interplay between the tumor cells and the surrounding tissues. Hanahan *et al.* have characterized the cellular and molecular events that enable the malignant transformation of cells harboring oncogenic alterations. These events include: uncontrolled cell proliferation, evasion of growth suppressors, inhibition/resisting of cell death, induction of angiogenesis by creating a particular microenvironment that contains blood vessels, the activation of invasion and metastasis, reprogramming of energy metabolism, evasion of immune detection, tumor promoting inflammation and genomic instability (1). Improve understanding of the genetic changes observed in cancer cells is leading to more effective treatment strategies.

There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected (2). Normally, the body forms new cells when they are needed and replacing old cells that die. If this process goes wrong due to genetic changes, new cells grow even if they are not needed and old cells do not die when they should. An oncogene is a gene that can become permanently turned on or activated when it is not supposed. When this happens, the cell grows out of control, which can lead to cancer. Tumor suppressor genes are responsible for slowing cell division, repairing DNA mistakes, or inducing apoptosis. When tumor suppressor genes do not work properly, cells can grow out of control, which can also lead to cancer. The accumulation of proliferated cells due to genetic alteration can form a lump or mass of tissue called a tumor. Tumors can be either benign or malignant. Malignant tumors are the ones that are considered cancerous. The core of a tumor is deprived of oxygen and nutrients due to its expansion, so growth of new blood vessels (angiogenesis) is required. Malignant tumors can invade nearby tissues (invasion). The cells can also break away and spread to other parts of the body (metastasis) (Figure 1). The transformation of cancer from a

localized solid tumor to a disseminated metastatic tumor is always associated with poor patient prognosis. Tumor invasion and metastasis requires orchestration of a myriad of proteins that play a role in cell migration and adhesion.

Protein kinases are one of the most frequently mutated families of genes that contribute to neoplastic malignancies (3). Protein kinases may act as tumor suppressors or proto-oncogenes. Mutations in protein kinases may lead to tumorigenesis due to their involvement in numerous molecular events that are essential for the development of the hallmark of cancer as described by Hanahan *et al* (1, 4).

The most common cancer treatments include surgery, chemotherapy and radiotherapy. However, these are not the only available cancer therapies, there are alternative cancer treatments including immunotherapy, hyperthermia, and the use of kinase inhibitors which is a class of chemotherapy.

Kinases have become one of the most attractive targets for drug development in cancer treatment. The first kinase inhibitor that was approved for clinical use was Imatinib (2001), it is used for the treatment of chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs), and a number of other malignancies. Currently, there are more than 25 kinase inhibitors that have been approved for cancer treatment and 130 kinase inhibitors that are in clinical trials (phase I-III) (5, 6).

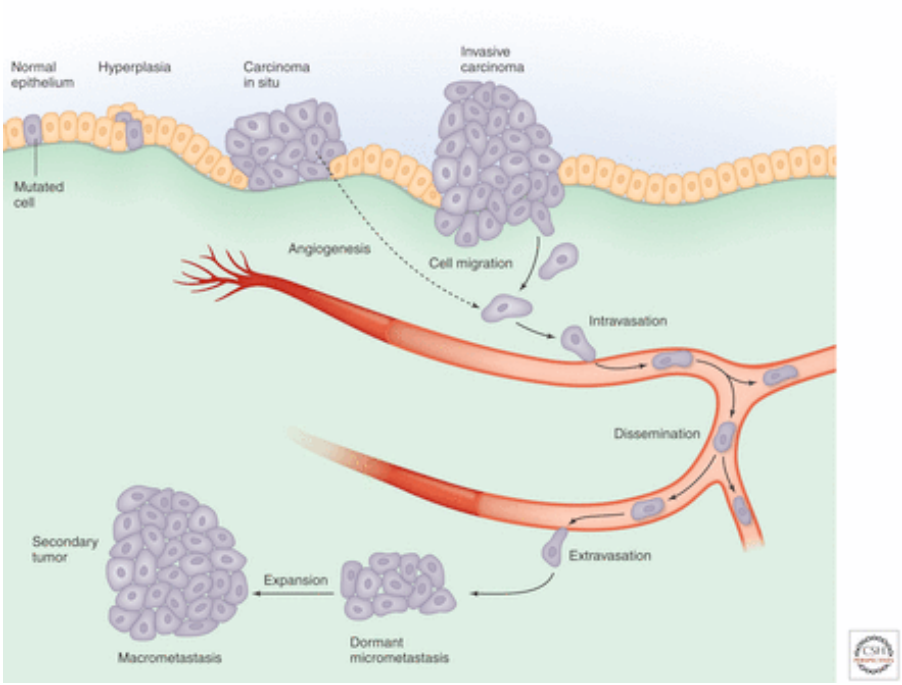

Figure 1. Simple illustration to show the Cancer progression that involves the cancer hallmark. (Reprinted from (7) with permission).

1. Kinases

Protein kinases are a large superfamily of enzymes that play crucial roles in most cellular processes. Kinases constitute one of the largest and most functionally diverse gene families. Many protein kinases are components of signal transduction pathways that regulate cellular processes such as division, differentiation, migration, and survival. Deregulation of human kinases has been causally linked to a variety of human malignancies. This is why inhibition of these deregulated kinases is crucial for the development of new cancer therapies (5, 6, 8).

Kinases orchestrate its activity by adding phosphate groups to substrate proteins; these phosphate groups direct the activity, localization, and function of the phosphorylated proteins. The process of phosphorylation is mediated via the action of kinases that catalyze the transfer of a phosphate group (PO3) from the high energy donor, adenosine triphosphate (ATP), or guanosine triphosphate (GTP), to a hydroxyl group on serine, threonine, or tyrosine residues of the substrate proteins (9). Autophosphorylation can happen when the phosphate acceptor is located within the same molecule (9).

The set of genes encoding protein kinases in the genome is called the kinome. The human kinome includes 518 protein kinases, where 478 of them are eukaryotic protein kinase (ePKs) and they belong to a single superfamily whose catalytic domains are related in sequence (10). The remaining 40 kinases lack sequence similarity to the ePK domain, and they are known as the atypical protein kinases (aPKs).

The ePKs are further classified into eight major groups based on sequence similarity within this domain (Figure 2). **1-** The protein tyrosine kinase (TK) group consists of two main groups, cytosolic tyrosine kinases (CTKs) or non-receptor tyrosine kinases (e.g. Src, JAK1/2/3) and receptor tyrosine kinases (RTK). RTKs are transmembrane proteins involved in signaling at the cell membrane. Examples of RTKs include the human epidermal growth factor receptor (HER/EGFR) family, the insulin receptor (IR) and the closely related insulin-like growth factor 1 receptor (IGF1-R). **2-** The tyrosine kinase-like (TLK), this group has similar sequences to TK group, but they are generally active on serine/threonine substrates. TKL is the most diverse group, containing both non-receptor and receptor kinases. Its members include the interleukin-1 (IL-1) receptor-associated kinase (IRAK), the RAF kinases, the LIM domain kinase (LIMK), and the transforming growth factor beta (TGFβ) receptors. **3-** The STE kinases are classified into three families based on their homology to the yeast proteins STE20 (MAP4K), STE11 (MAP3K), and STE7 (MAP2K). One of the prominent members of this family is the p21-activated kinase family, (Paks), which are a critical regulators of diverse signaling pathways. This group also includes the MAP4K, MAP3K and MAP2K components of the MAPK cascade. **4-** CSNK1 (casein kinase 1, also known as CK1) is one of the smaller protein kinase groups. The members of this group are serine/threonine kinases and they phosphorylate a wide variety of substrates involved in cytoskeletal function and transcriptional regulation. They are also involved in the regulation of membrane trafficking, DNA replication, Wnt signaling, and RNA metabolism. **5-** The AGC group is named for the following enzyme families: protein kinase A, protein kinase G, and protein kinase C related, Members of the AGC family are cytoplasmic serine/threonine kinases that are regulated by secondary messengers such as cyclic AMP (PKA) or lipids (PKC). **6-** The CMGC group is named after its constituent subfamilies: CDK, MAPK, GSK3 and CLK. Members of this group are essential and typically represent a large group of kinases found in all eukaryotes. CMGC group members are involved in cell-cycle regulation and signaling, cell communication, and cell growth. **7-** CAMK (Ca^{2+}/calmodulin-dependent kinases), is a family of serine/threonine kinases that mediate many of the second messenger effects of Ca2+. There are two main classes of CaMKs, mulitfunctional CaMKs (CaMKK,

CaMKI, CaMKII and CaMKIV), which have multiple downstream targets, and substrate-specific CaMKs (CaMKIII), which have only one known downstream target. **8-** Finally, the receptor guanylyl cyclase (RGC) group represents the smallest of the kinase groups. Members of this group are similar in domain sequence to tyrosine kinases.

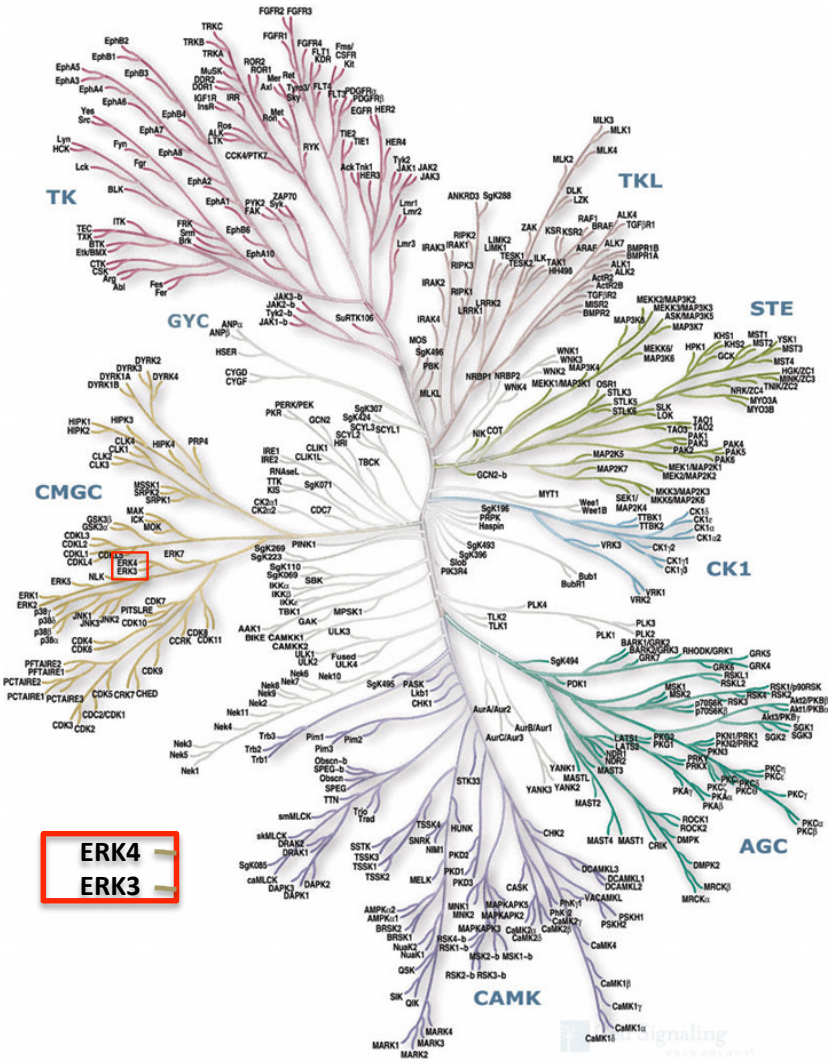

Figure 2. The human kinome. It comprises of 478 classical eukaryotic protein kinase (ePKs) and 40 atypical protein kinase (aPKs). The conventional and atypical MAPKs belong to CMGC group. ERK3 and ERK4 are atypical MAPKs. (Adapted from (10))

2. Signaling pathways

Cell signaling is a process where stimuli are transmitted via a signaling cascade to effector molecules that orchestrate the appropriate biological response. The stimuli could be in the form of extracellular molecules (e.g. hormones, growth factors, cytokines, chemokines or neurotransmitters), UV light, or high osmolarity. Specific cell membrane receptors recognize these extracellular molecules and activate intracellular molecules causing a cascade of signals within the cell. This cascade will end up with a biological response(s) such as proliferation, apoptosis, alteration in metabolism, or gene expression. There are many distinct signaling pathways (e.g. PI3K/AKT pathway, JAK/STAT pathway and MAPK pathway) (11).
These signaling processes make the living cells able to communicate, monitor, and respond to their external and internal environments. Aberrant signaling pathways are a hallmark of cancer (1). Therefore, it is important to investigate and understand how specific signaling pathways are turned on or off. Cascades of kinases that are commonly activated in cancer often belong to the Mitogen-activated protein kinases (MAPKs) pathway (12, 13).

3. MAP kinases

Mitogen-activated protein kinases (MAPKs) are protein Ser/Thr kinases that convert extracellular stimuli into a wide range of cellular responses. MAPK pathways are evolutionarily conserved kinase modules among eukaryotes that control fundamental cellular processes such as gene regulation, growth, differentiation, migration, apoptosis, and proliferation. MAPK pathways are comprised of a three-tier kinase module, which are sequentially acting kinases: MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKKs can be activated by small G-proteins of the Ras/Rho family. MAPKs have been divided into two groups, classical (conventional) and non-classical (atypical). Conventional MAPKs include the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), p38 isoforms (α, β, γ, and δ) and ERK5, whereas the atypical MAPKs comprise of ERK3, ERK4, ERK7, and Nemo-like kinase (NLK) (Figure 3). MAPKs will be dephosphorylated following their activation by broad-specificity phosphatases such as protein tyrosine phosphatases and PP2A, in addition to the MAPKs phosphatases (MKPs). The MKPs belong to the dual-specificity phosphatase family (DUSPs) (14, 15).

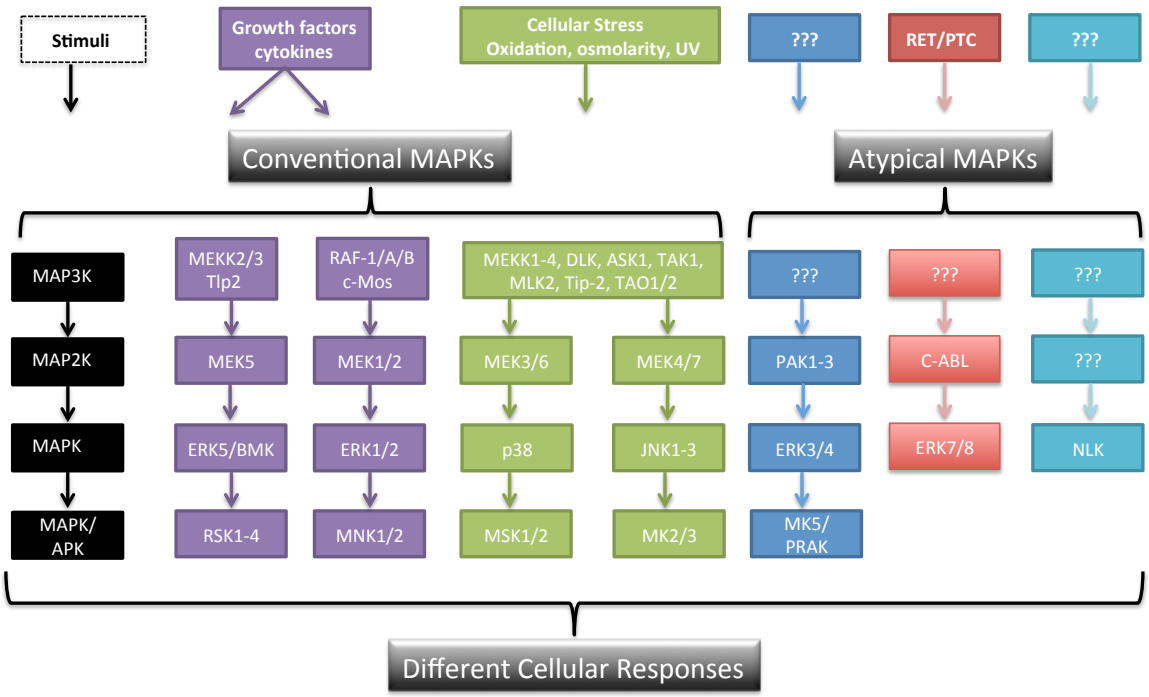

Figure 3. The different signaling cascade of Mitogen-activated protein kinases (MAPK). The conventional MAPKs have four subgroup pathways, while atypical MAPKs have three subgroup pathways. Growth factors, cytokines, cellular stress, oxidation, osmolarity, and UV can activate the conventional MAPK pathway. It is still unknown what activates the atypical MAPKs. (Adapted from (16))

3.1 The conventional Ras-Raf-MEK-ERK pathway

The Ras-Raf-MEK-ERK signaling pathway is one of the conventional MAPK pathways that controls several fundamental cellular processes (17) (Figure 3). The Ras-Raf-MEK-ERK signaling pathway orchestrates events starting from the binding of a growth factor (e.g. epidermal growth factor (EGF)) to its specific receptor tyrosine kinases (RTKs) at the cell membrane (18-20). This interaction leads to phosphorylation of appropriate transcription factors within the nucleus. Many studies link the deregulation of the Ras-Raf-MEK-ERK pathway to oncogenesis in humans. Approximately, 30% of all human cancers display

evidence of enhanced activation of the Ras-Raf-MEK-ERK pathway (21). In more than 50% of acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL), constitutive activation of the Ras-Raf-MEK-ERK pathway is observed (22, 23). In AML, activation of the Ras-Raf-MEK-ERK pathway can be the result of mutated class III RTKs (24).

Principal components of this pathway include the Ras family of small GTPases, which acts as molecular switches that control the activity of many signaling pathways. It has been reported that activating mutations in *Ras* subfamily *K-Ras*, *N-Ras,* and *H-Ras* occur in different types of cancer. It has also been shown that 90% of pancreatic adenocarcinomas harbor a *K-Ras* mutation. The *K-Ras* mutations are also involved in colorectal, lung, and biliary tract carcinogenesis. Additionally, a high percentage of *N-Ras* mutations are observed in melanomas, while *H-Ras* mutations are found in a majority of salivary gland tumors (25, 26). Upon stimulation by upstream receptors, Ras switches from the inactive GDP-bound form to the active GTP-bound form. Subsequently, Ras will bind to the downstream target, Raf. Members of the Raf family are A-Raf, B-Raf, and C-Raf or Raf-1. Approximately 8% of human cancers have been reported with activation mutation of the *B-Raf* gene (27, 28). In particular, mutations of *B-Raf* have been observed in over 60% of melanomas, around 30% of ovarian cancer cases, and approximately 20% and 50% in colorectal carcinomas and papillary thyroid cancer, respectively (27-30). The only known substrates of Raf are MEK1 and MEK2 (MEK1/2), and the only known MEK1/2 substrates are ERK1 and ERK2 (ERK1/2). Activated Raf phosphorylates and activates the MEK1/2, which then in turn phosphorylates and activates ERK1/2. Thus, constitutively active MEK1/2 and ERK1/2 proteins are present in a relatively high number of human tumors, particularly those from the colon, lung, pancreas, ovary, and kidney (21). Active ERK1/2 phosphorylates numerous cytoplasmic and nuclear targets, including kinases, phosphatases, transcription factors and cytoskeletal proteins (31).

3.1.1 The Ras-Raf-MEK-ERK inhibitors

The use of kinase inhibitors is a type of cancer therapy that inhibits or blocks the enzyme activity of the kinase. Gefitinib (ZD1839, Iressa) and erlotinib (OSI-774, Tarceva) are used to treat epidermal growth factor receptor (EGFR) mutated cancers (32). The EGFRs are part of the intracellular Ras-Raf-MEK-ERK pathway (33). The EGFR gene amplification was reported in a wide range of carcinomas. It was found that mutation of EGFRs enhanced

tumorigenicity in many cancers such as glioblastoma multiforme (GBM) (34), breast, lung, head and neck, ovarian, and prostate cancers (35). The three *Ras* genes (*H-Ras, N-Ras, K-Ras*) are the most commonly mutated oncogenes in human cancers (36, 37). Yet, after thirty years of research, no drugs that target Ras proteins directly or act on Ras-driven human cancers have been successfully developed (38). Indeed, cancers with Ras mutations remain the most difficult to treat. A few years ago farnesyltransferase inhibitors (FTIs) were considered as promising inhibitors. The addition of a 15-carbon farnesyl lipid tail in a reaction by the enzyme farnesyltransferase (FTase) is essential for Ras signaling due to the requirement of this posttranslational modification for the plasma membrane localization of Ras molecules (39). However, the results of clinical trials with FTIs were disappointing. Indeed, FTIs were effectively blocking the H-Ras farnesylation and membrane association, but FTIs did not effectively block N-Ras and K-Ras protein farnesylation and membrane association (40). Developing therapeutic agents against oncogenic Ras activity has been a challenging and unsuccessful. Therefore, efforts have been directed to developing therapies that target effector pathways downstream of Ras (e.g. Raf and MEK1/2).

As mentioned previously, 8% of tumors have been identified with activation mutations of B-Raf. More than 90% of B-Raf mutations in human cancer are in a single amino acid where valine was substituted by glutamate V600E (B-Raf-V600E) (28). There are two B-Raf inhibitors that have been approved for use in patients with metastatic melanoma and are positive for the B-Raf-V600E mutation (i.e. Vemurafenib (Zelboraf, PLX4032, RG7204) and Dabrafenib (Tafinlar)) (41).

MEK1/2 lies downstream of Ras and Raf, which makes it a potential target in tumors with Ras or Raf activation. Recently the MEK1/2 inhibitor Trametinib (Mekinist) was approved for clinical use. This inhibitor is used for the treatment of metastatic melanoma positive for B-Raf-V600E (8). The combination of two kinase inhibitors has been examined in clinical trails, and the combination of, for instance, Dabrafenib along with Trametinib has shown an improvement in progression-free survival (PFS) in B-Raf mutant melanoma (42, 43). Trametinib is the only approved MEK1/2 inhibitor, however, several other MEK1/2 inhibitors are at various stages of development: e.g. Cobimetinib (GDC-0973/XL518) is in Phase III trials for use in combination with vemurafenib for the treatment of advanced B-Raf mutant melanoma (44), Binimetinib (MEK-162) is in Phase II trials for patients with advanced melanoma harboring N-Ras or B-Raf mutations (45), Selumetinib/AZD6244 (ARRY-142886) is in Phase II trails for use with irinotecan as second-line therapy in patients with K-Ras mutated colorectal cancer (46) and RO5126766 (CH5126766) is in Phase I trials for patients

with advanced solid tumors (47). The ERK1/2 selective inhibitors have had limited development compare to Raf and MEK1/2 inhibitors. This could be due to the earlier assumption that ERK1/2 are the only known downstream targets of MEK1/2 thus no added benefit would result from ERK1/2 inhibitor compared to a MEK1/2 inhibitor. However, there has been interest in development of an ERK1/2 inhibitor due to the clinical observation of resistance to Raf and MEK1/2 inhibitors leading to the recovery of ERK1/2 signaling. It is believed that the inhibition of ERK1/2 along with Raf and/or MEK1/2 could increase the anti-tumor efficacy. Two ERK1/2 inhibitors have been reported, SCH772984 (Merck/Schering Plough) (48) and MK-8353/SCH900353, which is currently in Phase I clinical trials for patients with solid tumors (49, 50).

3.2 The atypical group I PAK-ERK3/ERK4-MK5 pathway

The p-21 activated kinases (PAKs) are serine/threonine kinases activated by the small GTPases Cdc42 and Rac in response to a variety of cell stimuli (51-53). They are comprised of six isoforms and grouped into 2 classes; group I, which includes PAK1-3 and group II, which includes PAK4-6 (54). PAK1 was first identified in 1994 as a target for Cdc42 and Rac1 (55). PAK family is regulating cellular processes such as motility, proliferation, and survival by phosphorylating a myriad of downstream effectors that are involved in these processes. PAKs were shown to signal downstream of these small GTPases to regulate cytoskeletal dynamics and cell motility via direct phosphorylation of substrates such as paxillin, cortactin, LIMK, and myosin light-chain kinase (52). PAK1 was also found to regulate the mitogen-activated protein kinase (MAPK) signaling pathway based on the ability of PAK1 to phosphorylate both Raf-1 and MEK1 (56). PAKs are overexpressed and/or activated in several human tumors such as breast cancer, neurofibromatosis, colon cancer and lung cancer (57). Stimulation of cell proliferation, survival, and motility are all hallmarks of cancer, that the PAK family is suggested to contribute to (Figure 4). The atypical MAP kinases, ERK3 and ERK4, were identified to be substrates of PAK1-3. ERK3 and ERK4 were constitutively phosphorylated in their activation loop by group I PAK, and a novel PAK-ERK3/ERK4-MK5 signaling pathway was suggested by authors (58, 59).

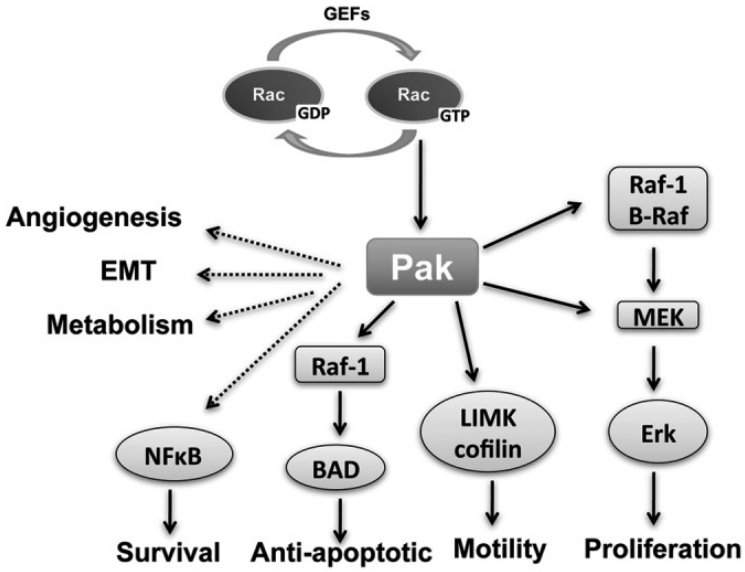

Figure 4. PAK family role in cancer hallmarks. This simple illustration shows the involvement of PAK in several cancer hallmarks due to its ability to phosphorylate many substrates. PAK can enhance cell motility by the phosphorylation of cytoskeletal targets LIMK, which in turn phosphorylates cofilin. PAK play a role in cell proliferation due to its ability to phosphorylate Raf-1 and/or MEK1/2. Raf-1 is also involved in resisting cell death. NFκB is regulated by PAK indirectly to promote cell survival. Other cancer hallmarks can be also promoted by the activation of PAKs (e.g. angiogenesis). (Reprinted from (57))

3.2.1 ERK3 and ERK4

Extracellular signal regulated kinase (ERK) 3 and 4 are atypical members of the mitogen-activated protein kinase (MAPK) family. Both *Erk3* and *Erk4* genes were identified in the early 1990s by homology cloning using probes derived from the MAPK *Erk1* (60, 61). The ERK3 and ERK4 proteins possess nearly 45% homology to ERK1/2 within their kinase domain (60) but have distinct structural features that make them atypical compare to the other MAPKs. These two kinases possess a single phosho-acceptor site in the S-E-G (Ser-Glu-Gly) motif instead of the conventional T-X-Y (Thr-X-Tyr) motif in their activation loop (Figure 5). In addition, in the kinase domain VIII, the A-P-E (Ala-Pro-Glu) motif is replaced by the S-P-R (Ser-Pro-Arg) motif, and they are the only protein kinases in the human genome to have an arginine residue at this position. Sequencing of the human cDNA revealed that ERK3 is a 721 amino acid protein with a molecular mass of ~100 kDa, whereas ERK4 is a 587 amino acid

protein with a molecular mass of ~70 kDa (62-64). Both proteins, ERK3 and ERK4, have a long C-terminal compared to the typical ERK1/2. They are further characterized by the presence of a catalytic kinase domain at the N-terminal end, for which they have 73% amino acid identity in their kinase domains (64). ERK3 and ERK4 define a subfamily of MAP kinases that are found in both vertebrates and invertebrates (64, 65).

Indeed, the regulation, substrate specificity, and physiological functions of atypical MAP kinases are not completely understood, as they do not share many characteristics of conventional MAPKs. However, genetic ablation of the *Erk3* gene has revealed that ERK3 plays an important role in fetal growth and lung maturation, while the disruption of the *Erk4* gene has no obvious effects on the morphology, viability, or physiology of the mice (66, 67). On the other hand, targeted inactivation of ERK4 in mice leads to a depression-related behavior in a forced swimming test (66). The first substrate identified for ERK3/ERK4 was the MAPK-activated protein kinase-5 (MK5 or PRAK). MK5 was demonstrated not only to act as a substrate for ERK3, but activated MK5 was shown to phosphorylate ERK3 both *in vitro* and *in vivo* (68). This interaction stabilizes ERK3 and excludes both ERK3 and MK5 from the nucleus (63, 69). ERK4 is mainly localized in the cytoplasm whereas ERK3 is localized both in the nucleus and the cytoplasm (62, 70, 71). Binding of MK5 to ERK4 will result in cytoplasmic accumulation of MK5 (63, 72, 73). The ablation of either ERK3 or ERK4 reduces the MK5 activity by 50% in cells; however, the ablation of both ERK3 and ERK4 causes an 80% loss of MK5 activity in cells (63).

Steroid receptor co-activator 3 (SRC-3) was recently identified as an additional substrate of ERK3 kinase (74). So far, no physiological targets of the ERK3/ERK4-MK5 signaling module have been identified. ERK3 and ERK4 seem to be constitutively phosphorylated at their activation loop by group I PAK, which suggested a PAK-ERK3/ERK4-MK5 signaling pathway (58, 59). Several studies have focused on ERK3 rather than ERK4 despite the fact that ERK3 is an unstable protein with a short half-life that is challenging to detect in both ectopic and endogenous settings. A role of ERK3 in regulation of lung cancer cell invasion, where ERK3 interacts with and phosphorylates SRC-3 specifically at serine 857 (S857), has been described. Phosphorylation of SRC-3 at S857 by ERK3 seems to be important for SRC-3 to interact with the transcription factor PEA3 and their subsequent regulation of matrix metalloproteinase (MMP) gene expression (74). ERK3 has also been found to be involved in regulating cell cycle progression, where ERK3 binds to Cdk1, Cdc14A and Cdc14B (75, 76). The phosphatases Cdc14A and Cdc14B were able to reverse ERK3 C-terminal

phosphorylation in mitosis (76). Moreover, a recent study has revealed the role of ERK3 in neuronal morphogenesis, where Brand *et al.* showed that Septin7, ERK3, and MK5 exist in the same complex along with the brain-specific nucleotide exchange factor kalirin-7 (77).

Several studies have also been aimed at discovering the physiological role of MK5. Recently, Stohr and colleagues suggested that MK5 is a substrate for ERK4 in cell migration (78). The study demonstrates that insulin-like growth factor 2 mRNA binding protein (IGF2BP1) promotes the velocity of cell migration by antagonizing MK5 activation (78). IGF2BPs in general were shown to facilitate the formation of polarized lamellipodia and regulate migration of tumor-derived cells in vitro (79, 80). Another study has suggested that MK5 plays a role in cell motility (81), although they did not agree whether MK5 is acting as a promoter or inhibitor in cell motility (78, 81). Other studies have found that MK5 phosphorylation of Hsp27 may lead to cytoskeletal rearrangement (82, 83). Additionally, the Hsp40, p53, Rheb, and FOXO3a proteins have been described as MK5 substrates (84-87). The phosphorylation of these substrates can result in many cellular responses such as Ras-induced senescence, proliferation, and nutrient starvation responses (84-86).

Recent studies have suggested the involvement of ERK3 in regulation of the immune system. ERK3 was recently describes as an important player controlling TCR-induced T-cell activation. Resting T-cells do not express ERK3, but the stimulation of T-cells leads to the transcription of the *Erk3* gene. Additionally, ERK3 plays an important role in sustaining T-cell activation to promote proliferation and differentiation (88). ERK3 expression is induced in both CD4+ and CD8+ T-cells following T-cell activation suggesting a possible role for ERK3 in T-cell response (88). Another study showed that during T-cell development, ERK3 expression in thymocytes was necessary to promote double positive thymocyte survival and thus allow successive TCRα gene rearrangements (89). ERK3 expression by thymic epithelial cells is essential for the differentiation of CD4+ T-cells (89). Moreover, it was shown that ERK3 controls thymocyte positive selection and that ERK3 deficiency affects thymic positive selection, which is a critical step of T-cell differentiation (90). In both studies, it was suggested that ERK3 might act as a direct downstream effector of the ERK1/2 signaling pathway in T-cells (88, 90). The discovery of ERK3's role in the immune system may help in designing better strategies for immune reconstitution in immunocompromised patients. To date, no data is available on the role of ERK4 in the immune system.

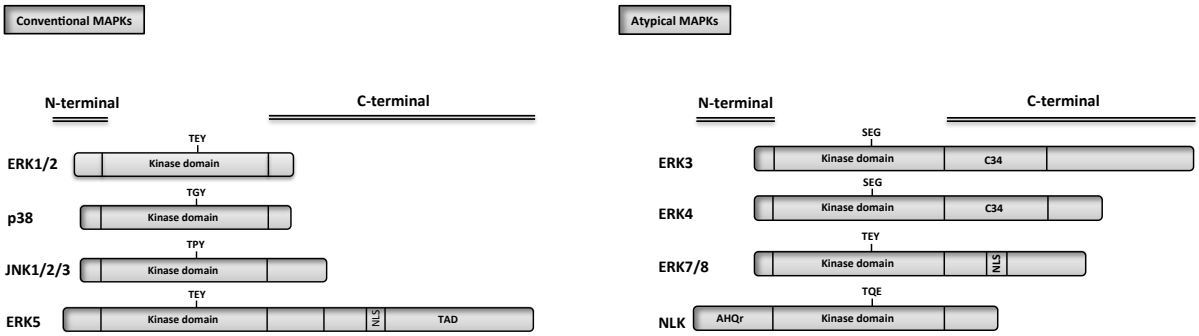

Figure 5. Schematic representation of the overall structure of the MAPKs. Both conventional and atypical MAPKs are composed of a kinase domain flanked with N- and C-terminus extensions of varying lengths. The activation loop phosphorylation motif is shown for each kinase. Different additional domains are also present in some MAPKs: transactivation domain (TAD); nuclear localization sequence (NLS); conserved region in ERK3/4 (C34); and Alanine, Histidine, and Glutamine rich domain (AHQr). ERK3 and ERK4 possess a single phosho-acceptor site in the S-E-G motif instead of the conventional T-X-Y. (Adapted from (91)).

3.2.1.1 ERK3 and ERK4 in Cancer

ERK3 has been shown to be upregulated in multiple cancers, and its upregulation is frequently associated with tumor metastasis (92). Some studies have observed an increased level of ERK3 in melanoma, breast cancer, and non-small cancer lung cells (74, 92, 93). In a recent review, Kostenko *et al.* have summarized almost all the available information regarding mutations in the *erk3* and *erk4* genes related to cancer (94). Either silent mutations or single amino acid substitutions have been found in ERK3 in different cancer types in lung, ovary and skin cancer tissue. The same types of mutations were also observed in ERK4 in lung and skin cancer tissue (95-100). ERK3 was found to play a role in cell proliferation of human carcinoma cells (101), cell migration, and invasion (74). The protein level of ERK3 was also found to be elevated in gastric cancer tissues compared with adjacent normal mucosa (102). In colorectal cancer, ERK3 kinase activity was increased in 10 of 21 cancers, while ERK1/2, JNK1, and p38 were downregulated (103). In addition, ERK3 facilitate lung cancer cell invasion by interacting with and phosphorylating SRC-3 specifically at serine 857 (74).

3.2.1.2 ERK3 and ERK4 interaction with Hsp90

According to a recent list of Heat shock protein 90 (Hsp90) clients by Picard, ERK3 and ERK4 were found to be one of these clients (104). The Hsp90 clients include receptors, transcription factors, kinases, and other unrelated proteins that share no common features in terms of sequence or structure. Hsp90 chaperones determine the stability and activity of their clients, many of which take part in signaling pathways and regulatory circuits that control cell homeostasis, growth, proliferation, differentiation, and cell death. In a study described in this thesis (Paper III), Hsp90 was found to be involved in ERK4, but not ERK3, stabilization.

Hsp90

Heat shock protein 90 (Hsp90) is a molecular chaperone that assists the folding and assembly of macromolecular structures. Hsp90 is an evolutionarily conserved molecular chaperone that is abundant in eukaryotic cells and its expression increases when cells are exposed to a variety of stresses. Different isoforms are expressed in different cellular compartments: Hsp90α and Hsp90β in the cytosol (105), 94 kDa glucose-regulated protein (Grp94) in the endoplasmic reticulum (106, 107), Tumor Necrosis Factor Receptor Associated Protein 1 (TRAP-1) in mitochondria (108), and Hsp90C in plastids (chloroplasts) (109, 110).

The Hsp90 chaperone transiently binds to substrate proteins that are called clients. Hsp90 is an essential partner for many signaling protein kinases that are required for the efficient cell growth and proliferation. In the last 30 years, over 200 Hsp90 clients have been identified using biochemical and biophysical methods (111). These clients include a wide range of protein classes (e.g. kinases, transcription factors, nuclear receptors, as well as, structural proteins such as actin and tubulin) (112). The Hsp90 clients include many oncogenic proteins that are involved in tumor cell proliferation, survival, and angiogenesis (113). The activity and stability of protein kinases are dependent on the molecular chaperone activity of Hsp90; therefore, inhibition of Hsp90 by specific inhibitors induces degradation or suppression of the activity of these kinases. Thus, Hsp90 has been considered as a target for cancer chemotherapy (112).

Hsp90 structure and function

Hsp90 is a dynamic dimer, and studies have suggested that dimerization is essential for its chaperoning function *in vivo* (114). Each homodimer is consisting of three main domains that have important functional interactions. First, the N-terminal domain (NTD) contains an ATP binding site, a drug-binding site, and a co-chaperone interacting motifs. Second, the middle domain (MD) is a central domain with docking sites for client proteins and co-chaperones. The third domain is a C-terminal domain (CTD) that contains the dimerization motif, second drug-binding region, and interaction sites for additional co-chaperones (113, 115). Some members of the Hsp90 family, such as cytosolic eukaryotic Hsp90 and Grp94, have a disordered region, termed the charged-linker, which separates the NTD and the MD (116). A long, highly charged linker is a characteristic feature of Hsp90s in eukaryotic organisms. Adjacent to the charged-linker, cytosolic eukaryotic Hsp90s have a C-terminal extension of pentapeptide MEEVD sequence (Figure 6) (115). The ATP binding site, located in the NDT domain of Hsp90, is unique and distinct from the ATP-binding cleft of Hsp70 and protein kinases, however, it is similar to those of bacterial type II topoisomerase and DNA gyrase B (117, 118). Hsp90 inhibitors, such as geldanamycin bind to the ATP-binding pocket of Hsp90 to inhibit the ATPase activity competitively (119). The NTD also contains a binding site for co-chaperones (e.g. Cdc37 and p23) (120, 121). The MD contains an Arginine in position 380, which is the key catalytic residue required for orientating and polarizing the phosphate of ATP (122). The MD also contains a binding site for clients such as Akt (123), endothelial nitric oxide synthase (eNOS) (124), and Cdk4 (125). As mention above, the CTD is essential for Hsp90 dimerization, but it is also involved in binding client proteins such as the tumor suppressor p53 (126). In the early 1990s, a number of co-chaperones were co-purified with Hsp90. These co-chaperones and Hsp90 had been found in complex with steroid receptors or protein kinases. Examples includes: Hop, Cdc37, and Aha1 that bind to Hsp90 C-terminus, N-terminus and middle, respectively (112). Cdc37 is one of the co-chaperones that help in the chaperone function of Hsp90. Cdc37, an essential protein, interacts with protein kinases through its N-terminal domain and recruits them to the Hsp90 system. The Cdc37 dimer binds between the two NTDs of an Hsp90 dimer, holding them in an open state, and therefore inhibiting N-terminal dimerization. In addition, Ccd37 physically interacts with the Hsp90 ATP-lid (Lid) to inhibit the progress of the ATPase cycle. The ATP-lid is open during the ADP-bound state and closed in the ATP-bound state (Figure 6). Cdc37 may hold Hsp90 in an open conformation for client loading (121).

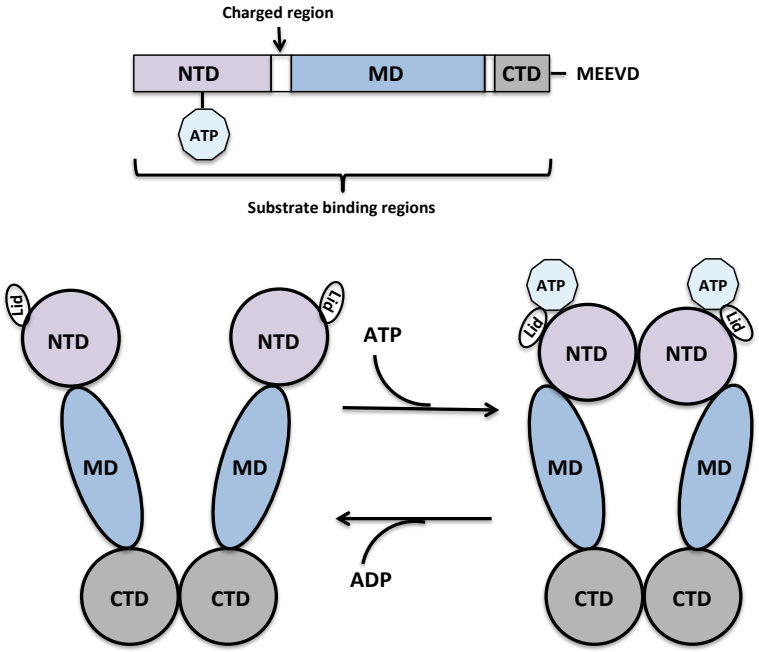

Figure 6. Hsp90 structure. Schematic drawing of human Hsp90. Hsp90 consists of three domains: an N-terminal ATP-binding domain **(NTD)**; a middle domain **(MD)**; and a C-terminal dimerization domain **(CTD)** with the pentapeptide **MEEVD** sequence. A charged region exists between the NTD and MD domains. ATP binding to the undimerized (open) NTD domain of HSP90 (lower panel, left) that leads to transient dimerization of the NTD (close) (lower panel, right). CTD is being constitutively dimerized. All three domains are reported to interact with substrate proteins.

3.2.1.3 ERK3 and ERK4 interaction with DUSP2

It has not previously shown that the family of dual-specificity phosphatases (DUSPs) dephosphorylates any of the MAP kinases other than p38s, ERK1/2 and the JNKs (127-130). Exclusively here in this study (paper I), we show that DUSP2/PAC1 interacts with and dephosphorylates both ERK3 and ERK4.

DUSPs

DUSPs (dual-specificity phosphatases) are a heterogeneous group of protein phosphatases that can dephosphorylate both the threonine/serine and tyrosine residues within the substrates (131). A subfamily of DUSPs is called mitogen activated protein kinase phosphatases

(MKPs). The main substrates for the dual-specificity MKPs are the MAPK family where they dephosphorylate MAPKs and attenuate their signaling (14) (Figure 7). DUSPs contain a highly conserved C-terminal catalytic domain and an N-terminal composed of a Cdc25-like domain (also termed CH2 domain for Cdc25 homology 2) and an intervening cluster of basic amino acids, known as kinase-interacting motif (KIM) or the MAP kinase-binding (MKB) motif. The KIM motif of DUSP is responsible for MAPK binding engage with a 'common docking' (CD) site of MAPKs (132). There are currently 25 genes in the Human Genome Organization database designated as DUSPs. Two groups of DUSPs were characterized, typical and atypical. Atypical DUSPs are characterized by the lack of KIM. The typical DUSPs are divided into three subclasses according to their subcellular localization: **1-** inducible nuclear DUSPs (DUSP1/MKP1, DUSP2/PAC1, DUSP4/MKP2, and DUSP5), **2-** cytoplasmic DUSPs (DUSP6/MKP3/PYST1, DUSP7/MKP-X/PYST2, and DUSP9/MKP4/PYST3), and **3-** nuclear/ cytoplasmic DUSPs (DUSP10/MKP5, DUSP16/MKP7, and DUSP8/M3/6). Regulating the function of DUSP-interacting proteins is not limited to DUSPs' phosphatase activity. It has been shown that DUSPs can modulate the function of MAP kinases by keeping them either in the cytoplasm or nucleus (133, 134). The ability of DUSPs to dephosphorylate proteins is not limited to the MAPK family. Several studies show that DUSP4, DUSP1, and DUSP22 could dephosphorylate STAT5, histone H3, and FAK, respectively (135-137). A recent review has summarized the different molecular mechanisms responsible for the cross-regulations between DUSPs and MAP kinases (Figure. 7) (138). The following section will focus on some of the deregulated DUSPs that are involved in cancer. However, the deregulation of DUSP is not limited to cancer, it could also be involved in other diseases (e.g. DUSP6 is downregulated in familial amyloidotic polyneuropathy). Moreover, some of the DUSPs play a role in the regulation of immune cell functions. DUSP1 was found to be a negative regulator for the production of inflammatory cytokines (139), while DUSP2 was found to positively regulate autoimmune responses in an arthritis animal model (140).

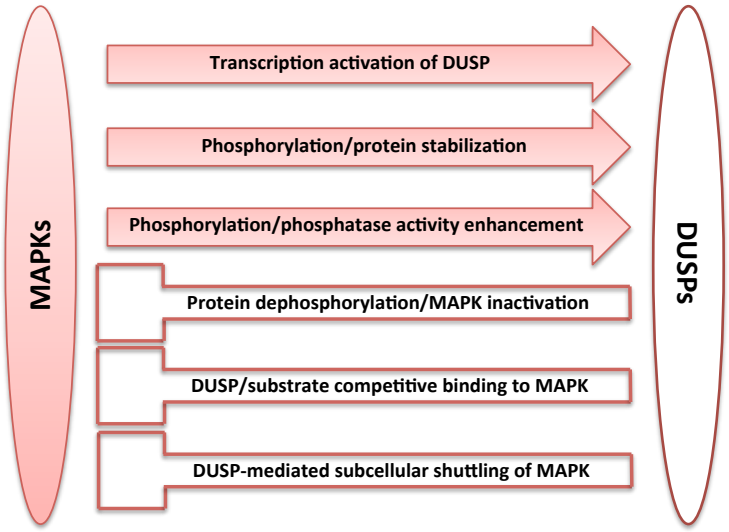

Figure 7. The cross-regulations between DUSPs and MAPKs. MAPKs act as activators for DUSPs, while DUSPs act as an inhibitors. (Adapted from (138)).

DUSPs and cancer

DUSPs as regulators of the MAPKs could function either as a tumor suppressor or oncogene. Numerous studies correlate loss of DUSP expression with progression of several tumor types. On the other hand, gain of DUSP expression is often associated with cancer progression. Several studies had reviewed the involvement of DUSP family in cancer (Table1). Overexpression of inducible nuclear DUSP1/MKP-1 was detected during the early stage in a range of human epithelial tumors, including prostate, colon, and bladder (141). However, the DUSP1/MKP-1 expression is lost as these tumors became more aggressive and invasive in theses cancers (141). DUSP1/MKP-1 overexpression was also detected in invasive ovarian carcinomas and was correlated with shorter progression-free survival (142). Another well-studied DUSP is the cytoplasmic DUSP6/MKP3/PYST1 (127, 143). DUSP6 was overexpressed in some cases such as in keratinocytes and breast cancer cells (144), glioma (145), and chronic myeloid leukemia (146), while it is downregulated in pancreatic cancer (147) and lung cancer (148).

DUSP	Cancer	References
DUSP1	Non-small cell lung cancer (NSCLC), gastric-, pancreatic-, breast-, hepatocellular-, colon-, prostate-, bladder and ovarian carcinomas	(141, 142, 149-154)
DUSP2	Ovarian carcinoma and acute leukemia	(155, 156)
DUSP4	Liver-, breast-, ovarian-, and rectal carcinomas, lung adenocarcinoma and pancreas cancer cells	(154, 157-160)
DUSP5	Mantle cell lymphoma (MCL), skin cancer and Burkitt´s lymphoma	(161-163)
DUSP6	Pancreatic and lung carcinoma, NSCLC, breast cancer, chronic myeloid leukemia, melanoma, glioma and ovarian cancer	(144, 145, 147, 148, 164-168)
DUSP7	Acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML)	(169-171)
DUSP8	Acute leukemia	(172)
DUSP9	Skin squamous cell carcinoma	(173)
DUSP10	Prostate and breast cancer	(174-176)
DUSP16	AML and Burkitt´s lymphoma	(177, 178)

Table 1. Expression of DUSPs in human cancer.

3.2.1.4 ERK3 and ERK4 role in cell migration

Several studies have suggested that ERK3 and ERK4 play a role in cell migration and invasion in relation to other proteins (e.g. SRC-3 and MK5) (74, 78, 81, 101). Here in this study (paper II), we have investigated the role of ERK3 in cell migration and adhesion.

Cell Migration

Cell migration is fundamental to cell and tissue dynamics in embryogenesis, inflammatory response, wound healing, as well as, in cancer invasion and metastasis. The ability of cancer cells to invade adjacent tissue, which could be followed by local or distant metastasis, is a hallmark of cancer. In metastasis, tumor cells migrate from the initial tumor mass (invasion) into the circulatory system (intravasation) and transit in the blood or lymph, followed by, extravasation and subsequent migration to a new site where cancer cells can grow. Cell motility is driven by cycles of actin polymerization, cell adhesion, and actomyosin contraction. Migrated cells can move either individually or collectively (Figure 8). In the case of individual cell migration, there are two distinct types of movement: amoeboid and mesenchymal (Figure 8). In order to migrate, cells must protrude their plasma membrane at the leading edge (the front). The actin cytoskeleton and regulators of actin dynamics are involved in all protrusion types. The assembly of actin cytoskeletal drives the initial extension of the plasma membrane at the cell front (i.e. leading edge protrusion) (179). Cells then form adhesions that link to the extracellular matrix (ECM) by recruiting signaling and cytoskeletal proteins to stabilize the protrusion (180). To pull the cell body forward, the contraction of actomyosin network will cause retraction of the cell rear and translocation of the cell body (181). The repetition of leading edge protrusion formation, actomyosin contraction and adhesions turnover of the cell rear completes the migratory cycle and allows the translocation of the cell in the direction of movement (182, 183).

Cells adhere to the ECM via integrins and the formation of focal adhesion complexes (184). Integrins are the predominant receptors that mediate cell adhesion to the ECM ligands (184, 185). The initial step of cell adhesion is the clustering of integrins. The cytoplasmic portions of the clustered integrins act as a platform for the recruitment of cellular proteins, such as adaptor and signaling proteins, to the inner surface of the plasma membrane, where they form

structures called focal adhesions (FAs) (186, 187). The integrin clustering will be linking to actin stress fibers in a process regulated by Rho/ROCK (185, 188). The proteins that localize to FAs, such as talin, paxillin, α-actinin, and tensin, provide strong linkages to the actin cytoskeleton and connect cells firmly to the ECM (189). Other proteins, including kinases or phosphatases, are also recruited to FAs, where they transmit ECM-derived signals to cellular pathways. Focal adhesion kinase (FAK) and Src are well characterized tyrosine kinases that play central roles in integrin-mediated signaling cascades (190). FAK and Src transmit signals from FAs to the cellular machinery by phosphorylating multiple integrin-associated proteins (190). A continuous, coordinated formation and turnover of FAs at the leading edge of the cell and disassembly of this attachment at the rear is required for directional migration (191, 192).

Many different molecules are involved in the coordination of cell migration and cytoskeleton rearrangement. RhoA, Cdc42 and Rac1 (Rho family of GTPases) are pivotal regulators of actin and adhesion organization, so they control the formation of protrusions (193). The Rho GTPases transmit signals from membrane receptors to the cytoskeleton and cell adhesions. High levels of actomyosin contractility, driven by Rho-ROCK (Rho-Rho-kinase) and JAK-STAT3, facilitate increased cells migratory speeds compared with elongated- mesenchymal cells (194-196). However, RhoA is also known to be involved in regulating leading edge membrane protrusions of migrating cells (197-199). Moreover, lower levels of contractility are associated with Rac-dependent elongated cell motility (200).

Amoeboid migration

Amoeboid movement mimics the single-cell behavior of the amoeba *Dictyostelium discoideum*. The shape of an amoeboid migrating cell is rounded or ellipsoidal. Amoeboid migration is characterized by low adhesion to the substrate and a lack of mature FAs and stress fibers (181, 201). The traction forces exerted by these cells are also low. There are two subtypes of amoeboid movement, blebby (rounded) and elongated (protrusion of actin-rich pseudopods (false feet)), depending on the mechanism of forward extension of the plasma membrane. The rounded amoeboid cells do not adhere or pull on the substrate but instead they use a propulsive, pushing migration mode (202, 203). The slightly elongated amoeboid cells generate actin-rich protrusions (pseudopodal) at the leading edge, however, these

protrusions are poorly defined and they have a weak adhesive interaction with the substrate (204, 205). Rounded amoeboid motility is characterized by squeezing movements that allow the cells to enter through matrix barriers and requires actin polymerization along the plasma membrane to stiffen and contract the cell cortex. It was suggested that rounded amoeboid motility does not require the MMPs to invade, however, a recent study shows that MMP-9 is upregulated in and promotes rounded amoeboid 3D migration (206). The small GTPase RhoA and its effector ROCK control the cortical actin dynamics and generate cortical tension and stiffness, and maintain the rounded cell morphology (207, 208). Amoeboid migration is a characteristic feature of lymphoma, invasive melanoma, and myeloic leukaemia cells, as well as, cells of certain neuroendocrine tumors, including small-cell carcinoma of the lung or prostate (209-211).

Mesenchymal migration

In mesenchymal migration, cells are elongated and characterized by high levels of attachment to the substrate and cytoskeletal contractility (212). The cycle of mesenchymal cell migration is defined by protrusion of the leading edge (213). Mesenchymal cells move via the steps described earlier (i.e. protrusion of the leading edge, actomyosin contraction, retraction of the cell rear, and translocation of the cell body) (181, 214).

Mesenchymal movement is predominantly found in cells from connective-tissue tumors, such as fibrosarcomas, gliomas and in epithelial cancers (215-217). Mesenchymal motility depends on the ECM proteolysis through production of MMPs. It is characterized by activation of Rac1 at the leading edge of the cell, which gives the migrated cells an elongated and polarized cell morphology (218). The mesenchymal cell migration is slow and characterized by nascent adhesions at the leading edge that mature into FAs; whereas, the amoeboid cells are highly motile and their movement is associated with high levels of actomyosin contractility (204, 212, 219).

Collective migration

In collective migration, cells do not migrate individually, but rather collectively, either as multicellular clusters, strands, or irregular shaped sheets or masses (220). It is also called chain migration or cell streaming (221). Collective cell movement is a well-described phenomenon that occurs during embryological development (222). The cells are characterized by the integrity of the cell-cell junction during movement. Migratory collective cells have multicellular polarity of the actin cytoskeleton that generate traction and protrusion force for migration and maintain cell–cell junctions. In most collective cell migration forms, the cells modify the tissue along the migration path, either by clearing the track or by causing secondary ECM modification, including the deposition of a basement membrane. Collective migration has been observed in metaplastic breast carcinoma and ovarian cancer (223, 224).

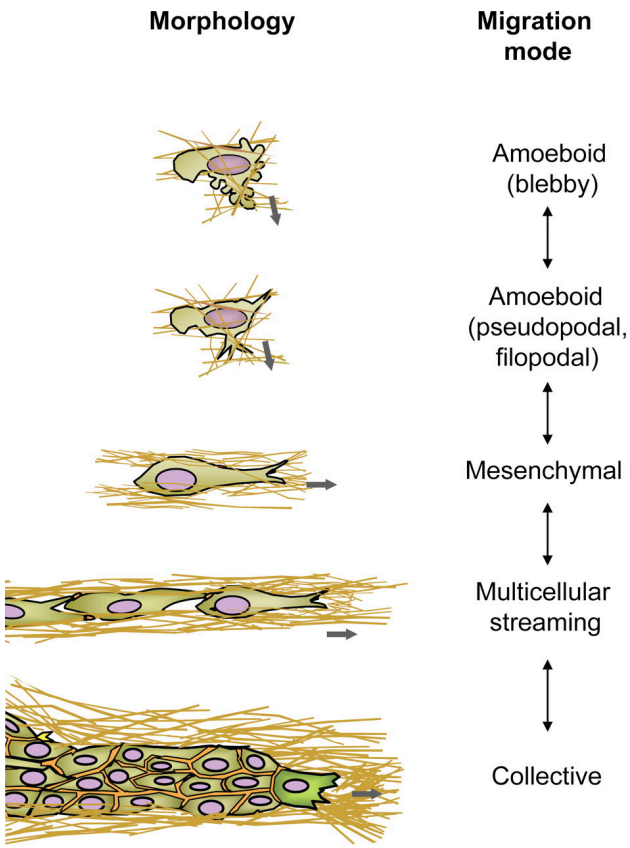

Figure 8. The different cell migration modes. Cells can either migrate individually or collectively. There are two distinct types of individual movement: amoeboid and mesenchymal. Amoeboid cells could be either rounded or slightly elongated (Adapted from (212)).

II. Aims of the study

This study had two aims:

- The first aim was to identify novel regulators for both ERK3 and ERK4 in order to gain more knowledge to understand their physiological function.

- The second aim was to investigate the physiological function of ERK3 regarding cell morphology and migration.

III. Summary of the papers

Paper I

The inducible nuclear phosphatase DUSP2/PAC1 specifically interacts with and dephosphorylates the atypical MAP kinases ERK3 and ERK4 and can regulate the output of the ERK3/4-MK5 signalling axis

Maria Perander, Rania Al-Mahdi, Thomas C. Jensen, Jennifer A.L. Nunn, Hanne Kildalsen, Bjarne Johansen, Mads Gabrielsen, Stephen M. Keyse and Ole-Morten Seternes

Abstract

The atypical MAP kinases ERK3 and ERK4 are activated by phosphorylation of a single serine residue lying within the signature sequence S-E-G in the activation loop. Thus far the regulation of ERK3 and ERK4 phosphorylation and thus activity is poorly understood. Here we have screened mammalian dual-specificity MAP kinase phosphatases for their ability to interact with ERK3 and ERK4 and report that the inducible nuclear phosphatase DUSP2, previously identified as a regulator of signaling through both the ERK1/2 and p38 MAPK pathways, binds specifically to both ERK3 and ERK4. This interaction is mediated via the conserved common docking (CD) domains within the carboxyl-terminal domains of ERK3/ERK4 and requires the kinase interaction motif (KIM) located within the non-catalytic amino terminus of DUSP2. The interaction between ERK3/ERK4 and DUSP2 results in the stabilization of DUSP2 and the dephosphorylation of ERK3/ERK4 both *in vitro* and *in vivo*. Moreover, the ability of ERK4 to stabilize DUSP2 is dependent on the catalytic activity of ERK4. Furthermore, DUSP2 is efficiently phosphorylated by ERK4 *in vitro*. Finally, we demonstrate that DUSP2 expression inhibits ERK3 and ERK4 mediated activation of its downstream substrate MK5. We conclude that the activity of DUSP2 is not restricted to the classical MAPK pathways and that DUSP2 is a physiological regulator of the atypical ERK3/ERK4-MK5 signaling pathway in mammalian cells.

Paper II

A novel role for atypical MAPK kinase ERK3 in regulating breast cancer cell morphology and migration

Rania Al-Mahdi, Nouf Babteen, Kiruthikah Thillai, Bjarne Johansen, Hilde Ljones Wetting, Ole-Morten Seternes and Claire M Wells

Abstract

In this study we precisely detected the morphological changes occurring on cells and cell motility in relation to the ERK3 (MAPK6) protein level in cells. Despite the fact that the *Erk3* gene was originally identified in 1991, its function is still unknown. MK5 (MAP kinase-activated protein kinase 5) also called PRAK is the only known substrate for ERK3. Recently, it was found that group I p-21 protein activated kinases (PAKs) are critical effectors of ERK3. PAKs link Rho family of GTPases to actin cytoskeletal dynamics and are known to be involved in the regulation of cell adhesion and migration. In this study we demonstrate that ERK3 protein levels are elevated as MDA-MB-231 breast cancer cells adhere to collagen I, which is concomitant with changes in cellular morphology where cells become less well spread following nascent adhesion formation. During this early cellular adhesion event we observe that the cells retain protrusive activity whilst reducing overall cellular area. Interestingly, exogenous expression of ERK3 delivers a comparable reduction in cell spread area, whilst depletion of ERK3 expression increases cell spread area. Importantly, we have detected a novel specific endogenous ERK3 localization at the cell periphery. Furthermore, we find that ERK3 overexpressing cells exhibit a rounded morphology and increased cell migration speed. However, exogenous expression of a kinase inactive mutant of ERK3 phenocopies ERK3 overexpression, suggesting a novel kinase independent function for ERK3. Taken together our data suggest that as cells initiate adhesion to matrix, increasing levels of ERK3 at the cell periphery are required to orchestrate cell morphology changes, which can then drive migratory behavior.

Paper III

Regulation of the atypical MAP kinases ERK4 by Hsp90

Rania Al-Mahdi, Bjarne Johansen, Hanne Kildalsen and Ole-Morten Seternes

Abstract

ERK3 and ERK4 are atypical MAP kinases that lack the canonical threonine and tyrosine residues of the conventional MAP kinases in their activation loop. However, they carry a single serine residue in the activation loop, which can be phosphorylated. Recently the group I p-21 activated kinases (PAKs) were discovered as an upstream activators for both ERK3 and ERK4. However, we still have little information into which physiological processes and biological functions of ERK3 and ERK4 as effectors for the PAK kinases. So far the only substrates that have been identified for ERK3/ERK4 are the MAPKAP kinase 5 (MK5) and the steroid receptor co-activator 3 (SRC-3). Hsp90 is a highly abundant and ubiquitous molecular chaperone located in the cytoplasm, which plays an essential role in many cellular processes including cell cycle control, cell survival, hormone, and other signaling pathways. Several protein kinases have been described as Hsp90 clients, while most of the MAP kinases have been show to be Hsp90-independent. Here we have analyzed the role of Hsp90 in regulation of ERK3 and ERK4, and find that ERK4 in contrast to ERK3 is able to form a complex with Hsp90 in mammalian cells. Treatment of cells with the Hsp90 inhibitors 17-AAG or Ganetespib results in a reduction in the level of ERK4 protein by more than 50%, but does not influence the abundance of ERK3 protein. Finally, we show that activation of MK5 by ERK4 or ERK3 is dependent on Hsp90 in HeLa cells.

IV. Discussion

General discussion

The atypical MAP kinases ERK3 and ERK4 are the main characters in this study. ERK3 and ERK4 were for long considered orphan proteins, without known upstream activators. However, in 2011, two studies detected that group I p-21 protein activated kinase (PAKs) can constitutively phosphorylate ERK3 and ERK4 in their activation loop (58, 59). The only well-characterized substrate for ERK3 and ERK4 at that time was MAP kinase-activated protein kinase 5 (MK5), also known by the name of PRAK. Recently, steroid receptor co-activator 3 (SRC-3) has been identified as a substrate for ERK3 (74). What was known about the biological function of ERK3 and ERK4 was limited and studying them was a challenge. In the last four years, more studies were focused on ERK3 than ERK4. However, the ERK3 studies on its physiological role were scattered in several different directions. Here, in this study we focused mainly on the interaction of ERK3 and ERK4 with two large protein families that are involved in cancer and cancer treatment (Figure 9). The first protein family we investigated was the dual-specificity MAPK phosphatases (DUSPs), where we have used a yeast 2-hybrid assay to analyze all ten members of the mammalian MAP kinase phosphatase (MKP) family for their ability to interact with ERK3 and ERK4. We found that these kinases specifically interact with DUSP2. In paper I, we investigated the interaction between ERK3/ERK4 and DUSP2 in depth. The second protein family that we were interested in was the heat shock protein family. Preliminary data from co-precipitation studies done in our lab to identify interaction partners for ERK3 and ERK4, followed by mass-spectrometry analysis suggested that Hsp90 could be an interaction partner for ERK3 and ERK4 (Bo Dreyer, unpublished data). This observation was later supported by results from several high throughput analyses (111, 225). The interaction between ERK3 and ERK4 with Hsp90 was investigated in paper III. In addition to the detection of new targets for ERK3 and ERK4, we chose to investigate the role of ERK3 in cell morphology and migration, as described in paper II (Figure 9). Our choice to study only the role of ERK3 in cell migration was based on previous studies (74, 101), however, we do not exclude the possibility that ERK4 may also have a role in cell migration, adhesion, and invasion.

Paper I

In this paper, we investigated the interaction between ERK3/ERK4 with Dual-specificity phosphatases 2 (DUSP2). The end result of our work strongly suggests that the atypical MAPKs ERK3 and ERK4 are *bona fide* substrates for the DUSP2. Among all the ten DUSPs, both ERK3 and ERK4 were found to only interact with DUSP2. It has been previously reported that DUSP2 interacts with and inactivates the classical MAPKs, ERK1/2 and p38, both *in vitro* and *in vivo* (226, 227).

The DUSPs are a subclass of protein tyrosine phosphatases that are able to dephosphorylate threonine/serine and tyrosine residues of their substrates. DUSP2 is involved in the immune system and plays an important role in innate immunity and inflammatory signaling (138, 228). High levels of DUSP2 along with DUSP1, DUSP4 and DUSP5 were found in activated immune cells (140). DUSP2 was found to be the most highly inducible DUSP in T-cells, B-cells, mast cells, eosinophils and macrophages after activation and was not detected in quiescent cells (140). Several previous studies have revealed the ERK3 role in cell differentiation (62). More recent studies have showed that ERK3 plays a role in the immune system (88-90). ERK3 kinase activity was required for normal thymocytes differentiation and to sustain the double positive thymocyte survival (89). Moreover, resting T-cells do not express ERK3, but the transcription of *Erk3* gene can be induced upon T-cell stimulation (90). The observed interaction between DUSP2 and ERK3 suggests a possible new area of research where their role in relation to T-cell differentiation or survival can be tested. Surprisingly, experiments with activated macrophages and mast cells derived from mice lacking DUSP2 show that ERK1/2 and p38 phosphorylation is reduced, while JNK activity is induced (140). The activation of ERK1/2 in DUSP2 deficient bone derived mast cells can be rescued when JNK inhibitors are used which suggests a negative cross-talk between the two MAPKs (140). ERK1/2 is required for the induction of ERK3 expression in activated T-cells (88). Consequently, it was suggested in Marquis and co-workers' studies that ERK1/2 phosphorylation is important for ERK3 protein expression in activated T-cells (88).

The decrease in ERK3 abundance after 1-2 hours, when DUSP2 expression peaks could be explained by the capability of ERK3 phosphorylated at S189 to interact with and be stabilized by MK5. When DUSP2 dephosphorylates ERK3 at S189 this may prevent the interaction between ERK3 and MK5 and thus affect ERK3 stabilization.

Indeed, we have tried several times to knockdown DUSP2 by siRNA and shRNA in order to investigate its direct effect on ERK3 activity in an endogenous setting, but we have not

succeeded. In our study, we revealed that DUSP2 binds to and dephosphorylates the serine phosphor-acceptor residue within the activation loop of both ERK3 and ERK4 *in vivo*. This binding depends on the integrity of the KIM motif, which is located in the N-terminal of the DUSP2 or the CD domain within ERK3 and ERK4. Since the interactions of MAPKs with both DUSPs and their substrates is mediated by the common docking site of the MAPKs, it has been suggested that one mechanism for how DUSPs might regulate MAPK signaling is by competing with the substrate-binding of MAPKs. In the case of ERK3/ERK4, DUSP2, and MK5 we know that ERK3/ERK4 use different motifs to interact with either DUSP2 or MK5. ERK3 and ERK4 bind to DUSP2 with the CD domain but to MK5 through the FRIEDE motif. This indicates that DUSP2 may regulate the PAK-ERK3/ERK4-MK5 signaling axis.

The dephosphorylation of ERK3 and ERK4 by DUSP2 results in a reduction in MK5 phosphorylation of T182 within the activation loop. The phosphorylation of MK5 depends on the presence of either ectopically ERK3 or ERK4 with ectopically MK5 and with the overexpressed DUSP2 a significant reduction in MK5 phosphorylation occurs due to the necessity of ERK3/ERK4 phosphorylation to phosphorylate and activate MK5. ERK3 phosphorylation was shown to be necessary for binding with MK5 in activated T-cells (88). The importance of ERK3 catalytic activity has been shown in a new study where it is required to sustain double-positive thymocyte survival (89). The catalytic activity of several DUSPs increases upon binding to their MAPK substrates. However, this is not always the case, for example, DUSP10 catalytic activity is not increased by binding to its substrates p38 or JNK (229). The catalytic activity of DUSP2 is induced by its binding to ERK2 (230), but it does not increase with binding to either ERK3 or ERK4.

Recently, a novel role for MK5 in the immune system has been revealed. MK5 was found to phosphorylate FOXO1 at serine S215, which in turn, leads to the activation of *Rag* transcription (231). The recombination-activating gene (RAG) is essential for the generation of mature B and T lymphocytes, and B-cell development was completely abrogated in *Rag1* and *Rag2*-null mice (231). To our best knowledge, the role of ERK3, ERK4, or DUSP2 in regulating RAG expression in B or T-cells has not been investigated.

Paper II

The ERK3´s substrate MK5 has been the focus of attention regarding cell migration and F-actin rearrangement (78, 81, 82). Several studies have described proteins that interact with ERK3 and have a role in cell migration and invasion. Accordingly, it was suggested that

ERK3 might also have a role in these functions (58, 59, 74). Our study was also inspired by the discovery of ERK3 as a substrate for the group I p-21 activated kinases (58, 59). To investigate the role of ERK3 in cell adhesion and migration we had to start from scratch and analyze how the cell shape, adhesion and speed changes in relation to ERK3 protein levels in the cell. MDA-MB-231 and MCF-7 were the cell lines that we chose for our study. One of the main reasons for using these cell lines, other than that they are breast cancer cells was the fact that they both express endogenous ERK3 protein. We started this study with the investigation of ERK3 localization. Previous studies reported that ERK3 is constitutively localized both in the nucleus and cytoplasm (62, 70). However one study has suggested a localization of ERK3 in the Golgi/ER-Golgi intermediate compartment (ERGIC) (232). In our study, we confirmed what others have observed with ERK3 expression in the nucleus and the cytoplasm, but we were also able to detect endogenous and ectopically expressed ERK3 in the cell periphery. These findings made us consider if the ERK3 protein has a role in either cell adhesion or cell motility. Moreover, we observed that cells plated on collagen I displayed increased levels of ERK3 protein, in fact, these levels were gradually increasing from 2 to 8 hours after plating. The increase of ERK3 protein level after plating on collagen was in agreement with another study (101). This prompted us to charactrize cell morphology changes in relation to the abundence of ERK3 protein from 2 to 8 hours after plating. Our detailed analysis shows that over time the MDA-MB-231 cells spread and polarized so that at 8 hours post-seeding the cells actually exhibit a reduced spread area suggesting an increased level of contractility. Further, overexpression of ERK3 in the same cells resulted in even smaller cells. Therefore, our hypothesis was that this rounded/amoeboid cell shape allows the cells to begin efficient 2D migration.

We speculate that the more rounded ERK3 phenotype could reflect an amoeboid-like cell. Amoeboid cell migration is characterized by cycles of expansion and contraction. Several studies have observed the amoeboid migration type in leukocytes (201, 233). Recently, ERK3 was found to have a role in T-cell differentiation and activation (88, 89). Based on these studies and our study, we speculate that ERK3 might have a subsequent role in T-cell migration to peripheral tissues.

We found that cells with a loss of ERK3 expression are unable to adopt the same morphological shape as control cells. Therefore, we suggest that ERK3 is required to protect the cell from hyper-elongation, and we speculate that this requirement is attributable to a role for ERK3 in cell contractility. The ERK3 knockdown cells exhibited more mesenchymal cell shape where the cells were bigger and more elongated. ERK3 is an unstable protein and its

abundance may be linked to more rounded cells, whereas its absence could be linked to a more mesenchymal shape.

The rounded/ amoeboid ERK3 overexpressing cells were found to have an increased cell migration speed. On the other hand, ERK3 knockdown cells exhibit normal migration speed. It has been previously suggested that the rounded/amoeboid like cells exhibit an elevated cell migration speed comparing to mesenchymal cells (212). In 3D matrices, the velocity of amoeboid cell migration ranges from 2μm/min on A375m2 melanoma cells (195) to 25μm/min in lymphocytes, representing the peak migration velocity (234), whereas mesenchymal cell movement velocity is around 0.1-0.5μm/min (235). The low speed of mesenchymal cell movement could be explained by the slow turnover of focal adhesions during migration. In our study, the mesenchymal ERK3 knockdown cells exhibit approximately the same cell migration speed as control cells. It might be that ERK3 knockdown cells would exhibit migration defects in a more constrained environment where increased cellular contractility is required, such as in migration through a 3D matrix (236-238). The behavior of cancer cells cultured in a 3D matrix is different from the behavior of those that are cultured in a 2D matrix. For example lamellopodia are rarely observed in 3D matrices because they depend on a planer substrate to spread across (239). Moreover, we observed that the actin cytoskeleton configuration was changed in cells overexpressing ERK3. Stohr and co-workers reported that the knockdown of IGF2BP1 (a RNA binding protein) increases the ERK4 protein level, MK5 activation, and induces the increase of needle-like F-actin structure (78). In agreement with this study, our results showed the same change in F-actin arrangement as a consequence of an increase in ERK3 protein level. Our results suggest a novel role for ERK3 protein in the early phase of cell migration, contractility, and protrusion. Furthermore, we were able to detect the loss of cell-cell adhesion in response to ERK3 transfection in MCF-7 cells. Malignant tumor cells must dissociate from the primary tumor mass by loss of cell–cell adhesion capacity and invade the surrounding stroma. ERK3 can regulate the expression of MMP2, MMP9, and MMP10 through phosphorylation of SRC-3 (74). The MMPs upregulation has the ability to degrade the extracellular matrix molecules, which results in disruption of cell-cell contacts (240-242). In a recent study, Orgaz and colleagues find that MMP-9 promotes rounded/amoeboid 3D melanoma cell migration (206). Our data also suggested that ERK3's function in changing cell morphology is kinase-independent. Similar kinase-independent function has been reported for many kinases, for example ERK1/2, CDK6, and FAK. ERK1/2 directly stimulate

cell cycle entry immediately after they translocate into the nucleus via a kinase-independent mechanism (243). The kinase activity independent function of CDK6 was also suggested in a recent study, where CDK6 is contributing in a transcription complex that induces the expression of the tumor suppressor p16INK4a and the pro-angiogenic factor VEGF-A (244). Moreover, FAK can drive cancer growth and metastasis through kinase-dependent or kinase-independent functions (245). The FAK kinase activity is not essential for proliferation of all cell types (246, 247).

To sum up, our data show that endogenous ERK3 is located in the cell periphery and plays a major role in cell morphology changes. Both overexpressing ERK3 cells and ERK3 knockdown cells showed significant changes in spread area and elongation ratio. Ectopic ERK3 cells show an elevation on the protrusion level that coincided with the increasing level of ERK3 during the first 8 hours after seeding. ERK3 also influences in the loss of cell-cell adhesion and F-actin rearrangement. Finally, ERK3 is able to enhance the speed of cell motility.

Paper III

Hsp90 is one of the most abundant chaperone proteins in cells. Hsp90 is highly conserved from prokaryotes to eukaryotes, and it is involve in the folding and conformational regulation of a wide range of proteins (clients). The Hsp90 protein has a wide variety of clients including protein kinases. Several studies, along with Picard list (104), have tried to map which of protein kinases are Hsp90 clients and which are not (111, 225). Hsp90 is responsible for the chaperoning and maintenance of several oncogenic kinases including Raf-1, Cdk4, v-src, and ErbB2 (112). Inhibiting Hsp90 is an attractive strategy for cancer therapy, as it leads to the destabilization and degradation of multiple oncoproteins (248). Both ERK3 and ERK4, the two proteins that we are interested in, have been suggested as Hsp90 clients (104, 111, 225). ERK4 was identified in a high throughput study as a weak client for Hsp90, while ERK5 and ERK7 were identified as strong clients and MK5 as a non-client kinase (225). Several other protein kinases were shown not to be associated with Hsp90, including ERK1/2, p38s, and JNKs (104, 111, 225). The binding of ERK4 to Hsp90 was confirmed in our study, however, we were not able to detect a direct interaction between ERK3 and Hsp90. In Wu and co-workers' study it was suggested that the ERK3 protein level is increased after adding the Hsp90 inhibitor galdanmycin (17-AAG) in MDA-MB-231 and K562 cells (111). We think

that the contrast between Wu and co-workers' results and ours might be due to specific cell type effects. We have tested the effect of Hsp90 inhibition on ERK3 protein levels in three different cell lines (i.e. HeLa, HEK293 and H1299). For testing the H1299 cell line (human non-small cell lung carcinoma), we used Ganetespib (STA-9090), a resorcinol derivative, which is shown to have a potent antitumor activity in both *in vitro* and *in vivo* models of human non-small cell lung carcinoma. Resorcinol and its derivatives, geldanamycin and its analogues, were identified as Hsp90 inhibitors that compete with ATP for the binding site in the Hsp90 N-terminal domain (249).

The effect of the Hsp90 inhibition on the stability of endogenous ERK3, ERK4 and MK5 was tested in our study. We found that only the endogenous ERK4 stability was affected by inhibition of Hsp90 using the geldanamycin analogue 17-Allylamino-17-demethoxygeldanamycin (17-AAG). The ERK4 protein level decreased in a time course from 0 to 8 hours and it was almost completely depleted 24 hours after of adding 17-AAG. An interesting finding was that the ERK3 protein stabilization by MK5 seems to be Hsp90 dependent in HeLa and H1299 cells. This could be explained by that the cytoplasmic translocation of ERK3 induced by MK5 binding, is required for MK5 to be able to stabilize ERK3. This might be a way to make the ERK3/MK5 complex more prone to bind to the Hsp90 protein, which is mainly present in the cytoplasm. The ERK3-induced MK5 phosphorylation was affected by the inhibition of Hsp90 in HeLa cells, however, it was less affected in H1299. The ability of ERK4 to induce MK5 phosphorylation was decreased due to a reduction of the ERK4 protein level after adding an Hsp90 inhibitor.

Both ERK3 and ERK4 possess a long C-terminal extension that is absent in classical MAP kinases (61, 250). The interaction of ERK3 and ERK4 with MK5 is controlled by the FRIEDE motif, which is located in the C-terminal extension of both kinases (73). We assumed that since the classical MAP kinases (e.g. ERK1/2, p38s, and JNKs) do not possess the C-terminal extension and they are not Hsp90 clients, that the C-terminal extension could play a role in the binding of ERK4 with Hsp90. However, most of the previous studies have shown that kinases (e.g. Raf-1, v-src, and MOK) require the kinase catalytic domain to bind to Hsp90 (251-253). The C-terminal deletions we made for ERK4 clearly demonstrate that Hsp90 did not require the ERK4 C-terminal extension in order to interact. The FRIEDE motif deletion had also no effect on the ERK4-Hsp90 binding. The Hsp90 protein was also able to interact with all ERK4 point mutations. Therefore, we conclude that neither the ERK4 catalytic activity nor the ATP binding site of ERK4 are required for the interaction of ERK4 with Hsp90. This pointed to either the N-terminal domain or the kinase domain of ERK4 as

the region necessary and sufficient for interaction, which is also the case for other kinases.

Paper I, II and III in relation to cancer

The conventional MAPK ERK1/2 has been a subject of cancer research for decades. The aberrant activation of the ERK1/2 pathway in many cancers is associated with chemotherapeutic drug resistance. Both ERK1/2, as well as p38, can be inactivated by DUSP2. In paper I, DUSP2 was also found to inactivate both ERK3 and ERK4. The upregulation/downregulation of ERK3, ERK4, and DUSP2 proteins has been reported in several types of cancers. The upregulation of ERK3 in cancer has been associated with tumor promoting function such as metastasis, cell proliferation, migration, and invasion. In paper II, we found also that overexpression of the ERK3 resulted in enhanced cell scattering and migration. Increased expression level of ERK3 protein is associated with both increased migration speed and morphological changes of cells into a rounded cell shape. The rounded/amoeboid cell migration has been known to be faster than the mesenchymal cell migration. Indeed, DUSP2 expression is markedly decreased in most solid cancers. The suppression of DUSP2 is associated with tumorigenesis and malignancy. Thus, decreased of DUSP2 levels coincide with increased ERK3 levels in cancer, which together could contribute to changes in the tumor cell morphology and thus cell motility and scattering.
In solid tumors with high levels of hypoxia inducible factor-1 (HIF-1) protein DUSP2 is suppressed, which leads to the prolonged phosphorylation of ERK1/2 and increased drug resistance (254, 255). The re-introduction of DUSP2 in these solid cancers results in the abolishment of ERK1/2-dependent cell survival and tumor growth. The loss of DUSP2 expression in acute leukemias correlates with high activity of ERK1/2, while high levels of DUSP2 expression correlated with poor survival in serous ovarian carcinomas (155, 156).
DUSPs and their aberrant regulation was detected in many cancers, and they have been associated with resistance of cancer cells to anti-tumor therapies (256). Take for example DUSP1, the most studied DUSP in cancer. Several siRNA studies have revealed that DUSP1 enhances resistance of ovarian, osteosarcoma, and NSCLC cell lines to cisplatin, doxorubicin or paclitaxel (257-259). In a clinical study, the overexpression of DUSP1 in breast cancer was repressed by doxorubicin and DUSP1 suppression led to cancer therapy resistance (260).

Further studies will be necessary to confirm the regulatory role of DUSP2 or DUSPs in general on ERK3/ERK4 or conventional MAPK cancer-related. In doing so, it would be interesting to determine the appropriate DUSP inhibitor for cancer therapy, if applicable. Even though there are not many studies on ERK4, we were able to show that DUSP2 has the ability to dephosphorylate ERK4 (paper I), as well as, detect ERK4 binding and its regulation by Hsp90 (paper III). In paper I, ERK4 was found to stabilize DUSP2 and increase its half-live, and in paper III, we observed destabilization of ERK4 following the inhibition of Hsp90. It would be interesting to determine the effect of Hsp90 inhibitors on the DUSP2 stabilization and if this is an ERK4-dependent process. Hsp90 inhibition is considered an exciting target for cancer therapy. Several studies have suggested that Hsp90 plays a role in cell migration and invasion (261-263). Unfortunately, we were not able to detect binding between ERK3 and Hsp90. It would be interesting if Hsp90 can bind and regulate ERK3, especially considering the recent findings reported in other studies and this study (paper II) showing the involvement of ERK3 in cell migration and invasiveness. However, the study by Wu and colleagues showed that the ERK3 protein level was upregulated after inhibition of Hsp90 in MDA-MB-231 and K562 cells (111). Moreover, it was shown that extracellular Hsp90α stabilizes MMP-2 and protects it from processing and subsequent inactivation in tumor cells in the MDA-MB-231 cells (264). Taking all these studies into account, along with Long and colleagues' study, which suggested that ERK3 regulates the expression of MMP2, 9 and 10 through phosphorylation of SRC-3 (74), may open a new area of research focus where we can investigate the co-relation between the extracellular Hsp90α and ERK3 in MDA-MB-231 cells.

Future perspective

Despite the frequent difficulties that are faced when working with less studied protein kinases such as ERK3 and ERK4, we have identified two proteins Hsp90 and DUSP2 that bind and interact with them. The interaction between Hsp90 and ERK4 has opened new prospects regarding the interaction between ERK4 and the Hsp90 co-chaperones (e.g. Cdc37, Hop, and Aha1). Cdc37 and Hsp90 form triple complexes with many proteins, in particular protein kinases. Currently, we are investigating whether Cdc37 binds to ERK4. It might be

worthwhile to detect the binding between ERK3 and Hsp90 in the MDA-MB-231 cell lines and accordingly their co-relation with MMPs.

Further investigation concerning paper II is in progress. We think that activation of RAC during nascent cell adhesion and subsequent cell migration promotes the localization of ERK3 at the cell periphery, which can also promote cell morphological changes. Experiments are ongoing to detect the ERK3 subcellular distribution to the cell periphery at different time points after seeding on collagen I. Further, knocking down group I PAK, the upstream activator for ERK3 and the downstream substrate of RAC, is an approach that would reveal if ERK3 localize in the cell periphery due to RAC activation. Moreover, we are performing a rescue experiment in shRNA ERK3 knockdown cells using a full-length wild type ERK3 from zebrafish to confirm our results. The molecular mechanism behind ERK3's role in cell adhesion, scattering, and migration still requires further investigation.

Since the discovery of ERK3 and ERK4, studies to discover their physiological role have been progressing slowly compared to the typical MAP kinase ERK1/2. More studies will be required to reveal the mystery around these proteins. Hopefully our novel step in finding the role of ERK3 in cell morphology and migration will direct more research into this field. In the end, I hope that these studies are efficiently contributing to discovering more about the roles of ERK3 and ERK4.

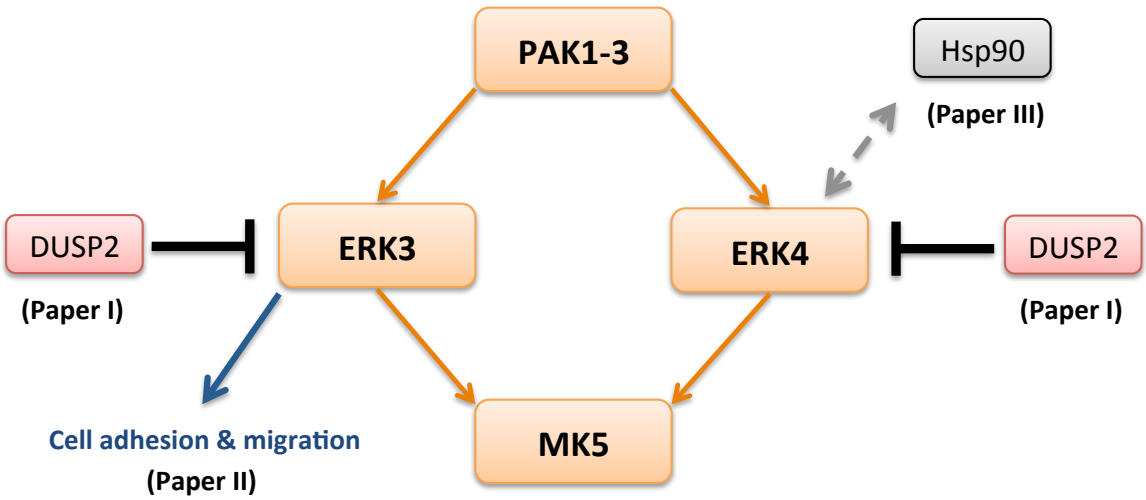

Figure 9. Schematic illustration of the project plans in this thesis.

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