Untersuchungen zur Regulierung und Funktion der Mitogen-aktivierten Proteinkinase ERK5

(Regulation and Function of the Mitogen-activated Protein Kinase ERK5)

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Phantasie ist wichtiger als Wissen,
denn Wissen ist begrenzt.

Albert Einstein

1 INTRODUCTION

The ability to coordinate complex physiological processes with a constantly changing environment is a prerequesite for any form of life. Every organism has to sense external parameters in order to react in an appropriate manner. Breaking down life to its unit – the cell, we can start to study and try to understand the molecular basis of the senses.

Each single cell of the approximately 10^{14} that make up a human organism has to communicate with its environment. Comparable to a computer a cell has to process input information and to convert it into an appropriate output. In the cellular context a broad variety of receptors sense external stimuli (input) such as hormones or contacts with other cells. We refer to the processing and transmission of information as signal transduction. Many individual pathways transmit signals along linear tracks from receptors to the nucleus and other other cell compartments and subsequently resulting in a discrete cell response. Since these pathways interact and interfere with each other, signal transduction resembles rather a network than a single chain of command (Downward, 2001; Pawson, 1995). Through amplification, desensitization, signal branching and integration a network-like structure allows the fine tuning of responses which is fundamental for embryonic development as well as in adult life (Bray, 1995; Gschwind et al., 2001; Hunter, 1995).

About one fifth of the estimated 32.000 human genes encode proteins involved in signal transduction including receptors, adaptors and effector enzymes. In this fundamentally important process particular importance is attributed to reversible protein phosphorylation which is a central theme in virtually all aspects of cellular function (Cohen, 2002). The addition of a highly charged phosphoryl group to an amino acid can modify protein function in every conceivable way, for instance by altering enzymatic activity and most important by facilitating or disrupting protein-protein interactions. The simplicity, flexibility and reversibility of phosphorylation coupled to the availability of ATP as phosphoryl donor explains why evolution has favoured this mechanism as general concept in regulation of cellular function.

Protein phosphorylation moved into spotlight of scientific interest as early as 50 years ago when phosphorylation was discovered to regulate glycogen phosphorylase (Fischer and Krebs, 1966). With the rise of molecular biology and techniques such as DNA cloning and sequencing, the number of identified kinases, which are enzymes able to transfer phosphoryl groups from ATP to target proteins, increased rapidly and led to the speculation that the vertebrate genome might encode as many as 1000 kinases (Hunter, 1995; Pawson, 1994).

Nowadays, completion of the human genome project revealed about 520 kinases (Manning et al., 2002b). Although the total sum does not reach earlier predictions, it is still a striking large number which further emphasizes the importance of protein phosphorylation. Taking also the 130 phosphatases into account that were identified so far, more than two percent of all human genes are directly involved in phosphorylation and dephosphorylation.

1.1 MAPKs

Almost all eukaryotic protein phosphorylation is conducted by a single superfamily of eukaryotic protein kinases (ePKs) sharing a conserved catalytic domain. ePKs are among the largest of protein families, comprising more than 1.5 % of all eukaryotic genes (Manning et al., 2002a). According to a recent extension of Hanks' and Hunter's taxonomy the human kinome can be divided into nine broad groups, 134 families and 196 subfamilies (Hanks and Hunter, 1995; Manning et al., 2002b). The STE group of kinases is one of these nine main groups and consists of the mitogen activated protein kinase (MAPK) families. The name STE refers to the first MAPK identified as a signal transduction enzyme in yeast.

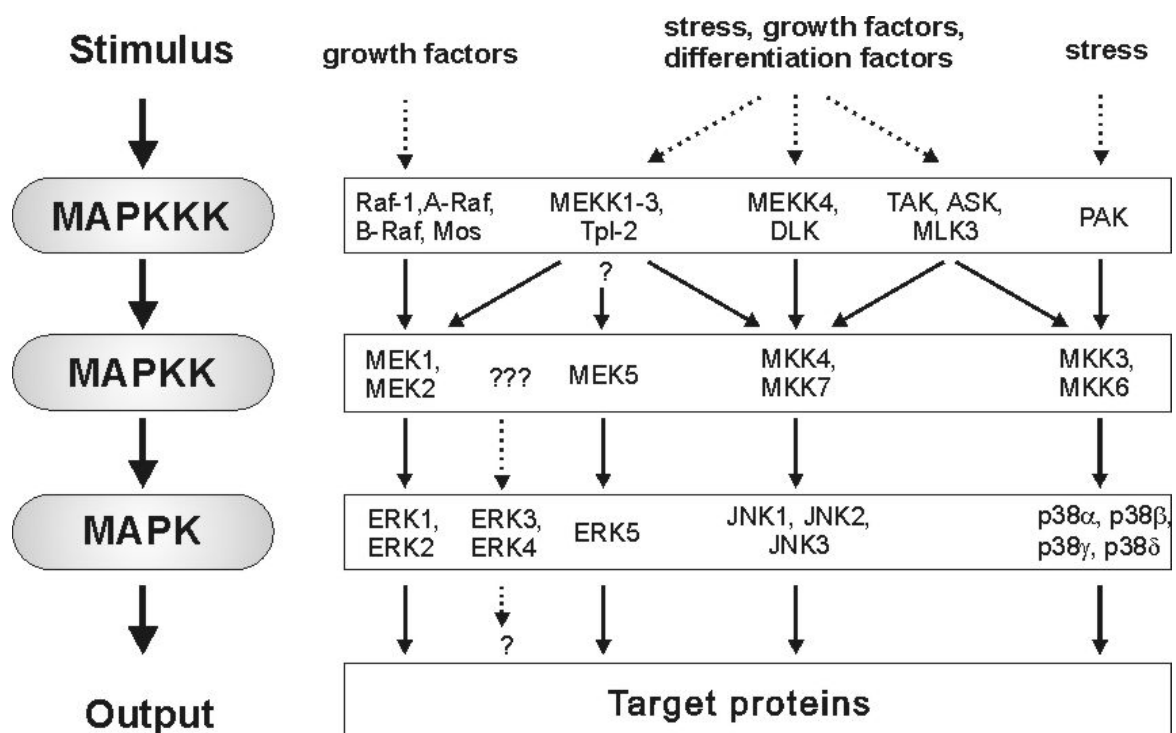

Figure 1: **MAPK cascades**. Overview of MAPK cascades according to Garrington and Johnson (Garrington and Johnson, 1999).

1.1.1 The MAPK Family

Mitogen-activated protein kinases are evolutionarily conserved enzymes found in virtually all eukaryotes (Manning et al., 2002a). A broad variety of stimuli elicit activation of MAPKs which regulate a large number of cellular processes in part by activating the transcriptional machinery (Kyriakis and Avruch, 2001; Pearson et al., 2001b). The activation of MAPKs is regulated through a three-tethered cascade composed of the MAPK itself, an activating MAPK kinase (MAPKK) and an upstream MAPK kinase kinase (MAPKKK) (Chang and Karin, 2001).

Mammals express at least four distinctly regulated groups of MAPKs, extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun amino-terminal kinases (JNK1-3), p38 proteins (α,β,γ and δ) and ERK5 (Garrington and Johnson, 1999) (Fig. 1). All of these are activated by specific MAPKKs of which each, however, can be activated by more than just one MAPKKK, increasing the diversity and complexity of MAPK signalling. Presumably each MAPKKK confers responsiveness to a distinct class of stimuli. For instance, activation of ERK2 by growth factors depends on the MAPKKK c-Raf, but others may activate ERK2 in response to pro-inflammatory stimuli (Chang and Karin, 2001). The situation is even more complex with JNK and p38 cascades which respond to many stimuli and can be activated by over-expression of at least a dozen of MAPKKKs whose physiological functions and specificities still remain elusive. At least four MAPKKKs, for example, were implicated in JNK activation by tumour necrosis factor and interleukin 1.

One of the most explored functions of MAPK signalling is the regulation of gene expression by direct or indirect phosphorylation and subsequent activation of transcription factors. In response to mitogens the concerted action of MAPKs has been shown to induce the immediate early genes fos and jun (Hazzalin and Mahadevan, 2002; Whitmarsh and Davis, 2000).

1.1.2 Signal Transduction by MAPK

The mechanism activated by mitogenic epidermal growth factor (EGF) illustrates some principles of signal transmission from the cell surface to the nucleus and the central role of MAPKs within this process (Fig. 2). By binding to its receptor EGF induces receptor dimerisation and autophosphorylation on tyrosines (Schlessinger, 2000a; Schlessinger, 2002). The phosphorylated and thus activated receptor binds adaptor proteins such as Grb2 and Shc.

The small adaptor proteins subsequently recruit the nucleotide exchange factor Sos to the membrane and thereby into proximity of Ras, a small monomeric, membrane-anchored GDP/GTP binding protein. It is well established that after Sos induced Ras activation which is accomplished by the induced exchange of GDP to GTP Ras attracts the kinase c-Raf to the membrane and subsequently contributes to its activation (Vojtek et al., 1993). C-Raf is a MAPKKK and thus represents the starting point of the MAPK cascade.

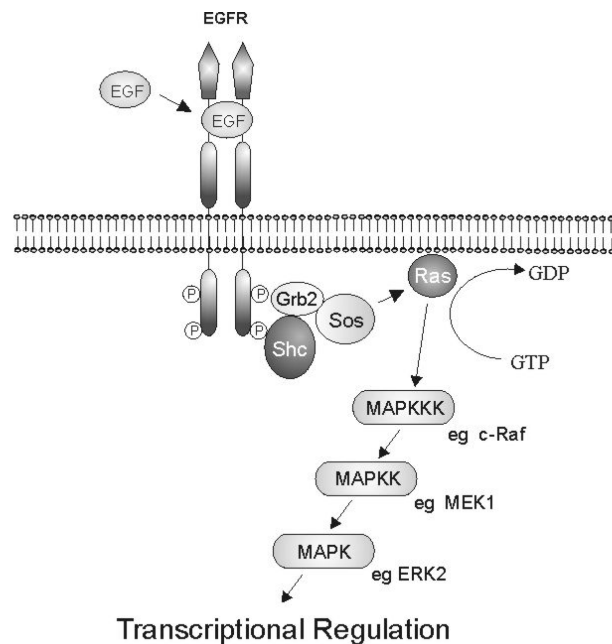

Figure 2: **MAPK activation by extracellular stimuli**. Epidermal growth factor (EGF) binds to its receptor (EGFR). Recruitment of adaptor proteins and Sos leads to the activation of Ras, which in turn activates the MAPK cascade.

Activated c-Raf, in turn, activates a second kinase MEK1 by phosphorylating it at two serine residues (Huang et al., 1993; Zheng and Guan, 1994). This modification activates MEK1 to phosphorylate both ERK1 and ERK2 on regulatory threonine and tyrosine residues in their activation loop (Derijard et al., 1995). The Raf-MEK1-ERK1/2 pathway is also called the 'classical' or 'mitogenic' MAPK cascade. Once activated ERK1 and ERK2 homodimerise, translocate to the nucleus and phosphorylate additional kinases, transcription factors and cell cycle regulators (Cobb et al., 1994; Khokhlatchev et al., 1998). An important downstream target of ERK is the Elk-1 transcription factor which participates in the transcriptional control of the fos proto-oncogene. Together with Jun proteins c-Fos forms the activating protein 1 (AP-1) dimer, an important transcription factor that induces several genes required for cell cycle progression (Shaulian and Karin, 2001).

1.1.3 ERK5

ERK5 is the only member of the fourth and least known MAPK subgroup. ERK5 differs considerably from other MAPKs in that it contains an unusually long carboxy-terminal tail of hitherto unknown function (Fig. 3) (Lee et al., 1995; Zhou et al., 1995). This 400 amino acid long extension also gave rise to its alternative designation as big MAP kinase 1 (BMK1). ERK5 is activated by diverse stimuli such as cellular stress and growth factors (Abe et al., 1996; Kato et al., 2000). The MAPKK MEK5 has been shown to specifically phosphorylate and thereby activate ERK5 (Kato et al., 1997; Lee et al., 1995). However, the other components involved in the signalling cascade that ultimately leads to the activation of ERK5 are only partly known.

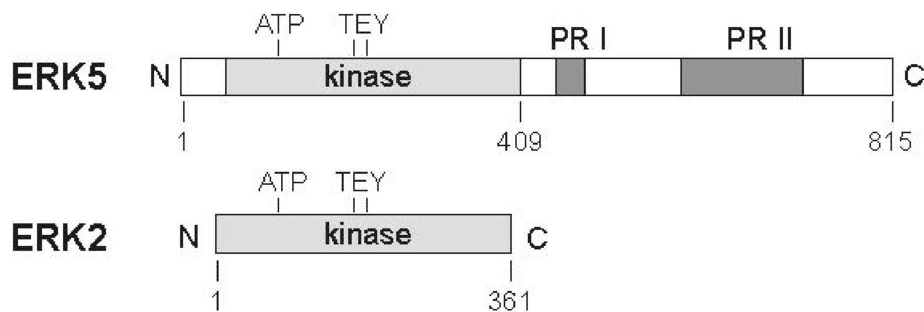

Figure 3 : **ERK5 contains a unique C-terminal tail**. The structures of ERK5 and its closest homologue ERK2 are presented schematically. The ATP binding sites and the TEY activation motives are indicated. The tail of ERK5 contains two proline-rich domains (PR I and II).

Many of the ERK5 activating stimuli also affect other MAPK family members. Synergistic actions of both the ERK1/2 and the ERK5 pathways have for example been reported in the induction of cell transformation (Pearson et al., 2001a). On the other hand, downstream effects exerted by activated ERK5 and ERK1/2 can in many cases be clearly distinguished from each other (Cavanaugh et al., 2001; Watson et al., 2001). Kato and colleagues (Kato et al., 1998) have, for example, demonstrated that ERK5 but not ERK2 is essential for proliferation and cell cycle progression in HeLa cells. More recently, Karihaloo et al (Karihaloo et al., 2000) have shown that ERK5 mediates EGF-induced morphogenesis in renal epithelial cells whereas ERK2 activity is critically involved in cell motility upon stimulation with hepatocyte growth factor.

ERK5 still remains one of the least characterised MAPKs and research has just started to shed some light on its functions. The embryonically lethal phenotype of ERK5 knock-out mice that die between day 10.5 and 12.5 with cardiovascular defects points to an essential role for

ERK5 signalling in development and especially in angiogenesis (Regan et al., 2002; Sohn et al., 2002).

1.1.4 The Paradox of MAPK Signalling

MAPK cascades pose a particular problem in signal transduction because they often serve as common conduit for myriads of diverse stimuli but still lead to highly defined and distinct cellular responses. Research of the last years has shed some light to the solution to this apparently paradoxical situation. Several distinct but not mutually exclusive mechanisms guarantee specificity of MAPK signalling on different levels. These include the process of activation and the interpretation of a certain activation profile. In yeast, the STE5 protein serves as a scaffold that organises the three required components of a pheromone-responsive MAPK cascade and its upstream activator into a module. Two analogous mammalian scaffold proteins were identified in the regulation of JNK (Whitmarsh et al., 1998).
Critical parameters in the determination of signal identity and ultimate cell fate are duration and amplitude of MAPK activation (Marshall, 1995). Almost a decade ago it was demonstrated that depending on the activation profile of MAPK rat phaeochromocytoma cells commit themselves either to differentiation or proliferation (Cowley et al., 1994; Traverse et al., 1992; Traverse et al., 1994). Just recently the immediate early gene product c-Fos was identified as a molecular sensor for the duration of MAPK activation. Only sustained MAPK activity leads to c-Fos activation because this requires MAPK-dependent transcriptional activation of the fos gene and subsequent stabilisation of the c-Fos protein by MAPK-mediated phosphorylation. Transient MAPK activity induces fos transcription to some extent but declines before appearance of the c-Fos protein which consequently remains unphosphorylated and instable (Murphy et al., 2002).
Since the profile of MAPK activation is critical for the definition of a specific cellular response a large group of phosphatases tightly regulates MAPK activity and in the same time allows fine tuning of the signal.

1.2 Phosphatases in the Regulation of MAPKs

Phosphatases are the main antagonists of kinase function. They can be roughly divided into three main groups according to their specificity: phosphotyrosine-specific, phosphothreonine/serine-specific or dual specific phosphatases. Among the superfamily of

eukaryontic protein kinases MAPKs are unique in that their activation loop must be phosphorylated on tyrosine and threonine to force the kinase into its active conformation (Canagarajah et al., 1997). Conversely, MAPKs are completely inactivated by dephosphorylation of either phosphoamino acid residue. Thus, all three types of phosphatases potentially neutralise MAPK activity (Keyse, 2000; Saxena and Mustelin, 2000).

1.2.1 Dual Specificity and Serine/Threonine-specific Phosphatases

The first type of phosphatases found to have specificity for MAPKs were the dual specificity phosphatases, a group whose founding member was the VH 1 protein encoded by Vaccinia virus (51). These so-called MAP kinase phosphatases (MKPs) differ from each other in their expression pattern and expression kinetics following stimulation. Most MKP genes are silent in resting cells, but highly inducible in response to growth factors and stress stimuli that both also activate MAPK family members (Fig. 4). Pac-1, for instance, is a lymphocytic MKP and becomes detectable 30 minutes after stimulation and MAPK activation. Pac-1 like most MKPs resides in the nucleus and is thus ideally suited for dephosphorylating activated MAPK and thereby resetting the MAPK pathway.
Activated MAPKs are also substrates for the purely serine/threonine-specific phosphatases PP2A and PP2C that merely remove phosphate from the phosphothreonine in the MAPK activation loop (Alessi et al., 1995; Millward et al., 1999; Takekawa et al., 1998). PP2A has been highly conserved in evolution as indicated by the comparison of homologues from yeast and man. PP2A has been implicated in a wide variety of cellular functions and in the regulation of more than 30 kinases (Millward et al., 1999). In contrast to MKPs PP2 phosphatases are localised in the cytosol. The first implication for PP2A in the regulation of MAPKs came from genetic studies on photoreceptor development in *Drosophila melanogaster* (Wassarman et al., 1996). PP2A does not only counteract MAPK but also MAPKKs activation. Noteworthy, the viral SV40 small t oncogene induces MAPK activation by presumably interfering with the action of PP2A (Sontag et al., 1993). However, the importance of PP2A in controlling MAP kinases seems to vary greatly between cell types and depends on the functional context. Moreover, PP2A which affects a large pool of diverse substrates is considered to act in concert with phospho-tyrosine specific phosphatases (PTPs). These PTPs contain a kinase-interacting motif (KIM) that confers a high intrinsic specificity for MAPKs (Pulido et al., 1998).

1.2.2 KIM-containing Tyrosine-specific Phosphatases

STEP, PTP-SL and HePTP form the family of PTPs containing a KIM. This short stretch of amino acids enables them to bind MAPKs. Interestingly, at least STEP and PTP-SL exist in many different forms ranging from short cytoplasmic to long variants that span the membrane up to three times (Hendriks et al., 1995; Lombroso et al., 1991; Ogata et al., 1995; Shiozuka et al., 1995). This diversity is caused by alternative splicing as well as variable start codon usage (Boulanger et al., 1995; Bult et al., 1997; Sharma and Lombroso, 1995). KIM containing PTPs (KIM PTPs) are generally characterised by their exclusively non-nuclear localization and a very restricted expression. HePTP, for instance, is expressed in bone marrow, spleen, lymph nodes and myeloid as well as lymphoid lineages, while PTP-SL is found in brain, lung and heart and STEP only in brain. The first indication for a role of KIM PTPs in cell proliferation and differentiation came from studies in haematopoietic cells that were obtained from patients with myelodysplastic syndrome. The HePTP gene was found to be located on the long arm of chromosome 1 which is often found in extra copies in this disease and correlates with reduced haematopoiesis and an increased risk of acute leukaemia (Boulanger et al., 1995; Bult et al., 1997; Zanke et al., 1994). Indeed, amplification of the HePTP gene was reported in a case of acute myeloid leukaemia. Conversely, deletions of the region comprising the HePTP gene have been reported in non-Hodgkin lymphomas and chronic lymphoproliferative disorders (Mitelman et al., 1990). Together these findings suggest that excess of KIM PTPs may correlate with reduced proliferation (e.g. HePTP in myodysplasia) and loss of KIM PTPs with increased cell proliferation and/or survival. The anticipated influence of these phosphatases would then directly reflect their effect on mitogenic MAPK signalling.

The KIM that targets the PTP to MAPK contains a serine which is subject to phosphorylation by protein kinase A (PKA). This modification was shown to interrupt MAPK binding (Blanco-Aparicio et al., 1999; Saxena et al., 1999b). Thus, pathways activating PKA can interfere with the activity of MAPKs by inhibiting KIM PTP-mediated negative regulation.

Tanoue *et al.* identified docking motifs in MAPKs that were highly conserved and that mediate interaction with substrates and activating MAPKKs (Tanoue et al., 2000; Tanoue et al., 2001). This domain was also shown to mediate the interaction of ERK2 with PTP-SL (Tarrega et al., 2002). Attention was drawn to this motif by the observation that the so-called *sevenmaker* gain-of-function mutation of ERK resulted in an increased and prolonged

activation which was later shown to be at least in part due to the abolishment of KIM PTP binding (Brunner et al., 1994; Oh-hora et al., 1999).
Taken together, KIM PTPs are specific inactivators of MAPKs and therefore able to participate in the regulation of diverse cellular functions.

1.2.3 'Sequential Phosphatase Model'

In order to integrate three different types of phosphatases acting on MAPKs in a single unifying hypothesis, Saxena and Mustelin proposed the following model (Saxena and Mustelin, 2000). All these enzymes act in a sequential manner and downstream of different external stimuli (Fig. 4). In the resting state MAPKs reside in the submembraneous cytosol, partly tethered to scaffolding proteins (Garrington and Johnson, 1999). Even under these conditions there would be some basal activity in the kinase cascade that would drive MAPK phosphorylation. The control of this background activity is attributed to the action of PP2A and PP2C in concert with KIM PTPs.

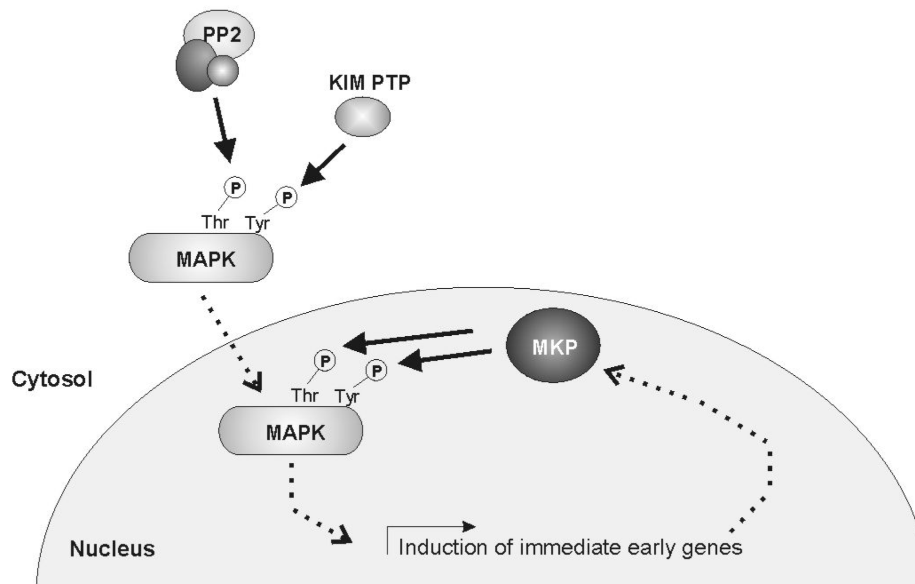

Figure 4: **Phosphatases in the regulation of MAPKs.** Activated MAPK is phosphorylated on tyrosine and threonine. The serine/threonine-specific phosphatase PP2 and KIM containing phosphotyrosine-specific phosphatases dephosphorylate and inactivate MAPK in the cytosol. Active MAPK having translocated to the nucleus induces immediate early genes including those coding for dual specificity phosphatases MKPs. Once translated MKP completely dephosphorylates MAPK and abrogates signalling.

Even upon stimulation the combination of serine/threonine and tyrosine-specific phosphatases might be able to control the activity of MAPKs during an initial phase in the cytosol. But

since PP2A/C as well as KIM PTPs are exclusively cytosolic those MAPKs that eventually manage to translocate to the nucleus, are free to phosphorylate their targets until MKPs are induced, synthesised and subsequently terminate signalling by dephosphorylating MAPKs. The existence of several MKPs with different modes of transcriptional activation, varying induction kinetics and distinct target preferences would further allow the cell to fine tune the activation profile of each MAPK and the balance between different MAPK family members.
This 'sequential phosphatase model' provides an explanation for the multitude of phosphatases involved in the regulation of comparatively few MAPKs. By acting in different compartments of the cell they represent an exquisite regulatory machinery that can control amplitude and duration as well as the localization of MAPK function. All these parameters together are critical for the determination of an adequate cellular response to a distinct stimulus.

1.3 Signalling by Tyrosine Kinases

For a long time tyrosine phosphorylation was considered to be functionally separate from serine/threonine phosphorylation. The addition of phosphate to a tyrosine residue was assumed to be mainly involved in the modulation of protein-protein interaction while the induction of conformational changes and the regulation of enzymatic activity were mainly attributed to serine/threonine phosphorylation. Although we today know that both kinds of phosphorylation are able to influence proteins in every and not mutually exclusive way, it is well established that protein tyrosine kinases play an important role in most fundamental cellular processes in part by their ability to regulate protein complex formation.

1.3.1 Receptor and Cytoplasmic Protein Tyrosine Kinases

In total the human genome contains 90 tyrosine kinase genes, of which 58 encode receptor tyrosine kinases (RTKs) distributed into 20 subfamilies (Blume-Jensen and Hunter, 2001; Manning et al., 2002b). RTKs contain a single transmembrane domain, a conserved cytoplasmic kinase domain and structural diverse ectodomains that determine their ligand specificity. The EGF receptor was the first surface signalling protein to be characterised by molecular biological methods and shown to be implicated in tumourigenesis (Ullrich et al., 1984). The EGF signalling pathway is highly conserved and was shown to be critically

involved in the developmental pattern formation of the nematode *Caenerohabditis elegans* as well as the fruitfly *Drosophila melanogaster* (Schweitzer and Shilo, 1997).

The human kinome further contains 32 cytoplasmic, non-receptor tyrosine kinases (NRTKs) grouped into 10 subfamilies: Src, Abl, Jak, Ack, Csk, Fak, Fes, Frk, Tec and Syk (Blume-Jensen and Hunter, 2001). In addition to a tyrosine kinase domain NRTKs possess multiple domains that mediate the interaction with proteins, lipids or DNA (Fig. 5).

The largest subgroup among NRTKs, with nine members, is the Src family of kinases. Depending on the context, members of this family were shown to regulate or at least to contribute to virtually all aspects of cellular function (Tatosyan and Mizenina, 2000; Thomas and Brugge, 1997). In fact, many NRTKs were first identified as cellular homologues of viral oncogene products with c-Src having been the very first to be described (Martin, 2001). The regulation of Src activity illustrates some principles of NRTK signalling (Bjorge et al., 2000; Schlessinger, 2000b). Src possesses two important regulatory tyrosine phosphorylation sites: The NRTK Csk phosphorylates a carboxy-terminal tyrosine of Src. This phosphotyrosine binds intramolecular to the SH2 domain and thus locks the kinase in its inactive conformation. The importance of this mechanism is underscored by the fact that viral, constitutively active v-Src lacks this regulatory site. Contrarily, autophosphorylation of another tyrosine in the activation loop leads to maximal activation. Deregulated Src signalling has been implicated in breast, lung and colon cancer (Biscardi et al., 1999; Frame, 2002; Irby and Yeatman, 2000).

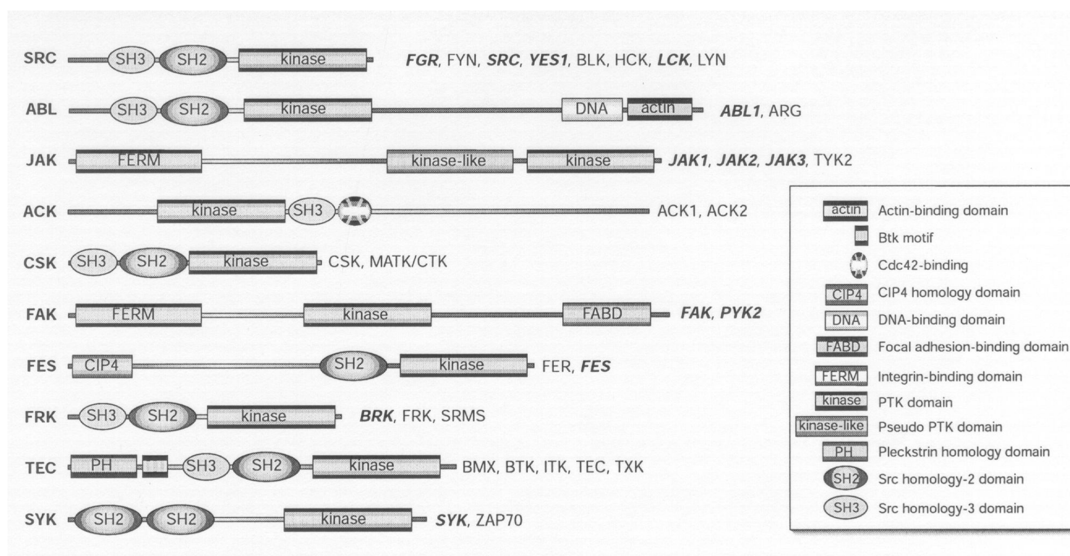

Figure 5: **Human cytoplasmic protein tyrosine kinases**. Adopted from Blume-Jensen and Hunter (Blume-Jensen and Hunter, 2001). The family members are indicated to the right and the family name to the left of each kinase. Kinases in bold and italic are implicated in human malignancies.

1.3.2 c-Abl

C-Abl and Arg are the members of a second family of NRTKs: c-Abl, which was in analogy to Src identified as the cellular homologue of a viral oncogene, has been implicated in many cellular processes (Shaul, 2000; Van Etten, 1999; Wang, 2000). Since the c-Abl protein contains many functional domains besides its tyrosine kinase including SH2 and SH3 domains, nuclear localisation and export sequences as well as binding sites for Actin, DNA and SH3 modules, c-Abl is likely able to simultaneously participate in or even to link numerous pathways by direct protein-protein interaction. Cytosolic and nuclear c-Abl kinases play distinct roles. For instance, one important function of nuclear c-Abl lies in the pathway linking DNA damage to the cell cycle machinery. It was shown that the induction of cell cycle arrest and apoptosis after UV-induced DNA damage involves the c-Abl-dependent stabilisation, acetylation and phosphorylation of p73, a member of the p53 tumour suppressor family (Agami et al., 1999; Costanzo et al., 2002; Gong et al., 1999). On the other hand, the cytoplasmic fraction of c-Abl and Arg were implicated in remodelling and stabilisation of the Actin cytoskeleton (Van Etten et al., 1994; Wang et al., 2001). In this context genetic studies in Drosphila suggested a role for the Abl orthologue Dabl as regulator of morphogenesis in the developing nervous system (Van Etten, 1999). Taken together recent findings have established a pivotal role for c-Abl in various and diverse cellular functions. However, most Abl research focuses on its oncogenic derivatives. The signalling of viral v-Abl and of the leukaemic fusion proteins Bcr/Abl and Tel/Abl will be discussed below in the context of chronic myeloid leukaemia.

1.4 Molecular Characteristics of Cancer

Cancer is the most common cause of death in the Western world affecting one third of its entire population. The development of tumours is a multistep process that involves genetic alterations which lead to the ‘loss of function’ of tumour suppressor genes and the ‘gain of function’ of oncogenes. These genetic changes which can be as subtle as point mutations or as striking as chromosomal rearrangements, consequently drive the progression of normal cells to malignant derivatives (Hanahan and Weinberg, 2000).
Studies on familial predisposition to cancer led to the first models attempting to describe the underlying mechanisms of tumourigenesis. According to the simple ‘two hit’ model, dominantly inherited predisposition to cancer entails a germ line mutation, while development

of cancer requires a second, somatic mutation (Knudson, 1996). A non-hereditary cancer of the same type requires the same two, though somatic hits. Although applicable in many cases to simplify and explain familial predisposition to genetic diseases, this models holds only fully true for the pathogenetically simple retinoblastoma tumour. The development of both hereditary as well as sporadic forms of retinoblastoma is exclusively caused by the inactivation of both alleles of the retinoblastoma (Rb) 1 gene (Knudson, 1971).

Observations of human cancers and animal models argue that tumour development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Cahill et al., 1999).

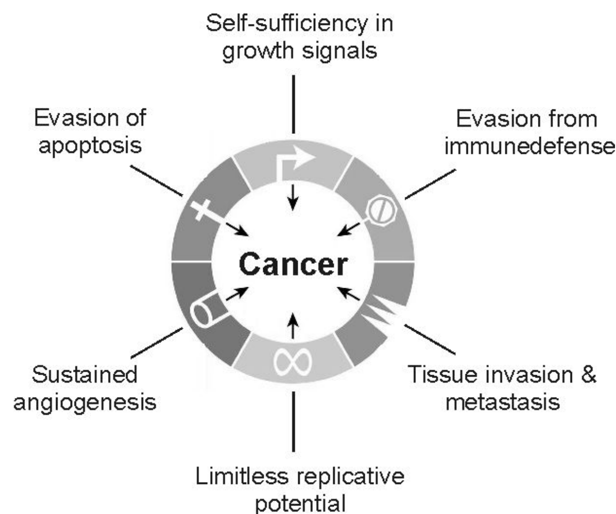

Figure 6: **Six hallmarks of cancer**. According to Weinberg and Hanahan most, if not all cancers have acquired the same set of functional capabilities during their development, albeit through various mechanistic strategies (Hanahan and Weinberg, 2000).

This multistep process is well illustrated by colorectal cancers, which typically develop over decades and appear to require at least seven genetic events for completion. Even so, inheritance of a single altered gene can result in a marked predisposition to colorectal cancer in two distinct syndromes, Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (Kinzler and Vogelstein, 1996). Recent evidence suggests that the genetic defect in FAP affects the rate of tumour initiation by targeting the proliferation control function of the APC gene. In contrast, the defect in HNPCC largely affects tumour progression by targeting the genome guardian function of DNA mismatch repair. According to their function we refer to these two types of affected genes as 'gatekeepers' and 'caretakers', respectively (Kinzler and Vogelstein, 1997). The inactivation of repair mechanisms as in HNPCC plays an important role in many different tumours (Fodde

et al., 2001; Harfe and Jinks-Robertson, 2000). This consequentially leads to an increase in the incidence of mutations and genetic instability and thus accelerates the progression from benign stages to carcinoma and metastasis.

The final cancer cell genotype and its resulting phenotype display six essential alterations in cell physiology that collectively dictate malignant growth. The six so-called hallmarks of cancer are self-sufficiency in growth signals, insensitivity to growth-inhibitory (cell cycle arrest) signals, evasion of programmed cell death, endless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Fig. 6). These six capabilities are presumably shared by most and perhaps all types of human tumours (Hanahan and Weinberg, 2000).

Cancer is not a single disease and tumours are indeed extremely diverse and heterogeneous, but underlying this variability is a certain number of 'mission critical' events including, for instance, the inactivation of the p53 gatekeeper gene product (Evan and Vousden, 2001). The challenge faced by the research community is to identify and understand the molecular anatomy of such pivotal steps in tumour progression and to develop therapies that directly target these points of convergence. The future combinational use of broadly applicable as well as cancer type-specific therapeutics might represent the most promising approach to suppression and cure of cancer.

1.5 Chronic Myeloid Leukaemia

1.5.1 Molecular Pathophysiology of Chronic Myeloid Leukaemia

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative expansion of transformed haematopoietic progenitor cells. It involves myeloid, monocytic, erythroid, megakaryocytic and rarely B-lymphoid and T-lymphoid lineages (Faderl et al., 1999). CML progresses through distinct stages. The earliest stage is characterised by the expansion of terminally differentiated neutrophils. Over several years disease progresses to an acute phase termed blast crisis, characterised by maturation arrest with excessive numbers of undifferentiated progenitor cells. Although heterogeneous, CML is the best-described leukaemia at the molecular level and was the first human disease in which a specific genetic abnormality could directly be linked to the pathogenic events of leukaemogenesis.

In 1960, Nowell and Hungerford identified an abnormal chromosome in 90% of chronic myeloid leukaemia patients (Nowell and Hungerford, 1960). This genomic abnormality was

later shown to be caused by the reciprocal chromosomal translocation of chromosomes 22 and 9 (Rowley, 1973). The resulting shortened chromosome 22, commonly termed Philadelphia chromosome encodes a Bcr/Abl fusion product (Ben-Neriah et al., 1986). The reciprocal *abl/bcr* gene on chromosome 9, although transcriptionally active, does not appear to have any functional role and no translated product has been identified so far (Diamond et al., 1995; Melo et al., 1993).

Depending on the breakpoint in the *bcr* gene three main types of resulting Bcr/Abl fusion proteins can be distinguished with the most common being the p210 Bcr/Abl protein (Melo, 1996). Numerous experimental models have established that Bcr/Abl is sufficient to produce CML-like disease in mice (Daley et al., 1990; Elefanty et al., 1990; Heisterkamp et al., 1990). The oncogenic capacity of Bcr/Abl is based on two features, first its exclusively cytosolic localisation (Ben-Neriah et al., 1986) and secondly its constitutive kinase activity (Van Etten et al., 1989). Several functional domains have been identified and shown to be required for the full transforming potential of the oncogene. In the Abl portion these domains are the SH1 tyrosine kinase, the SH2 and the Actin-binding domains. In the Bcr portion they include the coiled coil motif, the Grb2 binding site at tyrosine 177 and the phospho-serine/threonine-rich SH2 binding site (Fig. 7).

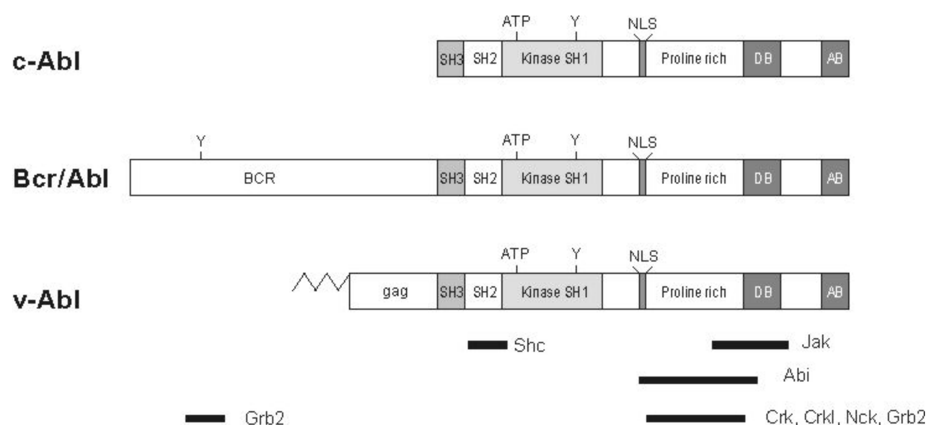

Figure 7: **Cellular c-Abl and its oncogenic variants**. Scheme adopted from Zou and Calame (Zou and Calame, 1999): SH2 and SH3, Src homology domains 2 and 3; SH1, tyrosine kinase domain; NLS, nuclear localisation sequence; DB, DNA binding domain; AB, Actin-binding domain. Binding sites of some interacting proteins are indicated by bars.

Oligomerisation mediated by the coiled-coil motif, membrane localisation and autophosphorylation of the Bcr/Abl kinase mimics an activated receptor tyrosine kinase. Bcr/Abl activates numerous signalling pathways by recruiting adaptor proteins such as Crkl and Grb2 as well as by directly phosphorylating target proteins. Thereby, Bcr/Abl interferes with basic cellular processes such as control of cell proliferation and differentiation (Jiang et

al., 2000; Puil et al., 1994), adhesion and cell survival (Cortez et al., 1995; McGahon et al., 1994). Among these activated signalling pathways are Ras/MAPK, PI-3 kinase, c-Cbl, Jak-Stat, and the Src pathway. Modulation of Crkl, Paxillin, Actin, Talin and Vinculin as well as Fak/Pyk2 is further considered to be responsible for adhesion defects and cytoskeletal abnormalities characteristically found in CML cells.

Malignant transformation by Bcr/Abl is characterised by altered adhesion to stromal cells and extracellular matrix, activation of mitogenic signalling and reduced apoptosis (Gishizky, 1996; Sattler and Griffin, 2001; Sawyers, 1997; Verfaillie, 1998). In haematopoietic cells Bcr/Abl was shown to induce mitogenesis (Deininger et al., 2000) and to protect cells from apoptosis (Cortez et al., 1995; McGahon et al., 1994; Nishii et al., 1996).

Although a massive amount of information on Bcr/Abl signalling has been accumulated so far, the single pathway through which Bcr/Abl causes leukaemia, if it actually exists, has not yet been identified. Koh and Daley (Koh and Daley, 2001) argued that Bcr/Abl does not activate a single critical signalling pathway but rather a multifaceted communication network. Therefore, they have called Bcr/Abl the ‘ultimate oncogene’. If the mechanism by which Bcr/Abl causes leukaemia is indeed based on the attack of the cell’s normal anti-cancer defences from various angels, it is conceivable that other oncogenes will operate using similar strategies.

The importance of the *bcr/abl* gene for CML progression was recently further exemplified by a study with transgenic mice and inducible expression of the oncogene. It was shown that besides initiating the disease *bcr/abl* was required for driving leukaemia progression throughout all stages, despite the appearance of numerous additional mutations in oncogenes as well as tumour suppressor genes (Huettner et al., 2000). Likewise, in comparable model systems Ras and Myc were also shown not only to be sufficient for tumour initiation but also essential for maintenance of the transformed phenotype (Chin et al., 1999; Felsher and Bishop, 1999).

1.5.2 Therapy of Chronic Myeloid Leukaemia

Therapy of CML is based on the use of standard chemotherapeutics as well as agents specifically targeting the Bcr/Abl kinase. Since the clinical course of CML is characterised by overproduction of differentiated myeloid lineages, it can be controlled for months to years by treatment with anti-proliferative agents. Hydroxyurea, for instance, is generally effective in normalizing peripheral blood counts. Unfortunately, cytogenetic responses are rare which

means that a significant percentage of bone marrow cells retain the Philadelphia chromosome. Hence, progression to blast crisis is not delayed. Allogenic transplantation of haematopoietic stem cells (HSCT) remains the only curative treatment of CML so far. But since substantial acute and chronic toxicities occur frequently, particular in older individuals, and the availability of HLA-matched donors is very rare, for most patients HSCT is not an option. Therefore, Interferon-α (IFN-α) has become the treatment of choice. IFN-α induces complete cytogenetic responses in 10-20% of treated patients and increases duration of chronic phase and long-term survival. A major disadvantage is the poor tolerance of IFN-α which may require discontinuation of treatment.

Several more recent lines of investigation focus on the development of therapies based on the direct inhibition of Bcr/Abl including several anti-sense and RNA interference approaches. A milestone in CML research was the development of Imatinib, a small molecule inhibitor of the Bcr/Abl kinase (Buchdunger et al., 1996, Druker, 2002; Druker et al., 1996). Imatinib is a 2-phenylamino pyrimidine and functions by binding to the highly conserved nucleotide-binding pocket of the catalytic domain of the Abl tyrosine kinase and constitutively blocking the binding of ATP (Schindler et al., 2000). Preclinical studies have established that Imatinib specifically inhibits proliferation and restores cytokine-dependence of Bcr/Abl positive leukaemia cells but only minimally affects growth of normal cells (Carroll et al., 1997; Deininger et al., 1997; Druker et al., 1996; Gambacorti-Passerini et al., 1997). The efficacy of Imatinib is based on the inhibition of proliferation and to a lesser extent on the induction of apoptosis (Holtz et al., 2002). A series of phase I and phase II trials demonstrated the remarkable activity of Imatinib in CML therapy (Druker et al., 2001a; Druker et al., 2001b; Ottmann et al., 2002; Sawyers et al., 2002; Talpaz et al., 2002). Imatinib was shown to induce remissions in early chronic phase as well as in blast crisis. Whereas responses in chronic phase have been enduring, remissions in blast crisis patients were transient over a period of 2 to 6 months. But since the majority of patients respond to Imatinib without showing any major side effects the Food and Drug Administration have recently approved it for CML treatment. Although patients in blast crisis benefit clinically from Imatinib application, treatment is hampered by the onset of resistance to the drug in virtually all cases.

1.5.3 Mechanisms of Imatinib Resistance

Resistance to Imatinib includes *de novo* resistance and relapse after initial response. Based on the mechanisms underlying drug resistance, patients can be divided into two subgroups: those

with persistent inhibition of the Bcr/Abl kinase and those with reactivation of the kinase. Patients with persistent Bcr/Abl inhibition would be predicted to have additional genetic abnormalities driving the growth and survival of leukaemic cells. In contrast, patients with unaffected Bcr/Abl kinase activity or those with reactivation of oncogenic Bcr/Abl signalling would be postulated to have developed mechanisms that prevent Imatinib from reaching its target or that render the target insensitive to the inhibitor. It can be further distinguished between systemic and cellular mechanisms of resistance. Enhanced drug metabolism or as recently shown sequestration of Imatinib by α1 acid glycoprotein (Gambacorti-Passerini et al., 2000; Gambacorti-Passerini et al., 2002) are two examples of systemic resistance. The most general mechanism for the development of cellular resistance is the increase of drug export rates which is often observed with various agents in cancer therapy. The latter is caused by an increased expression of membrane transport proteins such as the P-glycoprotein and the Multidrug Resistance Protein MRPI that act as real efflux pumps (Perek and Denoyer, 2002).

The majority of patients suffering from relapse after initial response to Imatinib, shows a reactivation of the Bcr/Abl kinase (Gorre et al., 2001). Two mechanisms have been shown to be responsible for cellular resistance, Bcr/Abl gene amplification and more important point mutations (Branford et al., 2002; Gorre et al., 2001; Hochhaus et al., 2002; le Coutre et al., 2000). The mutations found in the kinase domain or P loop of Bcr/Abl either interfere directly with Imatinib binding or destabilise the conformation required for Imatinib intercalation (Schindler et al., 2000; Shah et al., 2002). The IC50 of Imatinib for mutant Bcr/Abl is up to 10-fold higher than for the wild-type oncogene (Shah et al., 2002).

It is conceivable that different cellular and systemic types of resistance, rather than being mutually exclusive, complement each other to effect their final objective: the generation of cells that can survive despite Imatinib treatment. Attempts to overcome this problem in chemotherapy will have to consider all aspects of resistance. Therefore, the development of a multifaceted therapeutic approach to CML is of great interest and subject of intense research.

1.6 Aim of the study

The traditional view of MAPK function distinguished between three structurally distinct subfamilies, the ERKs, the JNKs and p38 kinases. With the cloning of ERK5/big MAP kinase 1, however, a yet untold chapter of MAPK signalling had been opened. Based on unique structural properties ERK5 could not be attributed to any of the three subfamilies. Since only

very limited information on ERK5 was available at the beginning of this study, the analysis of ERK5 expression, localisation and activation by diverse stimuli in various cell lines had to be approached.

MAPKs pose a particular problem in signal specificity in that they serve as common conduit for multiple upstream events and still regulate distinct cellular functions. The explanation to this apparently paradoxical situation alludes to a tight, multifaceted and finely tuned regulation of MAPKs. ERK5 is one of the least characterised members of the MAPK family and its only so far known regulator was its activating kinase MEK5 (Zhou et al., 1995). The central part of this study concentrates on the question how ERK5 signalling might be regulated.

First, in analogy to other MAPKs the activity of ERK5 was expected to be controlled by the phosphorylation state of its activation motif. In this context, the potential influence of the phosphotyrosine-specific phosphatase PTP-SL, a member of a PTP family previously implicated in the regulation of other MAPKs (Pulido et al., 1998; Saxena et al., 1998), was analysed.

Secondly, C-terminally of the conserved MAPK domain ERK5 contains a 400 amino acid long tail of so far unknown function. Since ERK5 differs from all other MAPKs in this tail the question whether and how these unique domains could contribute to the regulation of ERK5 or might affect its signalling had to be addressed.

Finally, MAPKs were suggested to be indirectly involved in tumour development by contributing to acquired capabilities of cancer such as growth self-sufficiency or evasion from apoptosis. Whether ERK5 would be involved in oncogenic signalling and how its involvement could contribute to tumourigenesis was a final question to be considered.

2 Materials and Methods

2.1 Materials

2.1.1 Laboratory Chemicals and Biochemicals

Acrylamide	Serva, Heidelberg
Agar	Difco, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine triphosphate)	Amersham Pharmacia, Freiburg
[γ-^{32}P] ATP (>5000 Ci/mmol)	Perkin Elmer/NEN, Köln
Batimastat	British Biotech, UK
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Chloroquin	Biotrend Chemikalien, Köln
Crystal Violet	Sigma, Taufkirchen
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Fibronectin	Calbiochem, Bad Soden
Formaldehyde	PolySciences, Eppenstein
Geneticin (G418, GibCo)	Invitrogen, Eggenstein
GF109203X	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid))	Serva, Heidelberg
Imatinib (Gleevec, STI571)	Dr. Keri, Vychem, Hungary
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Biomol, Hamburg
L-Glutamine (GibCo)	Invitrogen, Eggenstein
Leupeptin	Sigma, Taufkirchen
Lipofectamine® (GibCo)	Invitrgen, Eggenstein
Lysozyme	Sigma, Taufkirchen
MBP (Myelin basic protein)	Sigma, Taufkirchen
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
pNPP (p-Nitrophenyl phosphate)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
Polyfect®	Quiagen, Hilden
PD98059	Alexis, Grünberg
Ponceau S	Sigma, Taufkirchen
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Sigma, Taufkirchen
Superfect®	Quiagen, Hilden
T4 gene 32 protein	Pharmacia, Freiburg

TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
TPA (Tetradecanoyl-phorbol-13-acetate)	Sigma, Taufkirchen
Triton X-100	Serva, Heidelberg
Tween 20, 40	Sigma, Taufkirchen
Tyrphostin AG1478	Alexis, Grünberg
U0126	Calbiochem, Bad Soden

All other chemicals were purchased in analytical grade from Merck (Darmstadt).

2.1.2 Enzymes

Calf Intestine Alkaline Phosphatase	MBI Fermentas, St. Leon-Rot
DNAse I, RNAse free	Roche, Mannheim
Restriction Endonucleases	NEB, Frankfurt/ Main MBI Fermentas, St. Leon-Rot
T4-DNA Ligase	Roche, Mannheim
LA Taq-DNA Polymerase	Takara, Japan
Pfu DNA Polymerase	MBI Fermentas, St. Leon-Rot
Trypsin (GibCo)	Invitrogen, Eggenstein

2.1.4 „Kits" and Other Materials

Cell culture materials	Greiner, Solingen Nunclon, Dänemark Falcon, UK
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
ECL Kit	PerkinElmer/NEN, Köln
Dual-Luciferase® Reporter Assay System	Promega, Mannheim
Glutathione-Sepharose	Amersham Pharmacia, Freiburg
Hyperfilm MP	Amersham Pharmacia, Freiburg
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Poly Prep® Chromatography columns	Bio-Rad, München
Protein A-Sepharose	Amersham Pharmacia, Freiburg
Protein G-Sepharose	Amersham Pharmacia, Freiburg
QIAquick Gel Extraction Kit (50)	Qiagen, Hilden
QIAquick PCR Purification Kit (50)	Qiagen, Hilden
QIAGEN Plasmid Mini Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
Sephadex G-50 (DNA Quality)	Amersham Pharmacia, Freiburg
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Whatman 3MM	Whatman, Rotenburg/Fulda

2.1.5 Growth Factors and Ligands

EGF (murine)	Toyoba, Japan
NGF	Becton Dickinson, Heidelberg
αFGF	Pepro Tech, USA
Insulin	Lilly, Giessen

2.1.6 Media and Buffers

LB or 2xYT media were used for cultivation of all *Escherichia coli* strains. If and as required 100 µg/ml Ampicillin or 70 µg/ml Kanamycin were added to media after autoclavation. For the preparation of LB-plates 1.5% Agar were also added.

LB-Medium	1.0	%	Tryptone
	0.5	%	Yeast Extract
	1.0	%	NaCl
			pH 7.2
2xYT-Medium	1.6	%	Tryptone
	1.0	%	Yeast Extract
	1.0	%	NaCl
			pH 7.2

2.1.7 Cell Culture Media

The following Gibco™ media and additives were obtained from Invitrogen (Eggenstein):

Dulbecco's modified eagle medium (DMEM) with 4.5 mg/ml glucose supplemented with 2 mM L-Glutamine.

Minimum essential medium (MEM) with 2 mM L-Glutamine and 100 U/ml Penicillin/Streptomycin.

RPMI Medium 1640 with 2 mM L-Glutamine and 100 U/ml Penicillin/Streptomycin.

Nutrient mixture F12 (HAM) with 2 mM L-Glutamine and 100 U/ml Penicillin/Streptomycin.

Media were supplemented with heat-inactivated Fetal Calf, Bovine Calf or Horse Serum according to the requirements of each cell line. Following the supplier's instruction's 1 mM Sodium Pyruvate and Non-essential amino acids were added to some media.

Freeze medium: 90% heat-inactivated FCS, 10% DMSO.

2.1.8 Stock Solutions and commonly used buffers

BBS (2x)	50.0	mM	BES
	280.0	mM	NaCl
	1.5	mM	Na_2HPO_4
			pH 6.96
HEBS (2x)	46.0	mM	HEPES
	274.0	mM	NaCl
	1.5	mM	Na_2HPO_4
			pH 7.00
HNTG	20.0	mM	HEPES, pH 7.5
	150.0	mM	NaCl
	0.1	%	TritonX-100
	10 .0	%	Glycerol
	10.0	mM	$Na_4P_2O_7$
DNA loading buffer (6x)	0.05	%	Bromphenol blue
	0.05	%	Xylencyanol
	30.0	%	Glycerol
	100.0	mM	EDTA pH 8.0
Laemmli buffer (2x)	65.0	mM	Tris/HCl pH 6.8
	2.0	%	SDS
	30.0	%	Glycerol
	0.01	%	Bromphenol blue
	5.0	%	ß-Mercaptoethanol
Laemmli buffer (3x)	100	mM	Tris/HCl pH 6.8
	3.0	%	SDS
	45.0	%	Glycerol
	0.01	%	Bromphenol blue
	7.5	%	ß-Mercaptoethanol
NET	50.0	mM	Tris/HCl pH 7.4
	5.0	mM	EDTA
	0.05	%	Triton X-100
	150.0	mM	NaCl
PBS	137.0	mM	NaCl
	27.0	mM	KCl
	80.9	mM	Na_2HPO_4
	1.5	mM	KH_2PO_4 pH 7.4
SD-Transblot	50.0	mM	Tris/HCl pH 7.5
	40.0	mM	Glycine
	20.0	%	Methanol
	0.004	%	SDS
“Strip” buffer	62.5	mM	Tris/HCl pH 6.8
	2.0	%	SDS

	100.0	mM	β-Mercaptoethanol
TAE	40.0	mM	Tris/Acetate pH 8.0
	1.0	mM	EDTA
TE10/0.1	10.0	mM	Tris/HCl pH 8.0
	0.1	mM	EDTA pH 8.0
Tris-Glycine-SDS	25.0	mM	Tris/HCl pH 7.5
	200.0	mM	Glycine
	0.1	%	SDS

2.1.9 Bacterial Strains, Cell Lines and Antibodies

Names of people given as reference without further designation were members of this group.

2.1.9.1 E. Coli Strains

E. Coli strain	Genotype Description	Origin/ Reference
DH5αF'	F' endA1 hsd17 ($r_k^- m_k^+$) supE44 recA1 gyrA (Nal) thi-1 Δ(lacZYA-argF196)	Genentech, USA
CJ236	dut ung thi-1 relA	(Kunkel, 1985)
BL21 Codon+	F' ompT hsdS(r_B– m_B–) dcm+ Tet^r gal λ (DE3) endA Hte (argU ileY leuW Cam^r)	Stratagene, NL
SCS110	rpsL (Str^r) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44D (lac-proAB) (F' traD36 proAB $lacI^qZ\Delta M15$)	Stratagene, NL

2.1.9.2 Cell Lines

Cell Line	Description	Origin/ Reference
BV-173	Human B cell precursor leukemia	DSMZ, Braunschweig
COS-7	African green monkey, SV40-transformed kidney	Genentech, USA
EM-2	Human chronic myeloid leukaemia in blast crisis	DSMZ, Braunschweig
HEK 293T	Human embryonic kidney fibroblasts, transformed with adenovirus Typ V DNA	ATCC, USA
K562	Human chronic myeloid leukaemia, blast crisis	ATCC, USA
Ku 812	Human chronic myeloid leukaemia, blast crisis	DSMZ, Braunschweig
MEG-01	Human chronic myeloid leukaemia, blast crisis	DSMZ, Braunschweig

M1/LX	MEG-01 infected with pLXSN	this study
M1/WT	MEG-01 infected with pLXSN HA-ERK5 WT	this study
M1/AEF	MEG-01 infected with pLXSN HA-ERK5 AEF	this study
M1/KM	MEG-01 infected with pLXSN HA-ERK5 KM	this study
M1/pRS	MEG-01 infected with pRetroSUPER	this study
M1/pRS E5	MEG-01 infected with pRetroSUPER ERK5	this study
Phoenix A	Packaging cell line for the generation of helper free amphotropic retroviruses, based on HEK 293T	G. Nolan, Stanford, USA
Phoenix E	Packaging cell line for the generation of helper free ecotropic retroviruses	G. Nolan, Stanford, USA
PC12	Rat phaeochromocytoma cells	P. Cohen, UK
PC12/pc	PC12, transfected with pcDNA3	M. Sommer
PC12/WT	PC12, transfected with pcDNA3 PTP-SL WT	M. Sommer
PC12/CS	PC12, transfected with pcDNA3 PTP-SL CS	M. Sommer
Rat-1	Immortalised rat fibroblasts	R. Friedrich, Giessen
Rat-1/lx	Rat-1, transfected with pLXSN	this study
Rat-1/WT	Rat-1, transfected with pLXSN HA-ERK5 WT	this study
Rat-1/AEF	Rat-1, transfected with pLXSN HA-ERK5 AEF	this study
Rat-1/KM	Rat-1, transfected with pLXSN HA-ERK5 KM	this study
R1vA	v-Abl transformed Rat-1	this study
Swiss 3T3	3T3 - Swiss albino, mouse embryo fibroblasts, immortalised	ATCC, USA

All other cell lines were obtained from either the American Type Culture Collection (ATCC, Manassas, USA) or the German Collection of Microorganisms and Cell Lines (DSMZ, Braunschweig) and were cultivated following the supplier's recommendations.

2.1.9.3 Antibodies

The following antibodies were used for immunoprecipitations or as primary antibodies in immunoblot and immunofluorescence analysis.

Antibody	Description/ Immunogen	Origin/ Reference
Abl (SC-23)	Mouse, monoclonal, recognises the C-terminus of Abl kinases	Santa Cruz, USA
Actin	Rabbit, polyclonal, directed against a C-terminal peptide	Sigma, Taufkirchen
ERK2 (C-14)	Rabbit, polyclonal, peptide at C-terminus of rat ERK2, used for IP	Santa Cruz, USA
ERK2 (K-23)	Rabbit, polyclonal, peptide from sub-domain XI of rat ERK2, used for WB	Santa Cruz, USA
ERK5 (CT)	Rabbit, polyclonal, directed against a fusion of GST and human ERK5 aa 710 – 815	this study
ERK5 (PR1)	Rabbit, polyclonal, directed against a fusion of GST and human ERK5 aa 410 – 558	this study
HA.11	Mouse, monoclonal, recognises the influenza hemagglutinin epitope	BAbCo, USA
HA (12CA5)	Mouse, monoclonal, recognises the influenza hemagglutinin epitope	Roche, Mannheim
Histone H1	Mouse, monoclonal	Santa Cruz, USA
JNK (C-17)	Rabbit, polyclonal, directed against a C-terminal peptide of human JNK1	Santa Cruz, USA
p38 (C-20)	Rabbit, polyclonal/ peptide at C-terminus of murine p38	Santa Cruz, USA
P-Tyr (4G10)	Mouse, monoclonal, recognises phospho-tyrosine residues	UBI, USA
P-ERK2	Rabbit, polyclonal, recognises phospho-ERK1/2 (Thr-202/ Tyr-204) MAPK	NEB, Frankurt/M
P-p38	Rabbit, polyclonal, recognises phospho-p38 (Thr-180/Tyr-182) MAPK	NEB, Frankfurt/M
P-JNK	Rabbit, polyclonal, recognises phospho-JNK (Thr183/Tyr185)	NEB, Frankfurt/M
P-ERK5	Rabbit, polyclonal, recognises phospho-ERK5 (Thr218/Tyr220)	BioSource, Solingen
PTP-SL	Rabbit, polyclonal, direct against the peptide CHSMVQPEQAPKVLN coupled to Keyhole Limpet Hemocyanin	M. Sommer

RanGAP	Goat, polyclonal	F. Melchior, Martinsried (Pichler et al., 2002)
Tubulin	Mouse, monoclonal, ascites	Sigma, Taufkirchen
VSV (P5D4)	Mouse, monoclonal; recognises an epitope of eleven amino acids derived from the vesicular stomatitis virus glycoprotein VSV-G	Roche, Mannheim

For immunoblot and immunofluorescence analysis corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) or the fluorescent dye C2 were utilised.

Antibody	Dilution	Origin
Goat anti-mouse-HRP	1 : 20,000	Sigma, Taufkirchen
Sheep anti-goat-HRP	1 : 10,000	Sigma. Taufkirchen
Goat anti-rabbit-HRP	1 : 40,000	BioRad, München
Goat anti-rabbit-Cy2	1 : 1000	Jackson ImmunoResearch Labs, USA
Goat anti-mouse-Cy2	1 : 1000	Jackson ImmunoResearch Labs, USA

2.1.10 Plasmids and Oligonucleotides

2.1.10.1 Primary Vectors

Vector	Description	Origin/ Reference
pcDNA3	Mammalian expression vector, Amp^r, Neo^r, CMV promotor, BGH poly A, high copy number plasmid, F1+ origin	Invitrogen, USA
pcDNA3-Fc	Modified pcDNA3 containing cds for Fc chain 5' of the multiple cloning site	C.Cant
pBlueScript KS+	Cloning vector, F1 origin, Amp^r	Stratagene, USA
pGEX5X1-3	Prokaryotic expression vectors for the generation of glutathione-S-transferase fusion proteins, Amp^r, IPTG inducible	Amersham Pharmacia, Freiburg
pLXSN	Expression vector for retroviral gene transfer, Amp^r, Neo^r, ori from pBR322, 5'-LTR and 3'-LTR from MoMuLV, SV40 promotor	Clontech, USA

pLXSN-ESK	pLXSN vector with modified multipe cloning site	J. Ruhe
pLXSN-EKS	pLXSN vector with modified multipe cloning site	J. Ruhe
pRK5	Expression vector, Amp^r, CMV promoter, SV40 poly A	Genentech
pRetroSUPER	SiRNA generating vector, H1 RNA promotor, pBabe backbone	(Brummelkamp et al., 2002a)

2.1.10.2 Constructs

Vector	Insert description	Reference
pcDNA3-HA-ERK5	cDNA of human ERK5, N-terminal HA-tag	this study
pcDNA3-HA-ERK5 AEF	cDNA of human ERK5, TEY218-220 to AEF, uncoupled from MEK5 N-terminal HA-tag	this study
pcDNA3-HA-ERK5 KM	cDNA of human ERK5, K83 to M, kinase dead, N-terminal HA-tag	this study
pcDNA3-HA-ERK5 713	cDNA of human ERK5, truncated at codon for aa 713, N-terminal HA-tag	this study
pcDNA3-HA-ERK5 575	cDNA of human ERK5, truncated at codon for aa 575, N-terminal HA-tag	this study
pcDNA3-HA-ERK5 464	cDNA of human ERK5, truncated at codon for aa 464, N-terminal HA-tag	this study
pcDNA3-HA-ERK5 409	cDNA of human ERK5, truncated at codon for aa 409, N-terminal HA-tag	this study
pcDNA3-HA-MEK5	cDNA of human MEK5, N-terminal HA-tag	this study,
pcDNA3-HA-MEK5 AA	cDNA of human MEK5, S311, T315 to AA,	this study,

	constitutively inactive N-terminal HA-tag	
pcDNA3-HA-MEK5 DD	cDNA of human MEK5, S311, T315 to DD, constitutively active N-terminal HA-tag	this study,
pcDNA3-HA-ERK2/5	cDNA of murine ERK2 fused via GG linker to C-terminus of human ERK5 aa 410-815	this study
pcDNA3-HA-ERK5-Fc	cDNA of human ERK5, C-terminal Fc chain, N-terminal HA tag	this study
pcDNA3-HA-ERK5 409-Fc	cDNA of human ERK5, truncated at codon for aa 409, C-terminal Fc chain, N-terminal HA tag	this study
pcDNA3-VSV-ERK5	cDNA of human ERK5, N-terminal VSV-tag	this study
pLXSN-HA-ERK5	cDNA of human ERK5, N-terminal HA-tag	this study
pLXSN-HA-ERK5 AEF	cDNA of human ERK5, TEY218-220 to AEF, inactivatable, N-terminal HA-tag	this study
pLXSN-HA-ERK5 KM	cDNA of human ERK5, K83 to M, kinase dead, N-terminal HA-tag	this study
pGEX5X3 ERK5 PR1	cDNA of GST and human ERK5 aa 410 - 558	this study
pGEX5X2 ERK5 CT	cDNA of GST and human ERK5 aa 710 - 815	this study
pRetroSUPER ERK5	19 bp of human ERK5 cDNA	this study
pGEX5X1 MEK5	cDNA of GST and human MEK5 complete cds	this study
pcDNA3-HA-ERK2	cDNA of mouse ERK2, N-terminal HA-tag	(Daub et al., 1997)
pcDNA3-HA-p38	cDNA of human p38,	this study

	N-terminal HA-tag	
pRK5-PTP-SL	cDNA of murine PTP-SL, cytosolic form, aa 147-549,	(Pulido et al., 1998)
pRK5-PTP-SL CS	cDNA of murine PTP-SL, cytosolic form, aa 147-549, catalytic C480 to S, inactive	(Pulido et al., 1998)
pGEX-PTP-SL	cDNA of GST and PTP-SL aa 147-549, cytosolic form	(Pulido et al., 1998)
pGEX-SL juxta 1	cDNA of GST and PTP-SL aa 147-288, juxtamembrane	(Pulido et al., 1998)
pGEX-SL juxta 2	cDNA of GST and PTP-SL aa 147-255, juxtamembrane	(Pulido et al., 1998)
pGEX-SL juxta 1 ΔKIM	cDNA of GST and PTP-SL aa 147-288, ΔKIM aa 224-239	(Pulido et al., 1998)
pGEX-SL PTP	cDNA of GST and PTP-SL aa 289-549, PTP domain	(Pulido et al., 1998)
pGEX-IA2-β	cDNA of GST and IA2-β fusion aa 641-1015, cytosolic domain	this study
pGEX-STEP	cDNA of GST and PTP STEP complete cds	(Pulido et al., 1998)
pGEX-NC-PTP	cDNA of GST and NC-PTP aa 245-670, cytosolic form	J. Eickhoff
pGEX-HePTP	cDNA of GST and He-PTP aa 1-339, complete cds	K. Spiekermann
pcDNA3-HA-PTP-SL	cDNA of PTP-SL WT, cytosolic form, N-terminal HA	M. Sommer
pcDNA3-HA-PTP-SL CS	cDNA of PTP-SL C480S, cytosolic form, N-terminal HA	M. Sommer
pcDNA3-GFP-PTP-SL	cDNA of PTP-SL WT, cytosolic form, N-terminal GFP	J. Eickhoff
pcDNA3-GFP-PTP-SL CS	cDNA of PTP-SL C480S, cytosolic form, N-terminal GFP	J. Eickhoff
pEGFP-C2	cDNA of the enhanced green	Clontech

	fluorescent protein (EGFP)	
pGL-3-CMV	cDNA of luciferase, CMV promotor driven	B. Biesinger
pRK5-c-Abl	cDNA of murine c-Abl IV	(Jackson et al., 1993)
pRK5-c-Abl KM	cDNA of murine c-Abl IV K271 to M, kinase dead	R. Herbst
pRK5-v-Abl	cDNA of p160 v-Abl from Abelson leukaemia virus	(Gishizky et al., 1995)
pLXSN-v-Abl	cDNA of v-Abl	this study
pRK5-Bcr/Abl	cDNA of p210 Bcr/Abl, human fusion product of Philadelphia chromosome translocation in chronic myeloid leukaemia	(Hallek et al., 1996)
pcDNA3-Tel/Abl	cDNA of p145 Tel/Abl, human fusion product of a chromosomal rearrangement in leukaemia	(Spiekermann, 2002)
pRK5-c-Src	cDNA of p60 c-Src	(Luttrell et al., 1997)
pRK5-Lck YF	cDNA of murine p56 Lck constitutively active variant	(Marth et al., 1988)
pRK5-Fyn	cDNA of p59 Fyn	(Margolis et al., 1992)

Names given as reference without further designation were members of this group.

2.1.10.3 Important Oligonucleotides

Sequence (description)	Name
CGG GAT CCG CCG AGC CTC TGA AGG AGG AAG (Cloning of ERK5 into pc-DNA3 with HA tag, forward)	ERK5s/Norb2
GCT CTA GAA CTG TGG CAG CAA GGC ACA GG (Cloning of ERK5 into pc-DNA3 with HA tag, reverse)	E5as/Norb1
AGA AGG CAC AGT CGA GGC TGA (PCR subcloning from pcDNA3, reverse)	pc rev
ACC CAC TGC TTA CTG GCT TAT CG	pc fwd

(PCR subcloning from pcDNA3, forward)

GCC AAG CGG ACC CTC AGG (sequencing primer)	ERK5 seq1
CTG TGA GCT CAA GAT TGG (sequencing primer)	ERK5 seq2
CGG GAT CCC CCT GTG GCT AGC CCT TGG CCC C (Cloning of MEK5 into pGEX5X1, forward)	GST-M5 s/Norb4
CCG CTC GAG CTG GGC TTT CAG TGC CCT GCG (Cloning of MEK5 into pGEX5X1, reverse)	GST-M5 as/Norb3
GCC GGA TCC CTG TGG CTA GCC CTT GGC CCC (Cloning of MEK5 into pcDNA3 with HA tag, forward)	HaMEK5 s
CCG TCT AGA CTG GGC TTT CAG TGC CCT GCG (Cloning of MEK5 into pcDNA3 with HA tag, reverse)	HaMEK5 as
GCC CAA GCT TCC ACC ATG TAC ACC GAC ATC GAG ATG AAC CGG CTG GGC AAG GGA TCC GCC GAG CCT CTG AAG GAG (Cloning of ERK5 and addition of N-terminal VSV-tag, fwd)	VSV-ERK5 s
CTA CTA GAT ATT ACA CTG G (Sequencing primer)	M5seq
AGC ATT AGG GAT CTT CAT GAT GGC CAC CTG CTG (Mutagenesis of K83 to M of ERK5)	E5KM
CGG TAC CAG CGC GTG GCC ACA AAC TCA GCC ATG AAG TAC TGA TGT TCA G (Mutagenesis of T218AY220 to AEF of ERK5)	E5AEF
GCA TTT GTT CCA ACA TAC GCC TTG GCT ATA GCA TTC ACC AGC TGA G (Mutagenesis of MEK5 S311 and T315 to AA)	M5AA
GCA TTT GTT CCA ACA TAA TCC TTG GCT ATA TCA TTC ACC AGC TGA GTG C (Mutagenesis of MEK5 S311 and T315 to DD)	M5DD
GCGCTC GAG GGG GTC CTG GAG GTC AGG CAG (Cloning of Fc tagged ERK5, reverse)	E5Fc
GCG CTC GAG ACT AGC CAC AGG CTG TAG AGA AG (Cloning of Fc tagged ERK5 409, reverse)	E5kinFc
GGG GAT CCT CTC AGG AGA GGC CCA CG (Cloning of human p38, forward)	p38fwd

GCT CTA GAA ACC AGG TGC TCA GG (Cloning of human p38, reverse)	p38rev
GGT CTA GAT CAC TGG GTC ACA AGG TTG ACA TCA (Truncation of ERK5 at codon for aa713)	E5-713
GGT CTA GAT CAG GCC ATT CGA GTC CAG CGT TCC (Truncation of ERK5 at codon for aa575)	E5-575
GGT CTA GAT CAT GGT GGG GCA GGC TCA CTG AC (Truncation of ERK5 at codon for aa464)	E5-464
GCC TCT AGA TCA ACT AGC CAC AGG CTG TAG AGA AGG CTG G (Truncation of ERK5 at codon for aa 409)	HaE5kin as
GGC TCG AGC CTG GCT GTC CAG ATG TTG (Cloning of ERK2/5 chimera)	E5-CT fwd
GGC TCG AGC CCC CCA GAT CTG TAT CCT GGC TGG AAT C (Cloning of ERK2/5 chimera)	E2-rev
GAT CCC CAG CTG CCC TGC TCA AGT CTT TCA AGA GAA GAC TTG AGC AGG GCA GCT TTT TTG GAA A (Cloning of ERK5-specific siRNA generating pRS)	E5 1435 fwd
AGC TTT TCC AAA AAA GCT GCC CTG CTC AAG TCT TCT CTT GAA AGA CTT GACG CAG GGC AGC TGG G (Cloning of ERK5-specific siRNA generating pRS)	E5 1435 rev

2.2 Methods in Molecular Biology

2.2.1 Plasmid Preparation for Analytical Purpose

Small amounts of plasmid DNA were prepared from 2 ml of *E.Coli* culture using the Qiagen Plasmid Mini Kit.

2.2.2 Preparative Scale Plasmid Preparation

If larger amounts of DNA were required, 100 –1000 ml of bacteria culture were inoculated and DNA was prepared with the Qiagen Plasmid Maxi Kit following the manufacturer's instructions.

2.2.3 Enzymatic Manipulation of DNA

2.2.3.1 Specific Digestion of DNA Samples by Restriction Endonucleases

DNA samples were incubated with one or more restriction endonucleases. The ratio of Enzyme/DNA, the temperature, the buffer and the time of incubation were adjusted to each application. Usually, incubations for 1 hour at 37°C with a calculated 5-fold overdigestion and the buffers as supplied by the manufacturers were chosen.

2.2.3.2 Dephosphorylation of DNA 5'-Termini

In order to prevent self-ligation of vector termini generated by restriction digest, 5´-termini of vector were dephosphorylated with Calf Intestine Alkaline Phosphatase (CIAP). This phosphatase removes 5´-phosphate residues from DNA as well as RNA.
For dephosphorylation, 1 µg of cut vector DNA was incubated with 5 units CIAP in adequate reaction buffer (e.g. 50 mM Tris/HCl pH 8.0, 0.1 mM EDTA pH 8.5) at 37°C for 10 minutes. Either reactions were stopped by heat inactivation at 85°C for 10 minutes or DNA was directly purified using the QIAquick PCR Purification Kit.

2.2.3.3 Phosphorylation of Primer

The phosphorylation of synthesised oligonucleotides was required for direct ligation into vectors as well as for primer extension. For these purposes 200 pmol oligonucleotide were incubated with 5 units T4 Polynucleotide Kinase, supplied buffer and 10 mM ATP for 30 minutes at 37°C. The reaction was stopped by heat inactivation for 10 minutes at 70°C.

2.2.3.4 Ligation of Vector and Insert DNA

Catalyzing the formation of a phosphodiester bond between adjacent 5'-phosphate and 3'-hydroxyl termini in duplex DNA, T4 DNA Ligase is able to join double-stranded DNA with cohesive or blunt ends. Purified, digested and dephosphorylated vector DNA (40 ng), the designated insert DNA, 1 µl 10x T4 DNA Ligase buffer (0.66 M Tris/HCl pH 7,5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP) and 1 unit T4 DNA Ligase were combined. A molar ratio between insert and vector of 3 to 1 was usually chosen. Reactions were either left on 14°C over night or at 37°C for 2 hours and subsequently transformed into competent bacteria.

2.2.3.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for the separation DNA fragments ranging from as small as 200 bp to as big as several tenth kb. Depending on the size of the fragments of interest 0.7-2% agarose gels were prepared in horizontal chambers. TAE buffer was used for the electrophoresis. Voltage was usually set to 4-10 V per cm width of the gel. After separation DNA fragments were stained by gently agitating gels in TAE containing 0.5 µg/ml ethidium bromide and were subsequently viewed under UV light.

2.2.3.6 Isolation of DNA Fragments from Agarose Gels

Following gel electrophoresis gel slices bearing DNA fragments of interest were cut out of the gel. Agarose was dissolved and DNA was purified using the QIAquick Gel Extraction Kit following Qiagen’s protocol.

2.2.4 Introduction of Plasmid DNA into *E.coli*

2.2.4.1 Preparation of Competent Cells

The preparation of competent cells was according to the procedure described by Chung and Miller (Chung and Miller, 1993). Competent cells were shock frozen in liquid nitrogen and stored for up to one year at –70°C. Transformation frequency ranged between 10^5 and 10^7 colonies/µg DNA.

2.2.4.2 Transformation of Competent Bacteria

A 50 µl aliquot of competent bacteria was added to a 50 µl mixture of DNA usually ligation cocktails, 10 µl 5x KCM solution (500 mM KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$) and water. After thoroughly mixing, samples were incubated on ice for 20 minutes, 10 minutes at room temperature and after addition of 300 µl LB broth at 37°C for 1 hour while constantly shaking. Bacteria were streaked out on appropriate agar plates containing Ampicillin for the selection of transformants.

2.2.5 Enzymatic Amplification of DNA by Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA. With the dawn of the era of PCR a pleithora of applications has been developed and nowaday includes many standard techniques for analytical purposes. For long and accurate cDNA amplification Pfu Polymerase (MBI Fermentas) was used:

1 µl	template cDNA, 1-10 ng
1 µl	"forward" oligonucleotide, 10 pmol/µl
1 µl	"reverse" oligonucleotide, 10 pmol/µl
2.5 µl	10x PCR buffer II containing 20 mM $MgCl_2$
2 µl	dNTP-Mix, 2.5 mM each
0.5 µl	Pfu DNA Polymerase (2.5 U/µl)
ad 25 µl	H_2O

PCR reactions were carried out using an automated thermal cycler („Progene“, Techne). The following standard protocol was adjusted to each specific application:

3 min	94°C	(initial denaturation)
30 cycles:		
1 min	94°C	(denaturation)
1 min	58°C	(hybridization)
1.5 min/ kb	72°C	(extension)
5 min	72°C	

PCR products were either separated by agarose gel electrophoresis, excised and subsequently purified or directly purified with QIAquick Gel Extraction or PCR Purification Kit, respectively.

2.2.6 Oligonucleotide-Directed Mutagenesis

For the analysis of DNA and protein function it is often desirable to specifically alter the DNA sequence and thus in case of open reading frames also the amino acid sequence of the encoded product. The desired mutations are introduced in oligonucleotides. Two methods are commonly used to convert these oligonucleotides carrying the mutation into biological active circular DNA. The protocol established by Kunkel et al. (Kunkel, 1985) requires an Uracil-containing single-stranded template while for a nowadays more often used protocol (Braman et al., 1996) any circular double-stranded DNA can serve as template.

2.2.6.1 Preparation of Uracil-Containing, Single-Stranded DNA Template

CJ236 bacteria were transformed with circular DNA containing a F1 origin. Here only pcDNA3 and pBlueScript based DNAs were used. Samples of 2 ml 2xYT-medium were inoculated with several colonies of transformed CJ236 and grown at 37°C. When media appeared slightly hazy indicating that early log-phase was reached, cultures were infected with $2x10^7$ M13K07 phages/ml (Amersham Pharmacia). After furher incubation for 90 minutes 70 µg/ml kanamycin was added. Cultures were kept vigorously shaking at 37 °C over night.
Supernatants were cleared from bacteria by two centrifugations (13000 rpm). After addition of 200 µl 2.5 M NaCl, 20% PEG 6000 and incubation for 15 minutes at room temperature, phages were collected by centrifugation. The phage sediment was resuspended in 100 µl TE10/0.1 buffer. After several phenol extractions (50 µl each) single stranded DNA was precipitated by addition of 10 µl 8 M LiCl and 250 µl pure ethanol to the aqueous phase over night at –20°C. After washing with 70 % ethanol single-stranded uracil-containing phage DNA was dissolved in 15 µl TE10/0.1. Quality and concentration was checked by agarose gel electrophoresis and spectrophotometrically at 260 nm.

2.2.6.2 Primer Extension

The single-stranded uracil-containing DNA was used as a template for the generation of mutation carrying strand. 200 ng single-stranded template DNA, 2 pmol phosphorylated oligonucleotide, 1 µl 10x hybridization buffer (20 mM Tris/HCl pH 7,4, 2 mM $MgCl_2$, 50 mM NaCl) in a total volume of 10 µl were incubated for 2 min at 90°C and allowed to cool down to room temperature. The mixture was supplemented with 1 µl 10x synthesis buffer (5 mM dNTP-mix, 100 mM Tris/HCl pH 7.5, 50 mM $MgCl_2$ and 20 mM DTT), 5 units T4 DNA Ligase, 1 µg T4 Gene 32 Protein and 3 units T4 DNA Polymerase. Reactions were incubated for 5 min on ice, 5 min at 25 °C and then for 90 min at 37°C. Finally 66 µl TE were added to reactions and aliquots containing approximately 100 ng of double-stranded DNA products were used for transformation of *E.Coli*. DNA was prepared from randomly chosen clones analysed by restriction digest and sequencing. Oligonucleotides were designed to contain an additional restriction side compared to the wild type sequence in order to facilitate the screening of larger numbers of clones.

2.2.6.3 Site-directed Mutagenesis Using Double-stranded Template DNA

The major advantage of this protocol (also known as Stratagene's QuikChange Protocol) is that any double stranded DNA without any further requirements such as helper phage origins

can be used as template. Two complement oligonucleotides bearing the desired alterations are used in a PCR-like reaction. Differing from the above PCR protocol 5 ng template DNA and 2.5 units Pfu Polymerase were used. A few microliter of product after 12 to 18 cycles were checked by agarose gel electrophoresis. Subsequently, methylated template DNA was digested with methyl-sensitive restriction endonuclease Dpn 1. The persisting mutant DNA was used for transformation of competent bacteria. Clones were analysed as described above.

2.2.7 DNA Sequencing

Sequencing of DNA was performed following the “Big Dye Terminator Cycle Sequencing Protocol” (ABI). The following mix was subjected to a sequencing-PCR run:

	250	ng	DNA of interest
	10	pmol	sequencing primer
	4	µl	Terminator Ready Reaction Mix
	ad	20 µl	H_2O
25 cycles:	30	sec	94°C
	15	sec	45-60°C
	4	min	60°C

Sequencing reactions were diluted with 80 µl water and precipitated with 0.1 volume 3 M sodium acetate pH 4.8 and 3 volumes ethanol. Pellets were dissolved in 20 µl template suppression reagent, briefly boiled and analysed on a 310-Genetic Analyzer (ABI Prism).

2.3 Methods in Mammalian Cell Culture

2.3.1 General Cell Culture Techniques

All cell lines were grown in a humidified 95% air and 5 % CO_2 incubator (Heraeus, B5060 Ek/CO_2) at 37°C and routinely assayed for mycoplasma contamination using a bisbenzimide-staining kit (Sigma). Cells were splitted every 2 – 4 days and were counted with a Coulter Counter (Coulter Electronics) before seeding. Adherent cells were splitted by trypsinization and reseeding, while suspension cells were directly diluted into fresh media. Generally 15 cm dishes were used for maintenance culture.
The most often used cell lines were cultivated as follows: HEK 293T and COS-7 cells were grown in in Dulbecco's modified Eagle's medium (DMEM) and 10% FCS, while the medium for Swiss 3T3 cells was supplemented with 10% bovine calf serum. The rat pheochromocytoma cell line PC12 was cultivated with DMEM but containing 5 % FCS and 10 % horse serum. Plastic dishes for PC12 cells were coated with collagen (Sigma).
The leukaemic suspension cell lines K562, Ku 812, B-173, EM-2 and MEG-01 were grown in RPMI 1640 supplemented with 10-20 % FCS as recommended by DSMZ. All other cell lines used were cultivated according to the supplier’s instructions.

2.3.2 Transfection of Cultured Cell Lines

2.3.2.1 Calcium Phosphate Precipitation Method

4 hours prior transient transfection 600.000 HEK-293T cells were seeded in six-well dishes. Usually 1 to 4 µg DNA was transfected by using a modified calcium phosphate precipitation method as described by Chen and Okayama (Chen and Okayama, 1987). In this protocol, the capacity of cells to incorporate calcium phosphate-DNA complexes by a hitherto unknown mechanism is used for the transfer of genes in adequate expression vectors. Mostly, CMV promotor driven expression constructs were used. The calcium phosphate-DNA complexes were formed in water by mixing the components as follows:

dish	***6-well***	***6 cm***	***10 cm***
area	10 cm^2	21 cm^2	57 cm^2
Volume of medium	1 ml	2 ml	4 ml
DNA in H_2O_{bidest}	2 µg in 90 µl	5 µg in 180 µl	10 µg in 360 µl
2.5 M $CaCl_2$	10 µl	20 µl	40 µl
2 x BBS (pH 6.96)	100 µl	200 µl	400 µl
Total volume	200 µl	400 µl	800 µl

Formation of precipiates was induced by addition of the adequate volume of 2x BBS to the DNA and $CaCl_2$ containing aqueous solution. After throughly mixing reactions were incubated for 10 min at room temperature and then added to the cells. If required cells were starved on the day following transfection by serum withdrawal. Expression was occasionally analysed as early as 16 hours but usually 48 hours after transfection.

2.3.2.2 Transfection of COS-7 Cells Using Polyfect®

COS-7 cells were transiently transfected using Polyfect® (Qiagen) essentially following Qiagen's protocol. In brief, 160.00 cells were seeded in 6-well dishes 24 hours prior gene transfer. For transfection 1.5 µg DNA and 10 µl Polyfect were mixed and incubated in 100 µl serum-free and antibiotic-free medium for 10 minutes at room temperature. Reactions were diluted with 500 µl fully supplemented medium and added to the cells which were in 1.5 ml fresh medium. After 24 hours cells were starved by serum deprivation if assays requiring cell stimulation were to be performed.

2.3.2.3 Transfection of Rat-1 Cells Using Superfect®

Rat-1 cells were transfected with Superfect® (Qiagen). The day after seeding of 100.000 cells per 6-well dish, the medium was changed. Comparably to the Polyfect protocol 1 µg DNA and 2 µl Superfect were mixed in serum and antibiotics-free medium. Transfectable lipid-DNA complexes were formed during 15 minutes incubation at room temperature. The transfection mix was added to the cells, which were assayed after 48 hours for exogenous expression. For the generation of stably transfected Rat-1 lines cells were selected with 1 mg/ml G418 for 14 days.

2.3.2.4 Determination of Transfection Efficiency

Tranfection effciency was determined using reporter plasmids, such as CMV promotor driven GFP and Luciferase expression constructs. While GFP expression was easily monitored by fluorescence microscopy, relative luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega).

2.3.3 Retroviral Gene Transfer into Cultured Cells

High titer retrovirus was prepared as described previously (Pear et al., 1993). The packaging cell lines Phoenix A and Phoenix E were transfected with pLXSN or pRetroSUPER based expression vectors using calcium phosphate precipitation method. To enhance transfection efficiency cells were given 100 µM chloroquin. Target cell lines were seeded in 10 cm plates. After 48 hours supernatants of transfected Phoenix cells were collected, supplemented with 8 µg/ml polybrene and filtered through a 0.45 µm filter. Usually two donor plates of Phoenix cells were used per plate of target cells and viral supernatants were alternatingly collected every two hours. Acceptor cells were incubated with the viral supernatants three to four times for 2 h. Since virus titers are best after 4 hours but infection is almost complete after 1 hour of incubation with target cells, this modification of the standard protocol was used to reach a high multiplicity of infection. Retroviral supernatant was then replaced with fresh medium. Two days following infection, target protein expression was monitored by immunoblot. Cells were usually selected using either 2 µg/ml puromycin or 1 mg/ml G418 for 3 and 14 days, respectively.

2.4 Protein Analytical Methods

2.4.1 Preparation of Crude Cell Lysates

Cells were washed with PBS and lysed on ice by addition of ice-cold lysis buffer (50 mM HEPES pH 7,5, 150 mM NaCl, 1 mM EDTA, 10% glycerine and 1 % TritonX-100) supplemented with phosphatase and protease inhibitors (10 mM $Na_4P_2O_7$, 1 mM PMSF, 1 mM orthovanadate, 1 mM NaF and 0,5% aprotinin). Cellular debris were removed by centrifugation.

2.4.2 Determination of Total Protein Concentration in Lysates

The overall protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce, Sankt Augustin) and the supplied standard protocol.

2.4.3 Immunoprecipitation and *in vitro* Binding of Proteins

Lysates were pre-cleared with 20 µl sepharose slurry. For immunoprecipitations adequate amounts of antibodies were added together with 20 µl of mixed protein A and G sepharose

and one volume of HNTG. Fc-tagged proteins were directly precipitated with the mix of protein A and G sepharose.For *in vitro* binding studies, 1 µg of each Fc and GST fusion protein were incubated in 250 µl PBS (8 mM Na_2HPO_4, 1,5 mM KH_2PO_4, 137 mM NaCl, 2,7 mM KCl, pH 7,3) for 20 minutes at room temperature under constant shaking. After addition of 0.3 volumes of HNTG, samples were pre-cleared and finally precipitated with 20 µl GSH sepharose beads. In general, precipitation samples were incubated for at least 3 h on a rotation wheel at 4°C. Precipitates were washed three times with 0.5 ml HNTG buffer and were finally suspended in 2x Laemmli's buffer, boiled and processed to SDS-PAGE.

2.4.4 SDS-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE)

Separation of denatured proteins was accomplished using SDS-PAGE essentially as described by Sambrook et al (Sambrook et al., 1990). Depending on the size of proteins of interest gels with polyacrylamide content ranging from 7.5 – 15 % were prepared. The following proteins were used as molecular weight standards:

Protein	***MW (kDa)***	***Protein***	***MW (kDa)***
Myosin	205	Ovalbumin	29
ß-Galaktosidase	116	Carboanhydrase	43
Phosphorylase b	97	Trypsin-Inhibitor	21.5
BSA	66	Lysozym	14.4

2.4.5 Protein Transfer onto Nitrocellulose Membranes

For Western blot or immunoblot analysis proteins were transferred onto nitrocellulose membranes after separation by SDS-PAGE (Gershoni and Palade, 1982; Gershoni and Palade, 1983). Transfer was accomplished with Transblot-SD buffer in a 'Semidry'-blotting chamber (in house manufactured) applying 0.8 mA/cm^2 for 3 hours. Immobilised proteins on the nitrocellulose membrane were visualised with Ponceau S stain (2 g/l in 2% aqueous TCA solution) and bands of standard proteins were marked. Membranes were easily destained in water.

2.4.6 Immunoblot Detection

Membranes with bound proteins can easily serve as platform for immuno reactions. NET supplemented with 0.25% gelatine served as buffer throughout the whole process and was first used to block the remaining binding sites of the positively charged nitrocellulose membranes. Membranes were probed with adequate primary antibody directed against the protein of interest typically over night. Common dilutions for primary antibodies were around 1 to 1000. Membranes were then washed three times for 5 minutes and the Fc chain of bound primary antibody was detected by the appropriate secondary antibody coupled to horseradish peroxidase. After unbound antibody had been removed by washing three times for 10 minutes positive signals were detected by chemoluminescence using the ECL Kit (PerkinElmer/NEN). Membranes were either exposed to X-ray films or if quantification was necessary, filters were exposed to the LAS1000 chemiluminescence camera (Fujifilm) and analysed with the program Image Gauge 3.3 (Fujifilm). In the case the same membrane had to be used in a

second Western blot experiment, bound antibodies were removed by incubation with strip-buffer for 1 hour at 50°C. Then, membranes were thoroughly washed with water, blocked and the whole process was repeated.

2.4.7 Preparation of GST-fusion Proteins

The *E.Coli* strain BL21 Codon+ was used for the expression of proteins that do not require eukaryont-specific modifications. Bacteria were transformed with pGEX based expression contructs and first grown as 1 ml culture over night. Then 100 ml LB medium were inoculated with 0.1 ml of the bacteria culture and incubated under vigorous shaking for 8h hours. After addition of 900 ml LB medium containing 0.2 mM IPTG bacteria were grown over night at room temperature. Cells were collected by centrifugation, resuspended in PBS supplemented with 0.5 mM EDTA, 1 mM PMSF, 0.5 mM DTT and 10 µg/ml Aprotinin and lysed by sonication. The detergent Triton-X100 was added to a final concentration of 1% to avoid the formation of protein coagulation. The lysate was then incubated with 10 µg/ml DNAse 1 for 10 minutes at room temperature in order to degrade bacterial DNA. Lysates were then cleared by centrifugation and the desired fusion protein was bound to GSH-sepharose during 30 minutes of incubation at room temperature. Beads were transferred to Poly Prep® Chromatography columns and washed with PBS. GST fusion proteins were eluted with 50 mM Tris, pH 8.8 containing 15 mM GSH and 20 mM DTT. Positive fractions were identified by SDS-PAGE and Coomassie G250 stain. For long term storage, purified proteins were dialysed into PBS/ 30 % glycerol and kept at –70°C.

2.4.8 Purification of Fc-tagged Proteins

Transfected HEK 293T cells were collected and lysed. Fc-tagged proteins were precipitated with protein A sepharose at 4°C for two hours. Beads were transferred to Poly Prep® Chromatography columns and washed with PBS. Elution was accomplished with 1 ml aliquots of 100 mM glycine, 150 ml NaCl, pH 2.5. Elutes were collected in vials containing 20 µl 2 M Tris, pH 8.8 for neutralisation. Positive fractions were pooled and dialysed into PBS with 30% glycerol.

2.5 Biochemical and Cell Biological Assays

2.5.1 Stimulation of Cells

Cells were seeded in cell culture dishes of appropriate size and grown over night to about 80% confluence. After serum withdrawal for 24 hours cells were stimulated with various agonists as indicated in the legends. If inhibitors such as Imatinib were used to reduce signalling activity, cells were kept under optimal growing conditions. Stimulations were stopped by washing cells with PBS and immediate lysis.

2.5.2 MAPK Phosphorylation

MAPK are generally activated by phopshorylation of threonine and tyrosine residues in their activation motif. Therefore phosphorylation state-specific antibodies can be used to monitor the activity of MAPKs by Immunblot analysis. Crude cell lysates were mixed with half a volume 3x Laemmli's buffer and separated by SDS-PAGE. After transfer to nitrocellulose membranes rabbit polyclonal anti-Phospho-ERK2, Phospho-ERK5, Phospho-p38 and Phospho-JNK were used as primary antibodies. Membranes were usually reprobed with rabbit polyclonal anti-ERK2, anti-ERK5, anti-p38 and anti-JNK antibodies. In the case of ERK5, anti-ERK5 immunoprecipitates were used instead of crude cell lysates.

2.5.3 Kinase Assay

The activity of isolated usually immunprecipitated kinases can be measured *in vitro* using radioactive [γ-^{32}P]ATP and autophosphorylation and/or substrate phosphorylation as read out. For measuring ERK5 activity, exogenous HA-tagged ERK5 was immunoprecipitated from transfected COS-7 cells. Precipiates were washed twice with HNTG and twice with kinase assay buffer (20 mM HEPES pH7,5, 10 mM $MgCl_2$, 1 mM DTT and 0,5 mM orthovanadate). Samples were suspended in 30 µl kinase assay buffer supplemented with 50 µM ATP and 2 µCi [γ-^{32}P]ATP and incubated for 20 minutes at 30°C under constant shaking. For the measurement of additional substrate phosphorylation reactions were also supplemented with 1 µg of GST fusion proteins or 10 µg myelin basic protein. The reaction was extended to 30 minutes if 1 µg purified ERK5 protein was used instead of immunoprecipitated kinase. Assays were stopped by addition of 2x Laemmli's buffer and boiling. Samples were resolved on SDS-PAGE and transferred to nitrocellulose. Phosphorylation was detected by Phospho-Imaging using the BAS2500 Reader (Fujifilm) and quantified with Image Gauge 3.3 (Fujifilm). The amount of precipitated kinase was visualised by immunoblot analysis.

2.5.4 Phosphatase Assay

The easiest way to gain some insight into the activity of tyrosine-specific phosphatases is the use of para-nitrophenyl-phosphate (pNPP) as widely accepted and colorimetrically measurable substrate. For the measurement of PTP-SL activity, 1 µg of purified PTP-SL protein was diluted into 20 µl PBS and incubated with 1 µg of different proteins for 10 minutes at room temperature under constant shaking. Two 10 µl aliquots were then added to 100 µl pNPP buffer (25 mM HEPES pH 7,5, 1 mM DTT, 1 mM EDTA) containing 3,7 mg/ml pNPP. After 2 hours at 37°C, the absorption at 405 nm was determined. To analyse the influence of PTP-SL on the activity of ERK5 coupled *in vitro* phosphatase-kinase assays were performed. ERK5 precipitates were washed twice with HNTG and twice with PTP assay buffer (25 mM HEPES pH 7,3, 10 mM DTT, 5 mM EDTA). Samples were resuspended in 20 µl assay buffer containing the indicated amount of GST-PTP-SL fusion protein, and were incubated for 20 minutes at 30°C while shaking. As these reaction were followed by *in vitro* kinase assays they were stopped by washing with kinase assay buffer containing the phosphatase inhibitor orthovanadate. The kinase assay was carried out as described above.

2.5.5 Shift Analysis of Protein Phosphorylation

The addition of negatively charged phopshoryl groups to proteins reduces its mobility in SDS-PAGE. Hence, observed shifts can be used to deduce some information on the phopshorylation state of a protein. But since mobility shifts are not exclusively caused by phosphorylations, unspecific Calf Intestine Alkaline Phospatase (CIAP) can be used to remove any phosphoryl group and to clarify whether a certain shift is evoked by phosphorylation or not. The protein of interest was immunprecipitated and washed twice with HNTG and twice with CIAP buffer (50 mM Tris/HCl pH 8.0, 0.1 mM EDTA pH 8.5). Beads were resuspended in 20 µl CIAP buffer containing 20 units CIAP. Reactions were stopped after 20 minutes at 37°C by addition of 2x Laemmli's buffer.

2.5.6 Analysis of Subcellular Protein Localisation

Cells were seeded at 2 x 10^4 cells/cm^2 on glass cover slips and if required transfected as described above. Cells were processed for immunofluorescence after 24 hours of further culture. Cells were washed twice with PBS and fixed with methanol at –20°C for 5 min, rinsed once with –20°C cold acetone and washed twice with PBS. Alternatively, cells were fixed with PBS, 4 % formaldehyde, 0.125 M sucrose for 10 minutes at room temperature and subsequently permeabilised with ice-cold 0.2% TritonX-100 in PBS for 10 minutes.
All further steps were carried out at room temperature. Samples were incubated in PBG (PBS containing 0.5% BSA and 0.045% Teleostean Fish Gelatin) supplemented with 5% normal goat serum for 1 hour, washed twice with PBG and incubated with a 1:1000 dilution of anti ERK5 or anti-HA antibody for 1 hour. After three more washes with PBG, cells were incubated with the appropriate secondary Cy2-labelled antibody in a 1:1000 dilution for 1 h. After one more PBG and three more PBS washes, samples were rinsed in destilled water and mounted. DNA was stained for 10 min with 1 µg/ml bisbenzimid which was included in the penultimate washing step.

2.5.7 Analysis of Nuclear Proteins

Cells were trypsinated and collected by centrifugation at 500 × g. After washing twice with PBS cells were lysed in hypotonic lysis buffer (10 mM Tris pH 7,5, 10 mM NaCl, 3 mM $MgCl_2$, 0,5 % NP-40) on ice for 5 minutes. Nuclei were precipitated by centrifugation at 500 × g, washed once with hypotonic lysis buffer and finally dissolved in Laemmli buffer. The presence of a certain protein was tested by Immunoblot analysis. Since RanGAP exists in a nuclear 90 KDa and a cytosolic faster migrating form, anti-RanGAP (kindly provided by Frauke Melchior, Martinsried) immunoblotting was carried out to control equal loading and potential cytosolic contaminations.

2.5.8 Cell Cycle Analysis and Cell Survival

Leukaemic suspension cells were treated with various agents as indicated in the legends. Cells were collected by centrifugation and lysed in hypotonic buffer containing 0.1% sodium acetate, 0.1% Triton X-100 and 20 µg/ml propidiumiodide for 2 h on ice. Samples were

analysed on a Becton Dickinson FACScalibur flow cytometer. Cells with sub-G1 DNA contents were considered as dead and substracted to get the percentage of cell survival.

2.5.9 Growth Assay

Leukaemic suspension cells were seeded in duplicate at 200.000/ml in a total volume of 4 ml and were treated as indicated in respective figure legends. Every 24 or 48 hours cells were thoroughly mixed and aliquots were counted.

2.6 Statistical Analysis

Student's *t*-test was used to compare data between two groups. Values are expressed as mean ± standard deviation of at least three independent experiments. $P < 0.05$ was considered statistically significant.

3 RESULTS

3.1 Tyrosine-specific Phosphatase PTP-SL regulates ERK5 Signalling in multiple Ways

MAPKs including ERK5 are generally activated by phosphorylation of threonine and tyrosine residues in their activation motif, however, dephosphorylation of either residue is sufficient for kinase inactivation (Canagarajah et al., 1997). This part of the study concentrates on the question of whether and how PTP-SL might be involved in the downregulation of the big MAP kinase 1/ ERK5 signal. PTP-SL (Hendriks et al., 1995; Ogata et al., 1995; Sharma and Lombroso, 1995; Shiozuka et al., 1995), like STEP (Lombroso et al., 1991) and HePTP (Zanke et al., 1994), belongs to the kinase interacting motif (KIM)-containing phosphatases that have previously been shown to bind, dephosphorylate and thereby inactivate signalling by ERK1/2 (Pulido et al., 1998; Saxena et al., 1999a; Saxena et al., 1998). KIM-containing PTPs are characterised by a very restricted expression pattern. They are not found in the nucleus and are generally considered to play a role in the short-term inactivation of MAPKs (Saxena and Mustelin, 2000).

3.1.2 PTP-SL binds to ERK5

To address the relevance of PTP-SL function in the regulation of MAPK cascades, the potential interaction between ERK5 and the cytosolic form of the STEP-like phosphatase was investigated. Co-precipitation experiments with polyclonal anti-PTP-SL antibody and lysates from transfected human embryonic kidney (HEK) 293 cells were performed. Figure 8*A* (*first panel*) shows PTP-SL association with hemagglutinin-tagged ERK5 (HA-ERK5) in those cells co-overexpressing both proteins.

As can be further seen in the *second panel* of Fig. 8*A*, in addition to HA-ERK5 also endogenous ERK1/2 was detected in the immunoprecipitates in the presence but not the absence of overexpressed PTP-SL. It is conceivable that ERK1/2 might compete with ERK5 for PTP binding. As expected from earlier studies describing the expression of PTP-SL predominantly in cell lines of neuroendocrine origin (Augustine et al., 2000; Ogata et al., 1995; Shiozuka et al., 1995), we did not detect endogenous PTP-SL in HEK 293 cell lysates (Fig.8*A*, *bottom panel*).

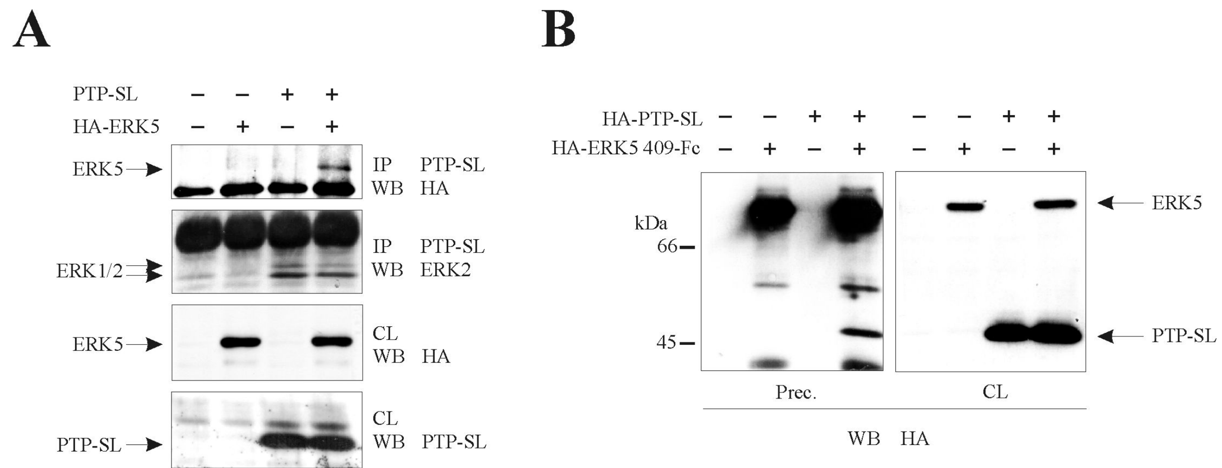

Figure 8: **Co-immunoprecipitation of ERK5 and PTP-SL in transfected HEK 293 cells**. Cells were transfected with expression constructs of ERK5 and PTP-SL or the empty vectors and lysed after 48 hours. *A*, pcDNA3 HA-ERK5 and pRK5 PTP-SL were transfected. PTP-SL immunoprecipitations (IP) and crude lysates (CL) were resolved by SDS-PAGE, followed by Western blot (WB) analysis using anti-HA, anti-ERK2 and anti-PTP-SL antibodies. The unspecific band migrating below ERK5 is due to the combination of antibodies used for precipitation and immunoblot (*top panel*). *B*, pRK5 HA-PTP-SL and pcDNA3 HA-ERK5 409-Fc were transfected. Protein A/G sepharose was used to precipitate Fc-tagged ERK5 409 (aa 1-409). Precipitates and crude lysates were immunoblotted with anti-HA antibody.

The interaction of ERK5 and PTP-SL was further demonstrated by performing the converse experiment as shown in Fig. 8*B*. When a truncated version of HA-ERK5 (HA-ERK5 409), that contained only the kinase domain but lacked the unique carboxy-terminal tail, was co-expressed with HA-tagged PTP-SL (HA-PTP-SL), we were readily able to pull down PTP-SL by precipitating ERK5 409 (Fig. 8*B*, *lane 4*). Addition of the IgG-Fc portion to the carboxy-terminus of the ERK5 409 construct was necessary to visualise the bound phosphatase, which would otherwise have been masked by the heavy chain of the precipitating antibody.

Having established that both proteins were able to co-immunoprecipitate with each other, the questions whether the proteins interact directly was addressed by performing *in vitro* binding experiments. Both proteins were tagged and purified. While a fusion protein of PTP-SL and glutathione S transferase (GST) was bacterially expressed, Fc-tagged ERK5 was isolated from lysates of transfected HEK 293 cells. Correct folding of the proteins was verified by testing their phosphatase or kinase activities (data not shown). Figure 9*A* shows that full length ERK5 as well as the truncated ERK5 409 protein directly interacted with both wild-type and catalytically inactive PTP-SL. Tanoue et al. and Tarrega et al. identified docking motifs in ERK2 that mediate interaction with substrates and regulators including phosphatases (Tanoue et al., 2000; Tanoue et al., 2001; Tarrega et al., 2002). These docking motifs are also present and conserved in the ERK5 kinase domain and thus are likely to mediate the binding to PTP-SL.

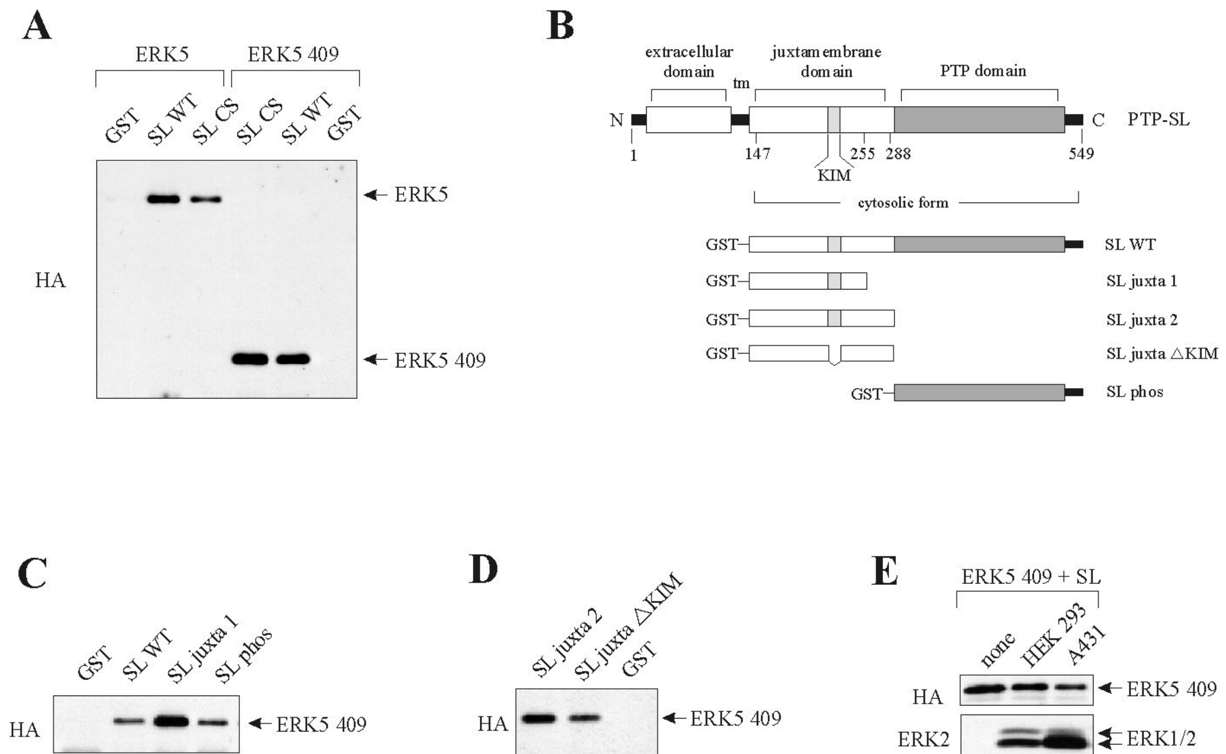

Figure 9: **Direct binding of ERK5 and PTP-SL.** Purified Fc- and HA-tagged ERK5 and ERK5 409 were incubated with fusion proteins of GST and PTP-SL (SL). After precipitation with GSH sepharose, samples were analysed by HA immunoblot. *A*, HA tagged ERK5 and ERK5 409 were incubated with PTP-SL WT or PTP-SL CS. *B*, schematic presentation of PTP-SL and used fusion proteins. Numbering of amino acids refers to the transmembrane form of PTP-SL and is according to Hendriks et al. (Hendriks et al., 1995). Kinase interacting motif (KIM) contains aa 224-239. *C* and *D*, ERK5 409 was precipitated with fusion proteins of GST and different domains of PTP-SL. *E*, ERK5 409 and PTP-SL WT were incubated in the presence of crude lysates (CL) of A431 and HEK 293T cells. Precipitates were additionally immunoblotted for ERK2 (*bottom panel)*.

In turn, to identify the domains of PTP-SL, which are involved and responsible for binding to ERK5, GST-fusion proteins that contained different portions of PTP-SL were prepared and tested for their ability to bind ERK5 409 (Fig. 9*B*). As shown in Figure 9*C*, not only the full cytosolic form of PTP-SL but also the juxtamembrane and the phosphatase domain alone were both interacting with ERK5. Interestingly, even the PTP-SL juxtamembrane construct lacking the KIM, a motif that was shown to mediate the interaction with ERK2 (Pulido et al., 1998; Saxena et al., 1999a; Saxena et al., 1998), was still able to bind to ERK5 (Fig. 9*D*). In further binding experiments crude lysates of HEK 293 and A 431 cells were used as source for proteins that might possibly compete for PTP binding sites. In the presence of these lysates associated ERK5 decreased to a degree that correlated with the amount of endogenous ERK1/2 interacting with PTP-SL (Fig. 9*E*)

Taken together, this set of data shows that the interaction between ERK5 and PTP-SL is direct and involves the kinase domain of ERK5 and – even though not exclusively – the KIM containing juxtamembrane region of PTP-SL.

3.1.2 ERK5 phosphorylates PTP-SL *in vitro*

To examine whether PTP-SL itself could serve as ERK5 substrate, *in vitro* kinase assays were performed with several GST fusion proteins containing different portions of PTP-SL as potential substrates. Activated ERK5 was immunoprecipitated from EGF stimulated COS-7 cells. As shown in Figure 10*A* wild type PTP-SL as well as those proteins containing the juxtamembrane domain was readily phosphorylated by ERK5. On the other hand, the phosphatase domain alone did not serve as a substrate. The identification of the juxtamembrane domain as site of phophorylation alludes to threonine 253 as modified amino acid in view of that it is also phosphorylated by ERK2.

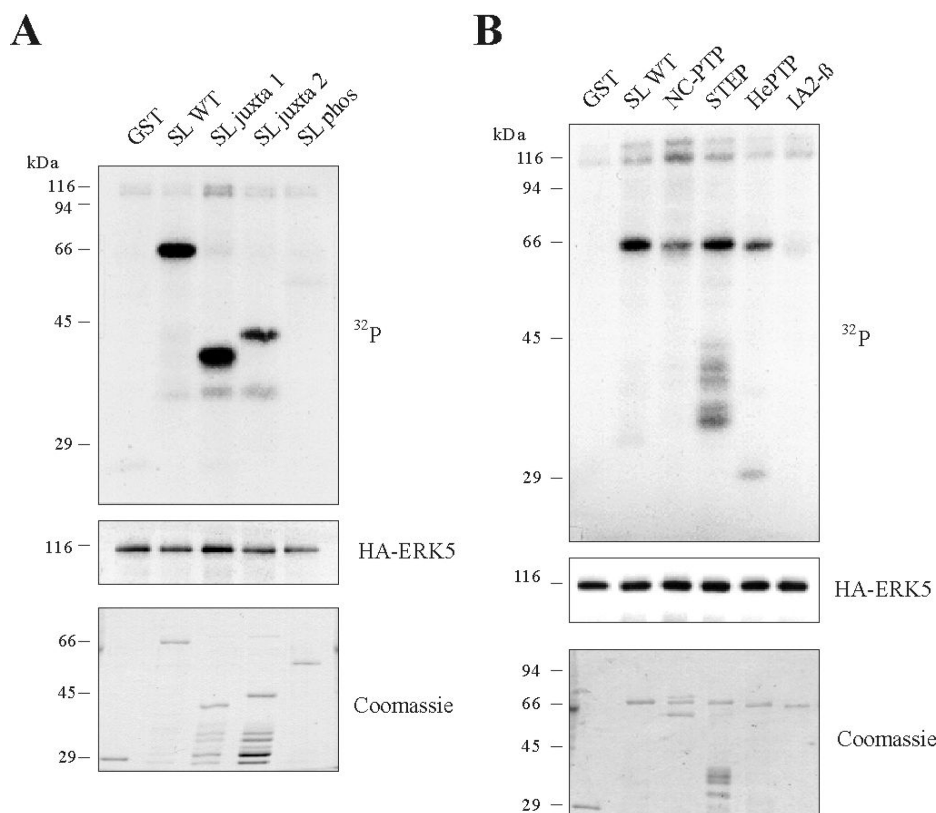

Figure 10: **PTP-SL is an *in vitro* substrate for ERK5**. HA-ERK5 was immunoprecipitated from lysates of transfected COS-7 cells that were stimulated with 15 ng/ml EGF for 5 min. Samples were subjected to kinase assays using 1 µg of different GST fusion proteins as substrate. After separation and transfer to a nitrocellulose membrane, samples were analysed by autoradiography and HA immunoblot. *A*, kinase substrates were GST-PTP-SL (SL) WT (aa 147-549), SL juxta 1 (aa 147-255), SL juxta 2 (aa 147 – 288) and SL phos (aa 256 – 547). *B*, GST fusion proteins of the full cytosolic forms of PTP-SL, NC-PTP, STEP, HePTP and the unrelated IA2-β were used in the kinase assay as in *A*. Amount and size of the used fusion proteins are shown in replica gels stained with Coomassie G250 (*A* and *B*, *bottom panels*).

Then the capability of ERK5 to phosphorylate additional KIM containing PTPs like NC-PTP, STEP (Lombroso et al., 1991) and HePTP (Zanke et al., 1992) was tested. According to the design of the GST-PTP-SL protein, the complete cytosolic forms of the phosphatases were

fused to GST. The resulting fusion proteins were of comparable size and migrated around 65 kDa on SDS-PAGE. All KIM containing phosphatases were phosphorylated in *vitro*, however, the cytosolic domain of the unrelated PTP IA2-β (Kawasaki et al., 1996) was – as expected – not modified by ERK5 (Fig. 10*B*).

3.1.3 Binding of ERK5 enhances the Activity of PTP-SL

Whether complex formation between ERK5 and PTP-SL would alter the enzymatic activity of the phosphatase was tested by measuring the activity of the bacterially expressed PTP-SL fusion protein upon binding to ERK5. As shown in Figure 11*A*, ERK5 as well as truncated ERK5 409 enhanced PTP-SL activity approximately 3.5-fold, whereas two control proteins namely GST and IgG had no effect. Although a similar increase in its activity was shown for the dual specific phosphatase MKP-3 in complex with ERK2 (Camps et al., 1998), the demonstrated influence of ERK5 binding on PTP-SL activity is the first ever observed for a PTP in association with a member of the MAPK family.

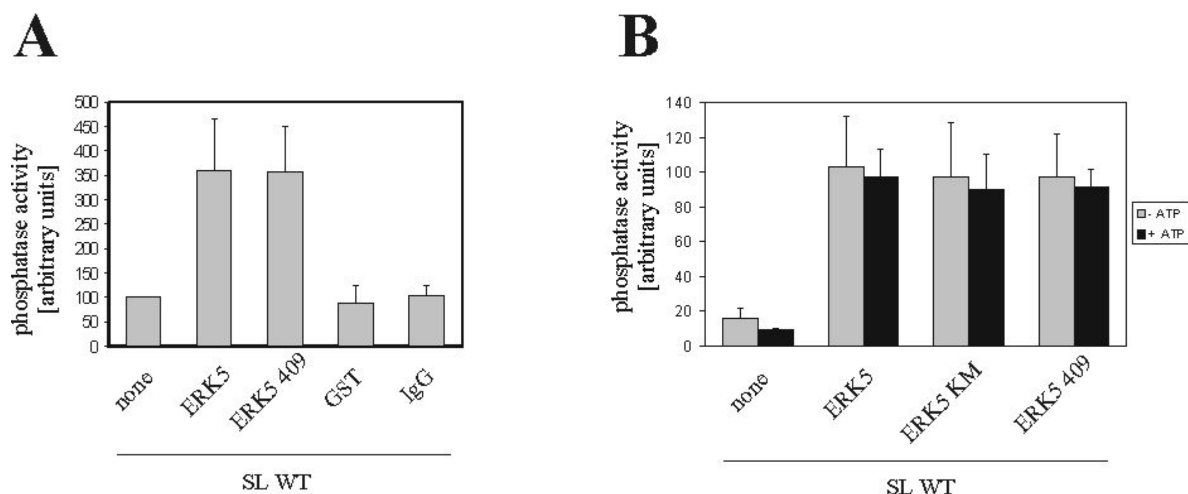

Figure 11: **Binding of ERK5 enhances the activity of PTP-SL.** *A*, 1 µg GST-PTP-SL (SL) WT was incubated with 1 µg Fc-tagged ERK5, ERK5kin or other proteins as indicated for 10 min at room temperature. Subsequently, PTP activity was measured with pNPP as substrate. Data represent the mean of three independent experiments ± SD. The activity of SL WT alone was set as 100% reference. *B*, *in vitro* kinase reactions in the presence and absence of ATP were performed with 1 µg each of purified Fc-tagged ERK5, ERK5 409 and SL WT. After 30 min at 30°C PTP activity was quantified in pNPP assays. Values are the mean of two separate experiments ± SD. PTP activity was generally lower under these conditions compared to the data presented in *A*.

Since PTP-SL was phosphorylated – although not quantitatively - by a preparation of ERK5 protein isolated from transfected HEK 293 cells (data not shown), we tested the influence of this phosphorylation on PTP-SL activity by performing *in vitro* kinase reactions in the presence and absence of ATP and subsequently quantified the resulting phosphatase activity.

As shown in Fig. 4*B* PTP-SL activity again was strongly enhanced in the presence of either wild-type ERK5, ERK5 409 or the kinase inactive mutant ERK5 KM, but was merely slightly decreased by addition of ATP in all cases. These results indicate that binding to – but not phosphorylation by – ERK5 makes a major impact on PTP-SL activity.

3.1.4 PTP-SL modulates ERK5 Activity *in vitro* and in intact Cells

To answer the question whether PTP-SL might regulate ERK5 activity, the influence of PTP-SL on ERK5 kinase activity was analysed. In vitro phosphatase reactions with various amounts of GST-PTP-SL fusion proteins combined with a preparation of activated ERK5 were performed and ERK5 activity was subsequently measured. COS-7 cells co-expressing the dominant active form of MEK5 were used as source for activated ERK5. MEK5 was shown to be the MAPK kinase specifically activating ERK5 and to possess constitutive activity if serine 311 and threonine 315 were mutated to aspartate (Kato et al., 1997; Zhou et al., 1995). Wild-type PTP-SL almost completely abolished ERK5 kinase activity, whereas the catalytically inactive CS mutant did neither affect autophosphorylation of ERK5 nor phosphorylation of the unspecific substrate myelin basic protein (Fig. 12*A*). As shown in Figure 12*B*, the degree of ERK5 inactivation correlated with the amount of utilised PTP-SL protein.

In order to test whether PTP-SL would be capable of inactivating ERK5 in transfected COS-7 cells, immuno complex kinase assays were performed after stimulation of cells with hydrogen peroxide or EGF. Whereas wild-type PTP-SL reduced ERK5 kinase activity to basal levels, the PTP-SL mutant lacking phosphatase activity seemed to further enhance ERK5 autophosphorylation (Fig. 12*C, upper panel*). ERK5 appeared as doublet resulting from a mobility shift of a small fraction of the enzyme that was hardly detectable in Western blot but clearly visible in the autoradiograph. This shift is probably due to phosphorylation of ERK5 since the amount of the upper band was found to correlate with the degree of kinase activation (Cavanaugh et al., 2001; Mody et al., 2001); when dominant active MEK5 was co-expressed this shift was virtually quantitative (Fig. 12*A* and *B*).

Since the relative activation of ERK5 after EGF and hydrogen peroxide treatment of cells was only moderate, we additionally expressed the constitutively active MEK5 construct to achieve a more pronounced ERK5 activation. Wild-type PTP-SL partially counteracted the effect of MEK5 and reduced ERK5 activity to a level that may reflect the balance between the opposing actions of activating kinase and inactivating phosphatase in the cell. In turn, co-

transfection of the catalytically impaired CS mutant of PTP-SL caused a profound increase in ERK5 activity which might be due to competition with endogenous phosphatases for ERK5 binding sites.

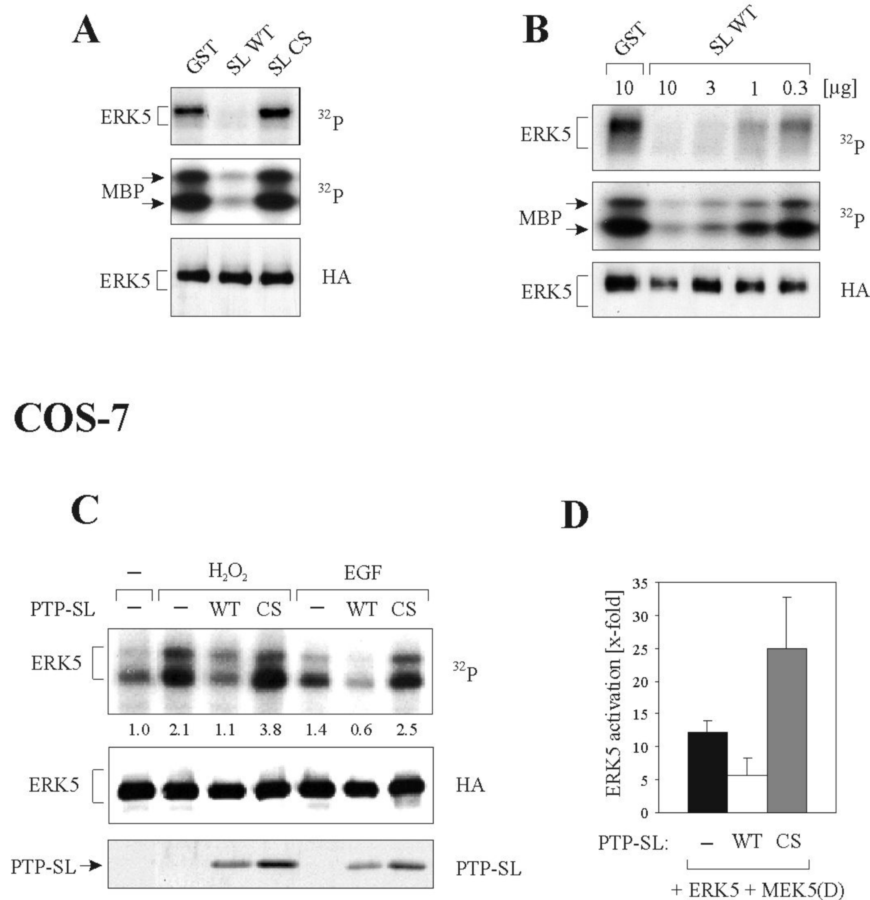

Figure 12: **PTP-SL inactivates ERK5 *in vitro* and in transfected COS-7 cells.** *A* and *B*, pcDNA3 HA-ERK5-Fc and pcDNA3 MEK5(D) were co-transfected into COS-7 cells. After 48 hours, Fc tagged ERK5 was precipitated with Protein A/G sepharose from crude cell lysates and subjected to *in vitro* phosphatase reactions in the presence of GST, GST-PTP-SL (SL) WT or SL CS. Residual ERK5 activity was measured in a kinase assay with MBP as additional substrate. The amount of SL protein was 3 µg if not indicated otherwise. Split samples were separated on two SDS-PAGEs and analysed by autoradiography (*upper and middle panels*) and by anti-HA immunoblotting (*bottom panel*). *C*, COS-7 cells were transfected with pcDNA3 HA-ERK5, pRK5 PTP-SL WT, CS or the empty vector. After serum starvation, cells were stimulated for 15 min with 200 µM H_2O_2 or 5 min with 15 ng/ml EGF. Anti-HA immunoprecipitates were subjected to *in vitro* kinase assays and resolved on SDS-PAGE . Autophosphorylation and the amount of precipitated ERK5 were visualised by autoradiography and anti-HA immunoblot (*upper and middle panel*). The small numbers below the lanes represent the relative autophosphorylation activities of ERK5. Expression of PTP-SL in crude lysates is shown by immunoblot using anti-PTP-SL (*lower panel*). *D*, pcDNA3 HA-ERK5-Fc was co-transfected together with pcDNA3 MEK5(D) and pRK5 PTP-SL WT, CS or the corresponding vectors into COS-7 cells. ERK5 was precipitated with Protein A/G sepharose followed by kinase assays. Samples were separated on SDS-PAGE and transferred to nitrocellulose membrane. The amount of precipitated and autophosphorylated ERK5 were quantified by HA immunoblot and phospho-imaging, respectively. Values represent the relative autophosphorylation activity normalised by the amount of precipitated ERK5 and are the mean of two independent experiments ± SD. ERK5 activity in the absence of exogenous PTP-SL and MEK5 was set as 1-fold.

3.1.5 Endogenous ERK5 is inactivated by PTP-SL in PC12 Cells

To evaluate the potential of PTP-SL to inactivate endogenous ERK5, PC12 cells stably expressing PTP-SL were stimulated with EGF and the activation state of immunoprecipitated ERK5 was analysed by Western blot. After stimulation, a reduced amount of active ERK5 was detected in cells overexpressing wild-type PTP-SL (Fig. 13, *top panel*), whereas in analogy to the effect seen in transfected COS-7 cells, the inactive CS mutant of PTP-SL further enhanced the activation of endogenous ERK5 (compare Fig. 12*C*, *D* and Fig. 13). Notably, two more or less equally phosphorylated forms of ERK5 were detected in PC12 cells. This indicates that additional modifications might be required for the mobility shift to the upper position. Figure 13 (*top panel*) further shows that PTP-SL reduced the amount of both activated forms of ERK5 to a similar extent. As it can be seen in the *third panel* of Figure 13, ERK1/2 were – as expected – also inactivated by wild-type PTP-SL. It has to be mentioned, that the expression of wild type PTP-SL when compared to its inactive mutant decreased with time during cultivation of cells indicating growth selection for cells with low phosphatase expression (Fig. 13, *bottom panel*).

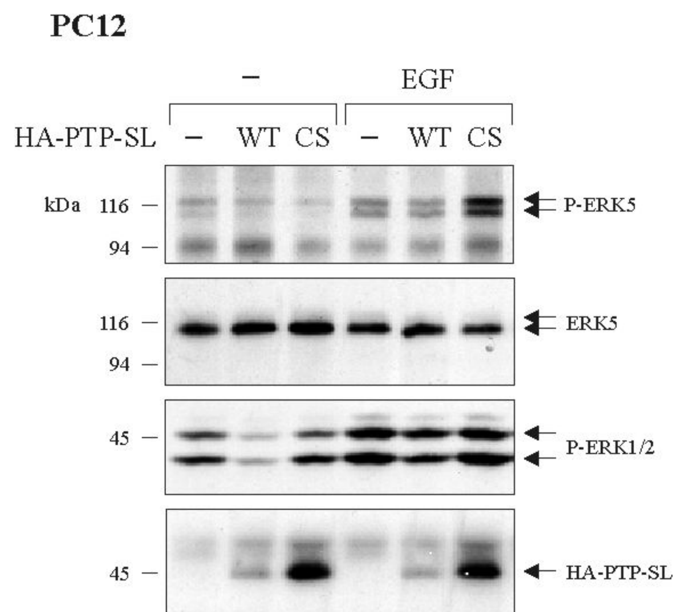

Figure 13: **Endogenous ERK5 is inactivated by PTP-SL in PC12 cells.** PC12 cells transfected with pcDNA3-HA-PTP-SL WT, CS or mock DNA were serum starved for 24 hours followed by stimulation with 30 ng/ml EGF for 5 min. Endogenous ERK5 was immunoprecipitated and split equally on two SDS-PAGEs. Samples were analysed by immunoblotting for ERK5 and for the active phosphorylated form phospho-ERK5 (*top two panels*). Activation of ERK2 was monitored by Western blot analysis of crude lysates using anti-Phospho-ERK1/2. The expression of HA-PTP-SL was visualised by HA immunoprecipitation and Western blot (*bottom panel*).

3.1.6 PTP-SL retains ERK5 in the Cytosol

When the subcellular localisation of endogenous ERK5 was examined in COS-7 cells by immunofluorescence, a major portion of ERK5 was found to reside in the nucleus (Fig. 14*B*, *C*). To test whether PTP-SL would have an influence on this subcellular localization, the phosphatase was fused to green fluorescent protein (GFP) and overexpressed.

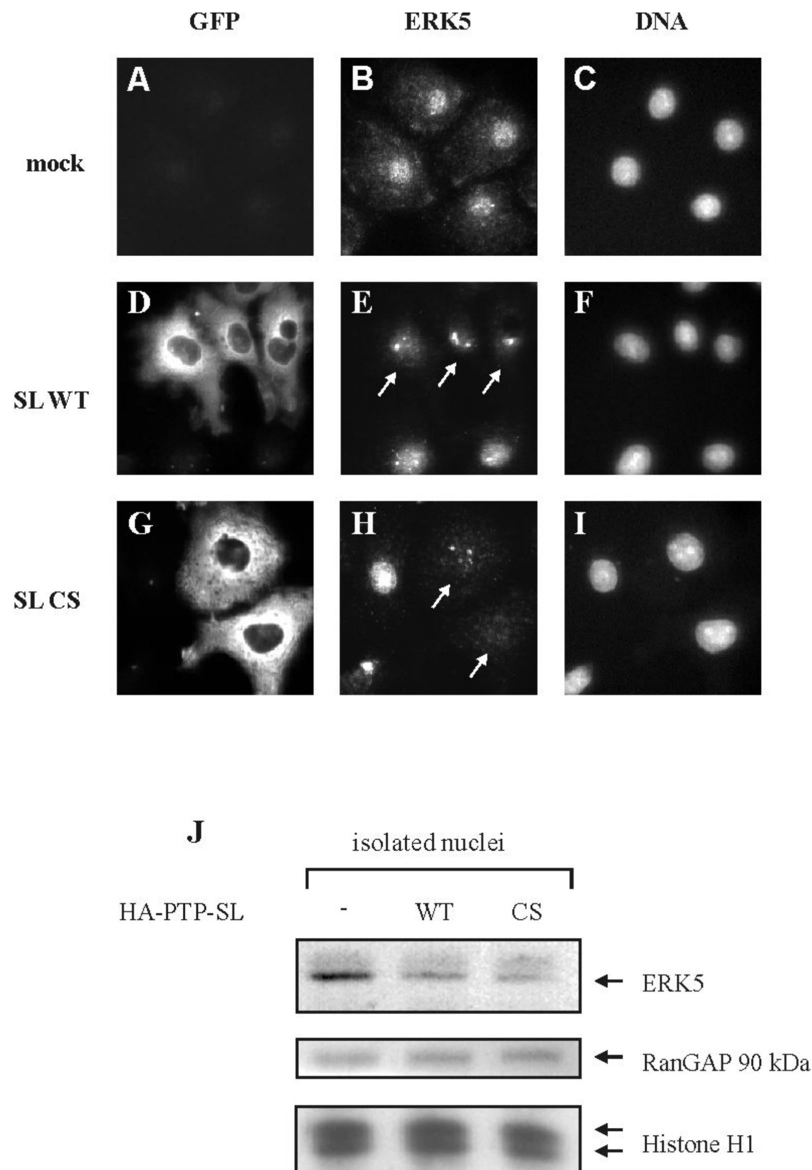

Figure 14: **PTP-SL retains ERK5 in the cytoplasm.** COS-7 cells were transiently transfected with pcDNA3 GFP-PTP-SL (SL) WT, CS or the empty vector and analysed by fluorescence microscopy after 24 hours. GFP autofluorescence is shown in the left panel (*A, D* and *G*). ERK5 was visualised by immunofluorescence using anti-ERK5 antibodies (*B, E* and *H*) and DNA was stained with bisbenzimid (*C, F* and *I*). Each horizontal panel shows identical sections of COS-7 cells. Arrows indicate nuclei with reduced ERK5 staining. *J*, isolated nuclei from the transfected COS-7 cells were separated on SDS-PAGE. The ERK5 content was analysed by anti-ERK5 immunoblot. Equal loading was assured by reprobing the membrane with anti-RanGAP and anti-Histone H1 antibodies.

It can be clearly seen in Figure 14*D - F* that only those cells that express PTP-SL as indicated by the autofluorescence of GFP show reduced ERK5 staining in the nucleus when compared with neighbouring untransfected cells. This reduction reflects the retention of ERK5 in the cytoplasm since expression of PTP-SL did not affect the overall levels of ERK5 protein in the cells (data not shown). Figure 14*G - I* further shows that not only the wild type PTP-SL but also its catalytically impaired CS mutant exerted this influence on ERK5. Accordingly, the amount of ERK5 protein was reduced in isolated nuclei from transiently transfected COS-7 cells expressing either form of PTP-SL (Fig. 14*J*). The influence of PTP-SL on the localization of ERK5 was independent of phosphatase activity which demonstrates that PTP-SL is able to modulate ERK5 action not only by dephosphorylating the kinase but also additionally by binding to it and thereby retaining it in the cytoplasmic department. Therefore the cellular PTP-SL expression level is an additional and critical parameter for MAPK regulation.

Taken together, PTP-SL is not only able to modulate the signalling of ERK5 by dephosphorylation and inactivation but also by imposing localisational constraints.

3.2 Oncogenic Abl Mediates Leukaemia Cell Survival by MEK5 independent Stabilisation of ERK5

Chronic myeloid leukaemia (CML) is a pluripotent haematopoietic stem cell disorder which is characterised by the Philadelphia chromosome translocation. This genomic abnormality gives rise to the Bcr/Abl fusion protein, a strictly non-nuclear protein tyrosine kinase with constitutive signalling activity. The Bcr/Abl oncogene is sufficient to initiate CML-like disease in mice (Daley et al., 1990; Elefanty et al., 1990; Heisterkamp et al., 1990), and is responsible for leukaemia progression (Huettner et al., 2000). Treatment of patients with the Abl-specific kinase inhibitor Imatinib (also termed Gleevec, Glivec and STI571) induced remission in the early chronic leukaemic phase as well as in late blast crisis (Druker et al., 2001a; Druker et al., 2001b). Whereas responses in the chronic phase were enduring, remission in blast crisis patients were only transient over a period of 2 to 6 months due to development of resistance to the kinase inhibitor. Point mutations in the Bcr/Abl gene can severely decrease the sensitivity for Imatinib and have been shown to be responsible for cellular drug resistance (Gorre et al., 2001; Shah et al., 2002). It is conceivable that due to selection during Imatinib therapy and after preliminary remission, clonal expansion of such

resistant cells will lead to relapse. Combinational therapy by additionally targeting survival pathways might thus be a powerful tool in preventing tumour progression that is caused by the expansion of resistant clones. One major survival signal is mediated by mitogen-activated protein kinases (MAPKs) (Ballif and Blenis, 2001) and inhibition of MAPK pathways by chemical compounds synergistically enhanced Imatinib-induced apoptosis (Yu et al., 2002).

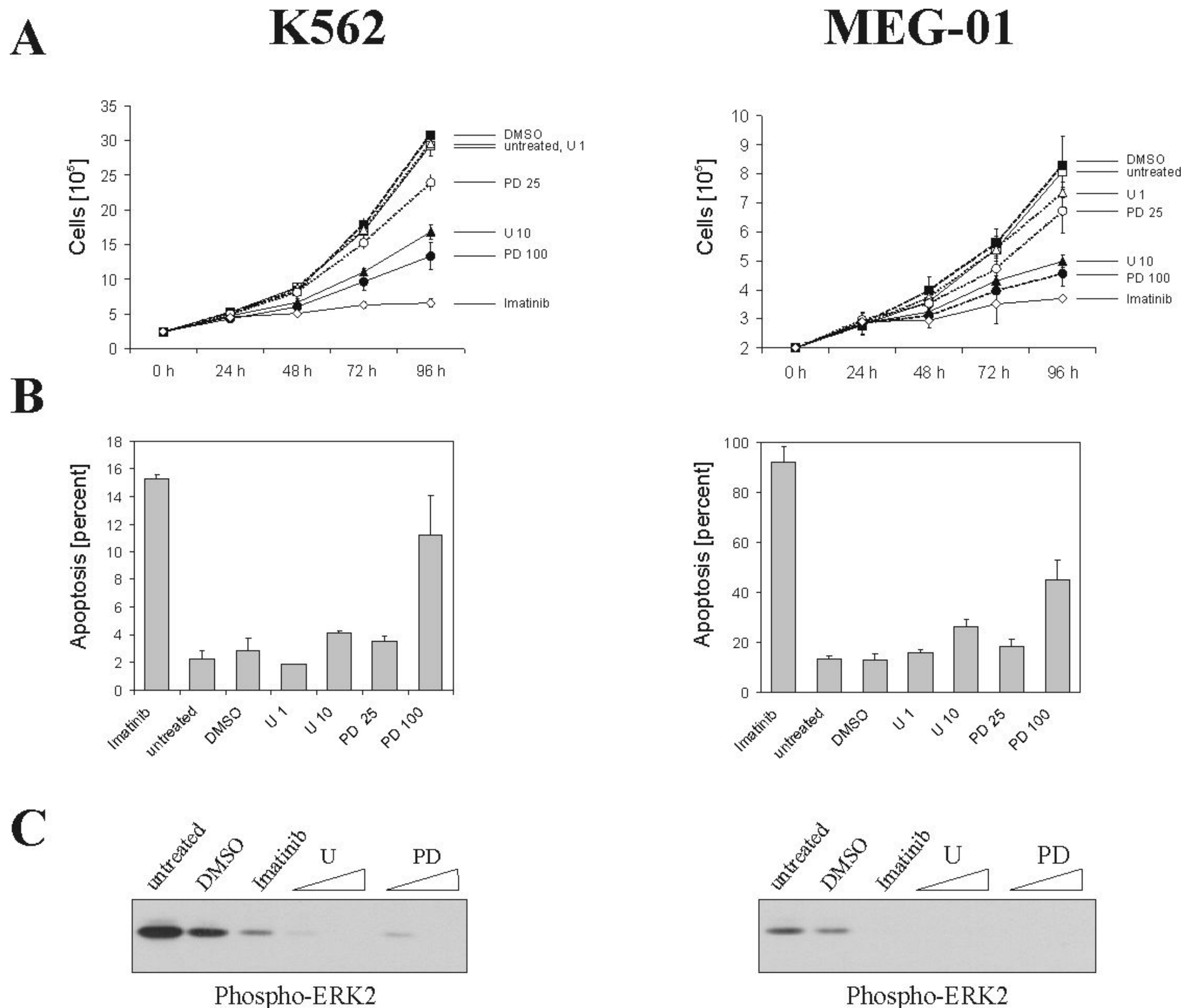

Figure 15: **Dose-dependent effects of MAPK pathway inhibitors on Leukaemia cells.** *A*, K562 and MEG-01 cells were grown in the presence of the MAPK inhibitors U0126 (1 and 10 µM, U1 and U10, respectively) and PD98059 (25 and 100 µM, PD25 and PD100, respectively). Control cells were grown in medium containing either no additive, the solvent DMSO alone or 1 µM of the Abl kinase inhibitor Imatinib. *B*, after 72 hours nuclear DNA content of cells was analysed by flow cytometry. The fraction of cells with less DNA than diploid cells was used as measure for apoptotis. *C*, the activity of ERK2 was monitored by anti-Phospho-ERK2 immunoblot of crude lysates. Cells were treated with the same concentrations of inhibitors as under *A* for four hours.

To gain some preliminary insight into the function of MAPKs in leukaemia, the two p210 Bcr/Abl positive cell lines MEG-01 and K562 were treated with the two most commonly used

inhibitors of the MAPK cascade PD98059 and U0126. Both compounds were known to interfere with MEK1 and MEK2 (English and Cobb, 2002) and thus to prevent the activation of ERK1 and ERK2 and downstream targets (Dudley et al., 1995; Favata et al., 1998).
The influence of the MAPK inhibitors on cell growth and survival was measured. Two concentrations of each inhibitor were used of which the lower had an only moderate effect on MEG-01 cell growth and virtually none on K562 cells (Fig. 15*A*). Raising compound concentration, however, strongly reduced proliferation as well as survival of both cell lines (Fig. 15*A* and *B*). Imatinib treated cells were used as control and showed strong induction of apoptosis and impaired proliferation. Interestingly, even lower inhibitor concentrations almost completely abolished the activity of ERK2 (Fig. 15*C*). Similar discrepancies in the dose-dependent influence of MAPK inhibitors on ERK2 activity on one hand and proliferation and survival on the other have been reported before (Woessmann and Mivechi, 2001). Noteworthy, the same inhibitors also – but less effectively – inhibited the MEK5-ERK5 pathway (Mody et al., 2001). This finding together with recent observations that implicated ERK5 in doxorubicin resistance of MCF-7 breast cancer cells (Weldon et al., 2002), led us to the question whether ERK5 might play a role in oncogenic Abl signalling in general and leukaemia cell survival in particular.

3.2.1 ERK5 interacts with oncogenic Abl Kinases

ERK5 expression was readily detected in the Bcr/Abl positive cell lines MEG-01 and K562 by Western blot (Fig. 16*A*, *bottom panel*). Signalling activity of both cell lines was highly sensitive to Bcr/Abl inhibition as monitored by the drastic reduction of overall tyrosine phosphorylation in crude lysates upon treatment with Imatinib (Fig. 16*A*, *right upper panel*). This correlates with the strong anti-proliferative effects of Imatinib seen above.
Immunoprecipitation of ERK5 indicated that ERK5 itself as well as several associating proteins were tyrosine phosphorylated in an Abl kinase dependent manner in both cell lines, albeit to a different extent. Tyrosine phosphorylation levels of ERK5 were more pronounced in K562 than in MEG-01 cells. Sizes of detected associating proteins ranged from approximately 160 to 210 kDa. A p210 protein was clearly enriched in ERK5 immunoprecipitates when compared to its relative abundance in crude cell lysates. Similarly, overexpression of Bcr/Abl, c-Abl, v-Abl and the less abundant leukaemic fusion protein Tel/Abl induced tyrosine phosphorylation of the protein complex precipitating with ERK5 in HEK 293T cells (Fig. 16*B*). Interestingly reprobing with pan-Abl antibody revealed some binding of different Abl forms to ERK5. Noteworthy, the p145 Tel/Abl protein was the most

potent in binding ERK5. The absence of Abl in precipitates with preimmune serum or control antibody demonstrated that the interaction of ERK5 and Abl is specific (Fig. 16*C*).

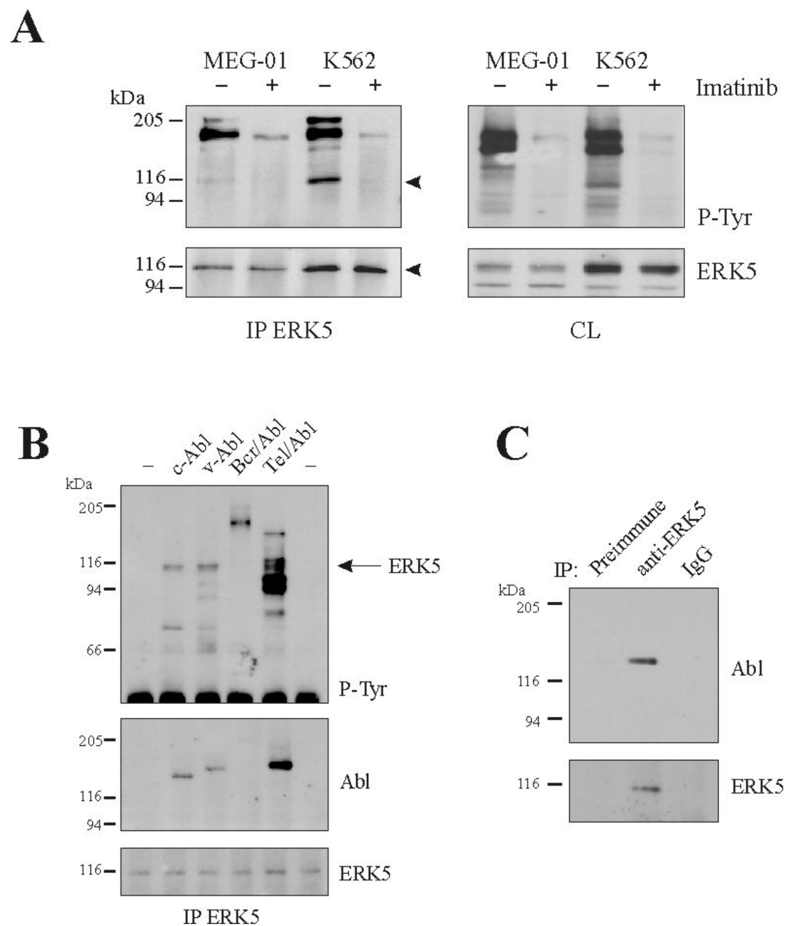

Figure 16: **Abl kinases bind and phosphorylate ERK5**. *A*, MEG-01 and K562 cells were treated with 10 µM Imatinib for one hour and lysed. ERK5 immunprecipitates (IP) and crude cell lysates (CL) were analysed by anti-phosphotyrosine and anti-ERK5 immunoblot. Arrows indicate ERK5. *B*, HEK 293T cells were transfected with expression constructs of different Abl kinases. ERK5 was immunoprecipitated and immuno complexes were analysed by anti-phosphotyrosine, anti-ERK5 and anti-Abl immunoblot. *C*, lysate from c-Abl expressing HEK 293T cells was prepared. Anti-ERK5 immunoprecipitates were compared to control antibody and preimmune serum. Bound c-Abl was visualised by anti-Abl immunoblot.

3.2.2 Abl Kinases activate ERK5

To test whether Abl activates ERK5, cells were co-transfected with different Abl kinases and ERK5. Immuno complex kinase assays were performed and ERK5 autophosphorylation was taken as read out. While Bcr/Abl and Tel/Abl over-expression had only minor effects (Fig. 17*A* and *B*), c-Abl and v-Abl strongly induced ERK5 activation to a similar level as the constitutively active mutant of the upstream kinase MEK5 (Buschbeck et al., 2002).

Src-family kinases, Fyn, Src and Lck, although much more potent in inducing overall tyrosine phosphorylation, did not activate ERK5 (Fig. 17*C*). As shown in Figure 17*D* Abl induced activation of ERK5 was blocked by Imatinib and is thus clearly dependent on intact Abl kinase activity.

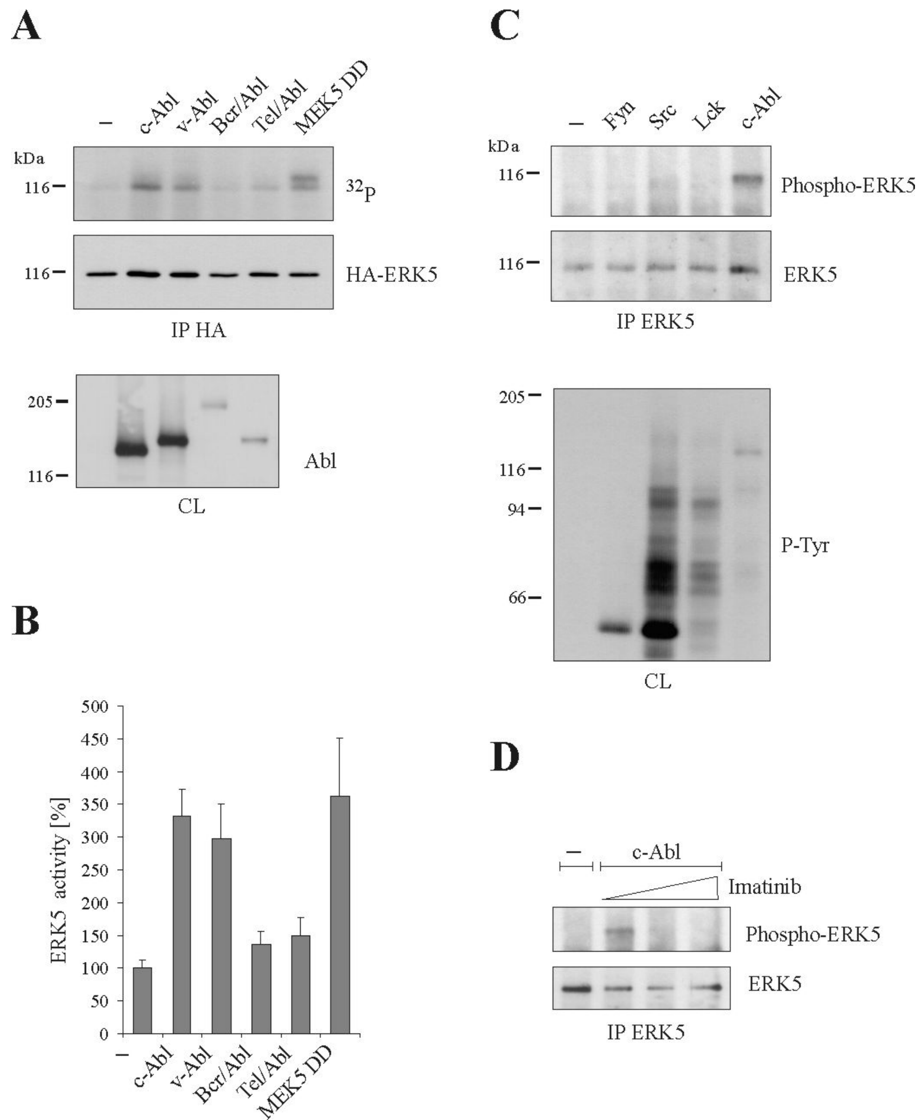

Figure 17: **Abl activates ERK5**. *A*, COS-7 cells were co-transfected with expression constructs for HA-tagged ERK5 and Abl kinases or constitutively active MEK5 DD. The activity of ERK5 was monitored by autophosphorylation in an anti-HA immuno complex kinase assay (*top panel*). The amount of immunoprecipitated (IP) ERK5 and the expression of c-Abl in crude cell lysates were analysed by anti-HA and anti-Abl immunoblot, respectively (*middle and bottom panel, respectively*). *B*, a quantitative view of ERK5 activation is shown. Autophosphorylation signals were normalised for the amount of precipitated ERK5. Data represents the mean of three experiments ± SD. *C*, HEK 293T cells were transfected with constructs for Src- family kinases or c-Abl. ERK5 was immunoprecipitated and its activation state was analysed by anti-Phospho-ERK5 immunoblot which specifically detects the activated form of the kinase (*top panel*). Membranes were reprobed with anti-ERK5 and the induction of overall tyrosine phosphorylation was monitored by anti-phosphotyrosine immunoblot of crude cell lysates (*middle and bottom panel*). *D*, HEK 293T cells transfected with c-Abl were treated with 0.1, 1.0 and 10 µM Imatinib. ERK5 activity was analysed as in *C*.

The roles of the many different MAPK family members in oncogenic Abl signalling are somewhat controversial. It has been convincingly demonstrated that the proto-oncogene product c-Abl acts in the pathway downstream of DNA damage that includes activation of the pro-apoptotic kinases p38 and JNK (Kharbanda et al., 2000; Kharbanda et al., 1998). However, the exact function and the subset of MAPKs preferentially targeted by deregulated Abl signalling remain widely elusive.

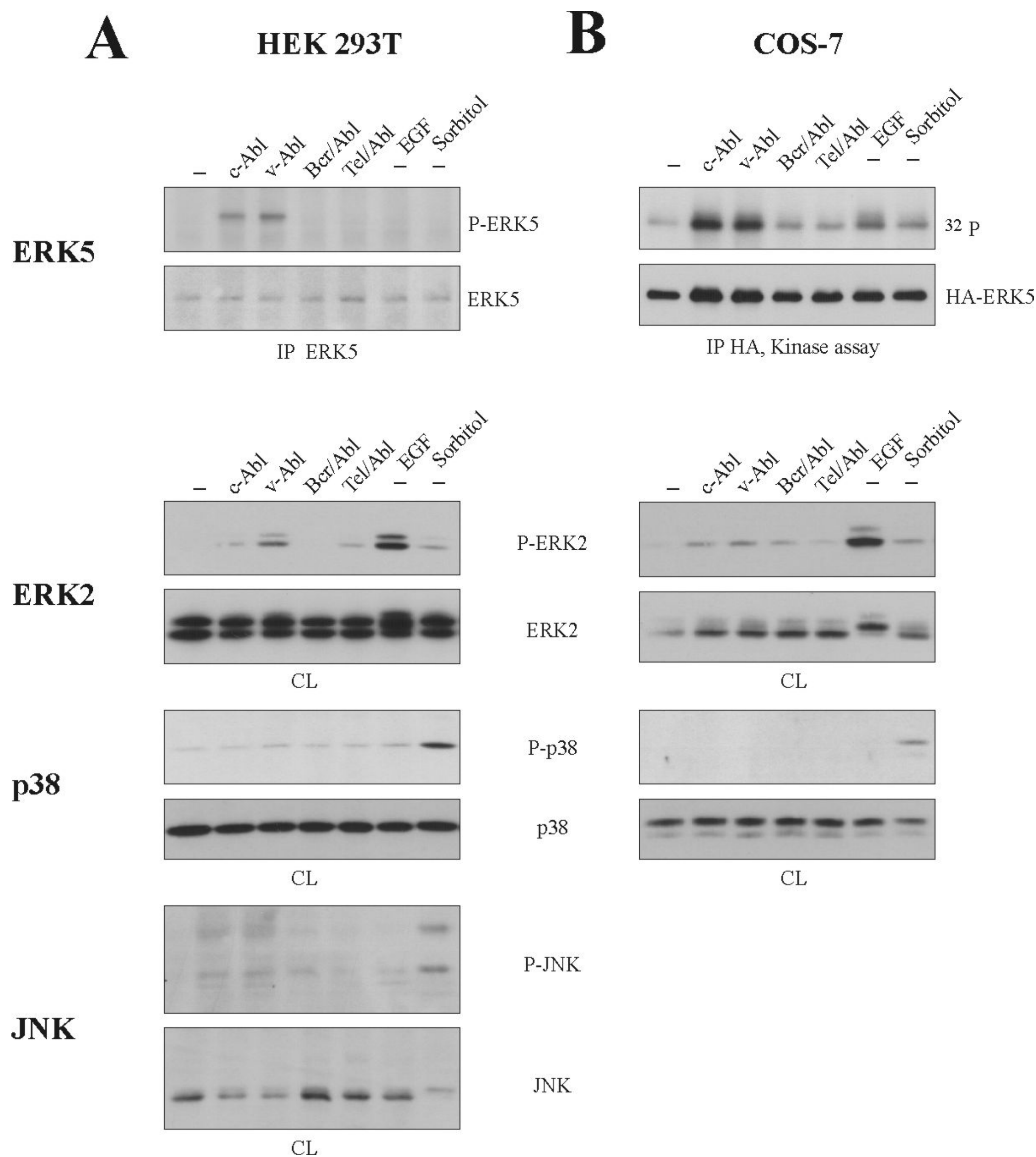

Figure 18: **Abl preferentially activates ERK5 among MAPKs.** The activation of different MAPKs was analysed in cells that were either overexpressing Abl kinases or that were treated with 20 ng/ ml EGF for 15 minutes and 0.5 M sorbitol (osmotic stress) for 30 minutes. *A*, HEK 293T cells were used. ERK5 immunoprecipitates (IP) and crude cell lysates (CL) were analysed by immunoblot using anti-ERK5, anti-ERK2, anti-p38, anti-JNK and the corresponding phosporylation state-specific antibodies, anti-P-ERK5, anti-P-ERK2, anti-P-p38 and anti-P-JNK. *B*, for analysis of MAPK activation in COS-7 cells HA-tagged ERK5 and p38 were co-overexpressed with Abl kinases. ERK5 was immunprecipiated and its autophosphorylation activity was assessed in an immuno complex kinase assay (*top panel*). The corresponding anti-HA immunoblot is shown below. Activation of ERK2 and p38 was analysed by immunoblot of crude cell lysates as in *A*.

The MAPK activity induced by overexpression of Abl kinases was compared to the activity reached upon stimulation of cells with EGF and osmotic stress. Known to activate MAPKs these two stimuli were included as internal reference for the degree of MAPK activation. First, activaty of ERK5, ERK2, p38 and JNK in HEK 293T cells was analysed by immunoblot (Figure 18*A*). As expected, in addition to ERK5 the other MAPKs were also activated by overexpression of the Abl kinases but to a significantly lower degree than by either stimulus. Not surprisingly, JNK and p38 were potently activated by osmotic stress, while ERK2 strongly responded to EGF. In contrast ERK5 was most potently activated by exogenous Abl but not detectably by either stimulation. In a second set of experiments COS-7 cells were taken as overexpression system (Fig. 18*B*). ERK2 and p38 activation was analysed as before while ERK5 activity was measured in an immuno complex kinase assay. ERK5 was again found to be more strongly activated by overexpression of Abl kinases than by EGF or Sorbitol. According to the results obtained from HEK 293T cells ERK2 as well as p38 were best activated by standard stimuli. In order to amplify the otherwise weak signal the level of p38 was enhanced by moderate overexpression. Conclusively, these results indicate that at least under overexpression conditions Abl kinases preferentially activate ERK5 in comparison to other MAPKs.

3.2.3 Abl Kinases regulate the Protein Level of ERK5

To extend the results from leukaemic cells ERK5 was further analysed in MEG-01 cells. Surprisingly endogenous ERK5 levels were reduced after extended treatment with Imatinib while the control protein Tubulin was not affected (Fig. 19*A*). To generate a v-Abl transformed cell line, Rat-1 fibroblasts were infected with a retroviral expression construct for v-Abl and grown until the appearance of multilayered cell aggregates indicated the loss of contact inhibition. These so called foci were isolated and further cultivated. In these particular fibroblasts ERK5 levels also decreased with time of Imatinib exposure (Fig. 19*B*), whereas ERK2 and p38 were not or only moderately affected. Since inhibition of Abl kinase activity resulted in a decrease of ERK5 protein, we used again COS-7 cells as expression system to conduct the converse experiment. As shown in Figure 19*C*, increasing Abl kinase activity by over-expression enhanced the level of ERK5 whereas expression of wild-type Abl in the presence of Imatinib or expression of the kinase dead Abl KM construct had no effect.

The amount of a certain protein in the cell reflects the balance between production and degradation. Thus many factors controlling promoter activity, mRNA stability, rate of

translation and of course the protein stability itself, define the abundance of a protein which can be observed in a simple immunoblot experiment.

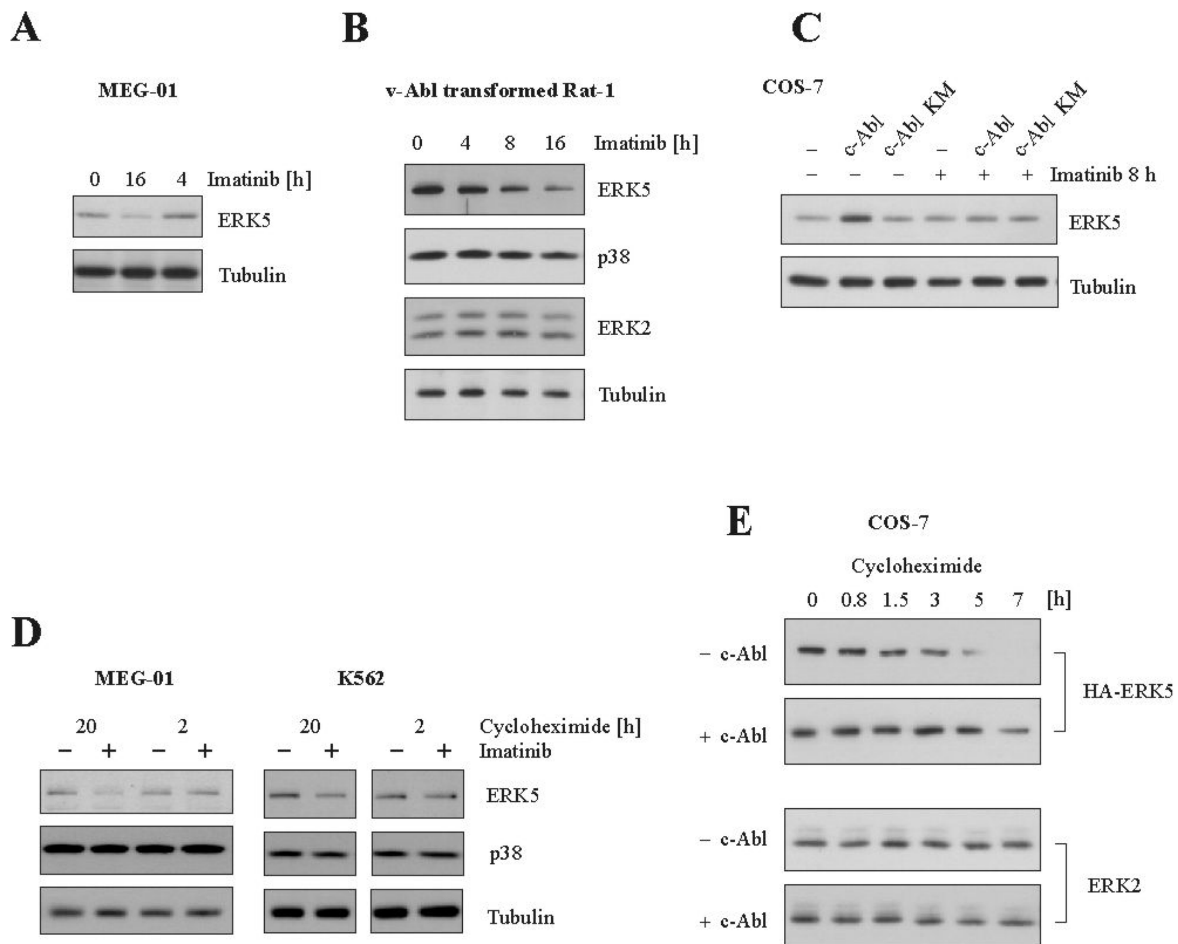

Figure 19: **Abl kinase stabilise ERK5**. *A-E*, Protein levels of ERK5, Tubulin, p38 and ERK2 were analysed by immunoblot of crude cell lysates. *A*, prior lysis MEG-01 cells were incubated with 10 µM Imatinib. *B*, v-Abl transformed Rat-1 cells were treated with 5 µM Imatinib. *C* and *E*, COS-7 cells were co-transfected with constructs for HA-tagged ERK5 and wild-type or kinase dead (KM) c-Abl. Cells were treated with 10 µM Imatinib or 100 µM translation inhibitor cycloheximide as indicated. *D*, MEG-01 and K562 cells were treated with 10 µM Imatinib in combination with 100 µM cycloheximide as indicated.

The fact that not only endogenous but also exogenous ERK5 which is expressed at a relatively constant rate were sensitive to Abl kinase activity, led us to speculating that the stability of the ERK5 protein was somehow affected. In order to test this hypothesis we blocked translation in MEG-01 and K562 cells by addition of cycloheximide and checked whether co-administration of Imatinib would still be able to influence the cellular level of ERK5. As shown Figure 19*D*, ERK5 but not p38 was reduced in cells treated with the inhibitor. Accordingly, co-expression of Abl in COS-7 cells prolonged the half-life of exogenous ERK5 but did not affect the protein levels of ERK2 (Fig. 19*E*). Abl kinases thus do not only regulate ERK5 by classical MAPK cascade activation but also by stabilisation of the protein itself.

3.2.4 Abl induced Stabilisation of ERK5 is MEK5-independent and involves the C-terminal Tail of ERK5

ERK5 differs from all other MAPKs in that it contains a 400 amino acid long C-terminal tail which gave rise to its additional designation as big MAP kinase 1. Although this portion of ERK5 is its most striking feature its function remains largely elusive.

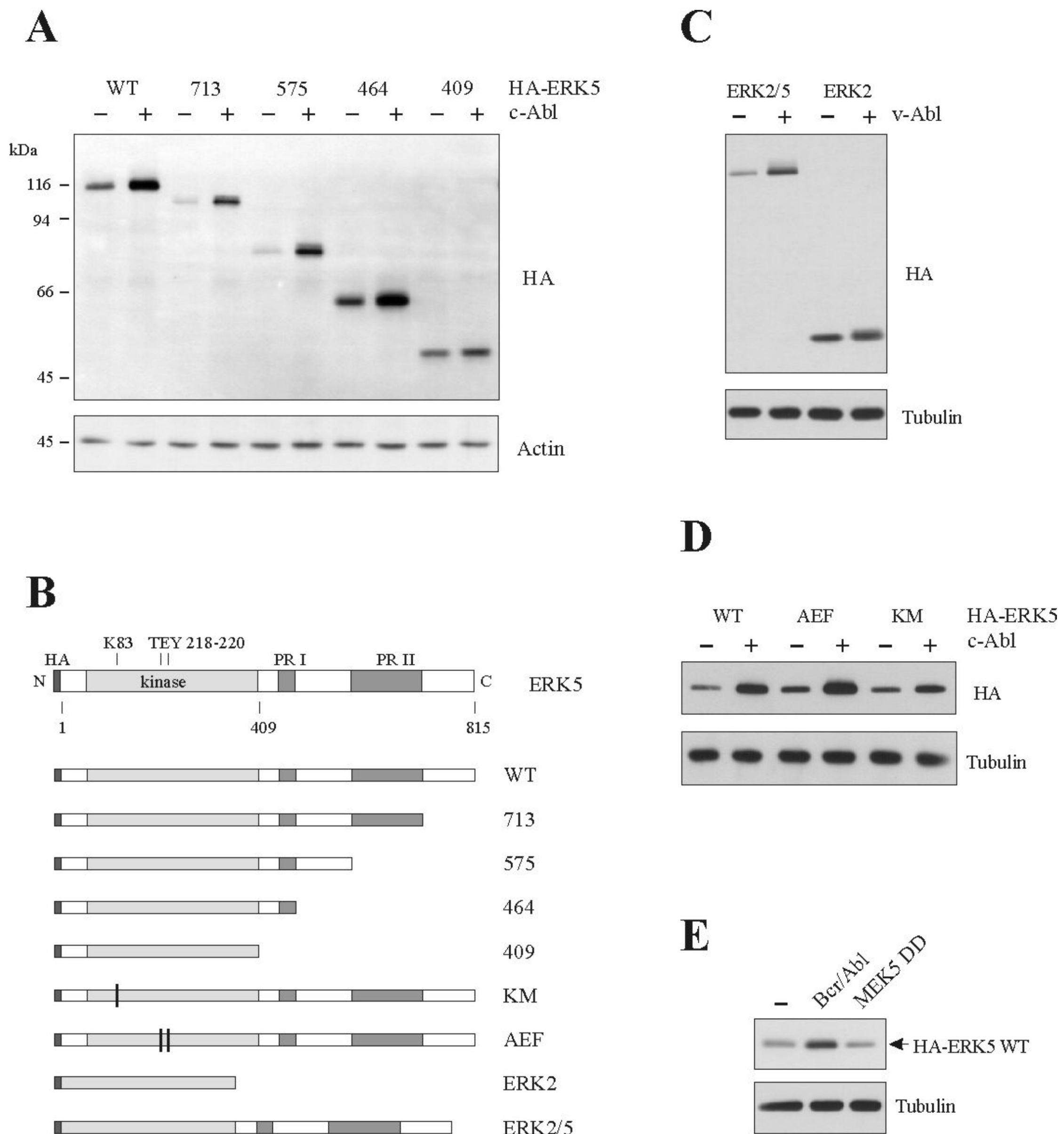

Figure 20: **The C-terminus of ERK5 is involved in Abl mediated stabilisation.** *A* and *C-E*, requirements for Abl mediated stabilisation were analysed in transfected COS-7 cells expressing various HA-tagged ERK5 mutants in combination with Abl kinases. Crude cell lysates were analysed by anti-HA, anti-Tubulin and anti-Actin immunoblot. *B*, schematic presentation of used mutants: PR, Proline rich; WT, wild-type; AEF, not activatable but basally active - mutation of MEK5 phosphorylation sites; KM, kinase dead; numbers indicate the last amino acid of truncated mutants; ERK2/5, fusion of ERK5 C-tail to full length ERK2.

Since the ERK5 C-terminus is highly conserved among mammals it has been speculated for a long time that it might possess a regulatory role. Here, the ability of c-Abl to stabilise various C-terminally truncated mutants of ERK5 was tested. All ERK5 mutants except for the shortest ERK5 409 construct were effectively stabilised by Abl over-expression (Fig. 20*A*). The 409 mutant lacks the entire C-terminal tail and reduces ERK5 to its MAPK domain that shares high homology with ERK2 (Fig. 20*B*). Fusion of the complete ERK5 C-terminus to ERK2 results in potent stabilisation of the chimeric protein by v-Abl as shown in Figure 20*C*. Scanning the ERK5 sequence with the Scansite algorithm (Yaffe et al., 2001) identified a potential Abl SH3 binding site in the proline rich domain I which is absent in the 409 mutant. Together these results suggest that the C-terminal tail of ERK5 might be able to modulate kinase action by mediating the interaction with Abl which would subsequently stabilise the ERK5 protein. However, a precise dissection of the parameters that determine this interaction between ERK5 and Abl requires further studies.

Figure 20*D* shows that over-expression of Abl elevated the amount of ERK5 AEF, a mutant which cannot be activated and is thus uncoupled from MEK5, and affected, although to a lower extent, also the level of kinase dead ERK5 KM. Moreover, constitutively active MEK5 DD – although potent in ERK5 activation (Fig. 17*A* and *B*) – did not enhance ERK5 protein levels (Fig. 20*E*). Stabilisation of ERK5 is thus independent of activation by MEK5 and represents a novel aspect in MAPK regulation besides the classical kinase cascade-mediated control.

3.2.5 Synergy of v-Abl and enhanced ERK5 Levels in Transformation

The oncogenic capacity of v-Abl is easily monitored in cell culture by the induction of foci in monolayers of rodent fibroblasts. To get some preliminary information about the functional connection of ERK5 and oncogenic Abl signalling, we compared the efficacy of v-Abl in transforming Rat-1 fibroblasts stably over-expressing either wild-type ERK5 or the AEF and KM mutants. Elevated ERK5 levels synergistically enhanced the amount of foci induced by v-Abl (Fig. 21). Strikingly, ERK5 AEF was as potent as the wild-type form, whereas kinase dead ERK5 KM had no influence at all. Although impaired in MEK5 dependent activation, the AEF mutant still harbours basal activity which is required and possibly sufficient for the observed potentiation of v-Abl induced transformation. In the absence of v-Abl, overexpression of any form of ERK5 alone did not lead to any transformation (data not shown).

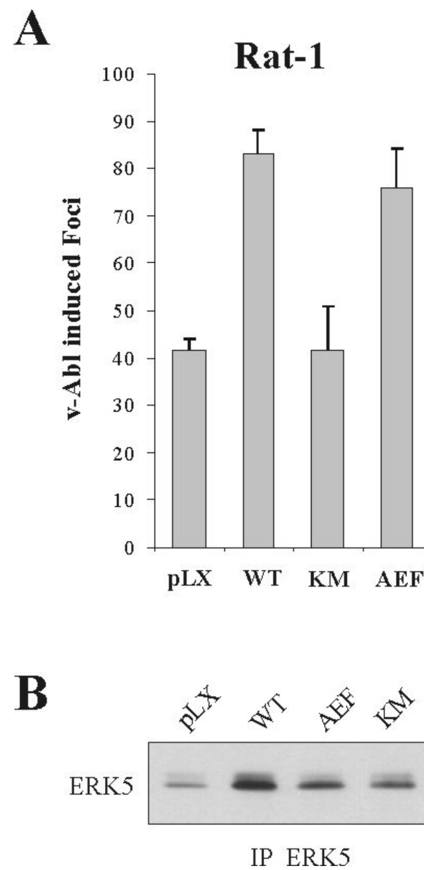

Figure 21: **Enhanced ERK5 levels enforce the transforming capacity of v-Abl.** *A*, polyclonal Rat-1 fibroblasts overexpressing either wild-type (WT) or mutant (AEF and KM) ERK5 and control cells transfected with empty vector (pLX) were used. Monolayers of cells were infected with ecotrophic pLXSN-v-Abl retrovirus and further cultivated for 14 days. The number of induced foci was determined. Data represents three independent experiments + SD. Cells infected with control virus did not produce any foci (not shown). *B*, anti-ERK5 immunoprecipitates were analysed by anti-ERK5 immunoblot.

3.2.6 ERK5 mediates Leukaemia Cell Survival

To approach the question whether ERK5 has a role in leukaemogenesis, the expression of ERK5 was analysed in five Bcr/Abl positive and ten Bcr/Abl negative leukaemia cell lines. ERK5 expression was readily detected in all Bcr/Abl positive and all but two Bcr/Abl negative cell lines (Fig. 22). The erythroleukaemia HEL and the histiocytic lymphoma U937 cell lines contained the lowest ERK5 levels. As expected ERK2, p38 and JNK as representatives of the three other main subgroups of MAPKs were ubiquitously expressed. Most interestingly, levels of JNK and even more pronounced of ERK5 differed much more between different cell lines than the levels of ERK2 or p38.

Having established the presence of ERK5 in Bcr/Abl positive lines, MEG-01 and K562 cells were stable transfected with expression constructs of either wild-type ERK5 or interfering

AEF and KM mutants in order to gain some information on the function of ERK5. Overexpression of any form of ERK5 did not affect the growth nor the cell cycle distribution of either cell line (not shown).

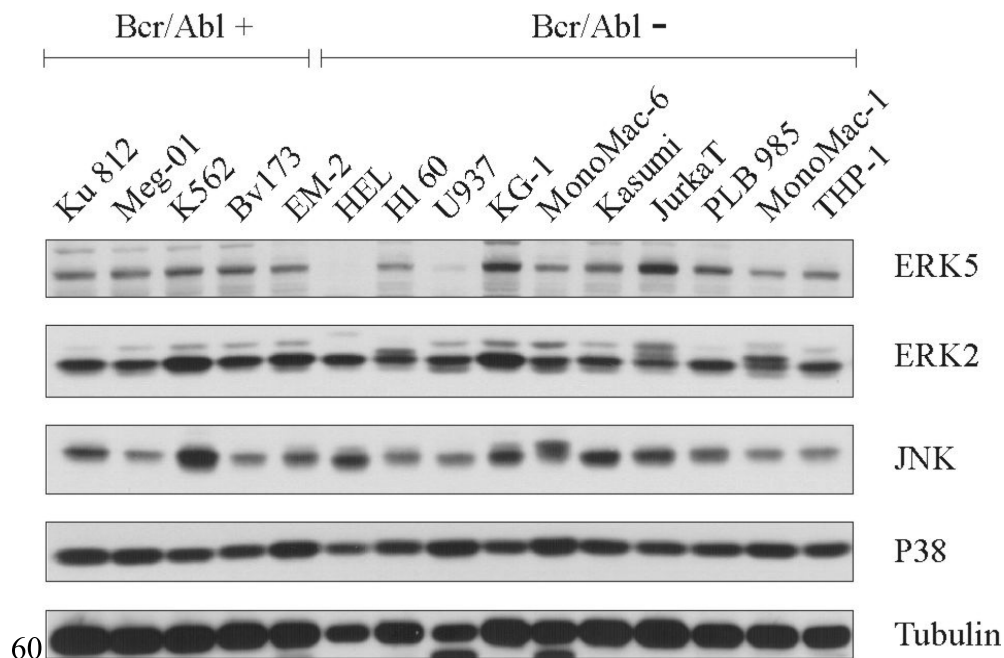

Figure 22: **MAPK expression in Leukaemia.** Crude cell lysates of Bcr/Abl positive and negative leukaemia cell lines were analysed by immunoblot using anti-ERK5, anti-ERK2, anti-p38, anti-JNK and anti-Tubulin antibodies.

This indicated that under normal conditions ERK5 is most likely not involved in the control of proliferation. However, taking recent findings into account implying ERK5 in chemoresistance (Weldon et al., 2002) the question was raised whether ERK5 might have a role in challenged cells, for instance during therapy. Therefore, the dose-dependent effects of the Abl kinase inhibitor Imatinib on the cell cycle distribution of all five Bcr/Abl positive cell lines were assessed. Cells were treated with Imatinib or control vehicle for three days, lysed and the DNA content of their nuclei was analysed by flow cytometry. The direct correlation between DNA content of a cell and its position in cell cycle allows to draw conclusions on the actual distribution among G1, S and G2/M phase and on the percentage of living cells by excluding sub-G1 cells. Under optimal growth conditions more than half of all cells were in S or G2/M phase indicating the highly proliferative character of these leukaemic cell lines (not shown). Treatment with Imatinib induced a strong cell cycle arrest monitored in the relative increase in G1 cells (Fig. 23, *middle and left column*). Since degradation of cellular DNA is a hallmark of apoptosis the appearance of a sub-G1 peak moreover indicated the induction of apoptosis in a significant fraction of cells. MEG-01 and Ku 812 were most sensitive to Imatinib treatment in terms of showing the largest percentage of sub-G1 cells.

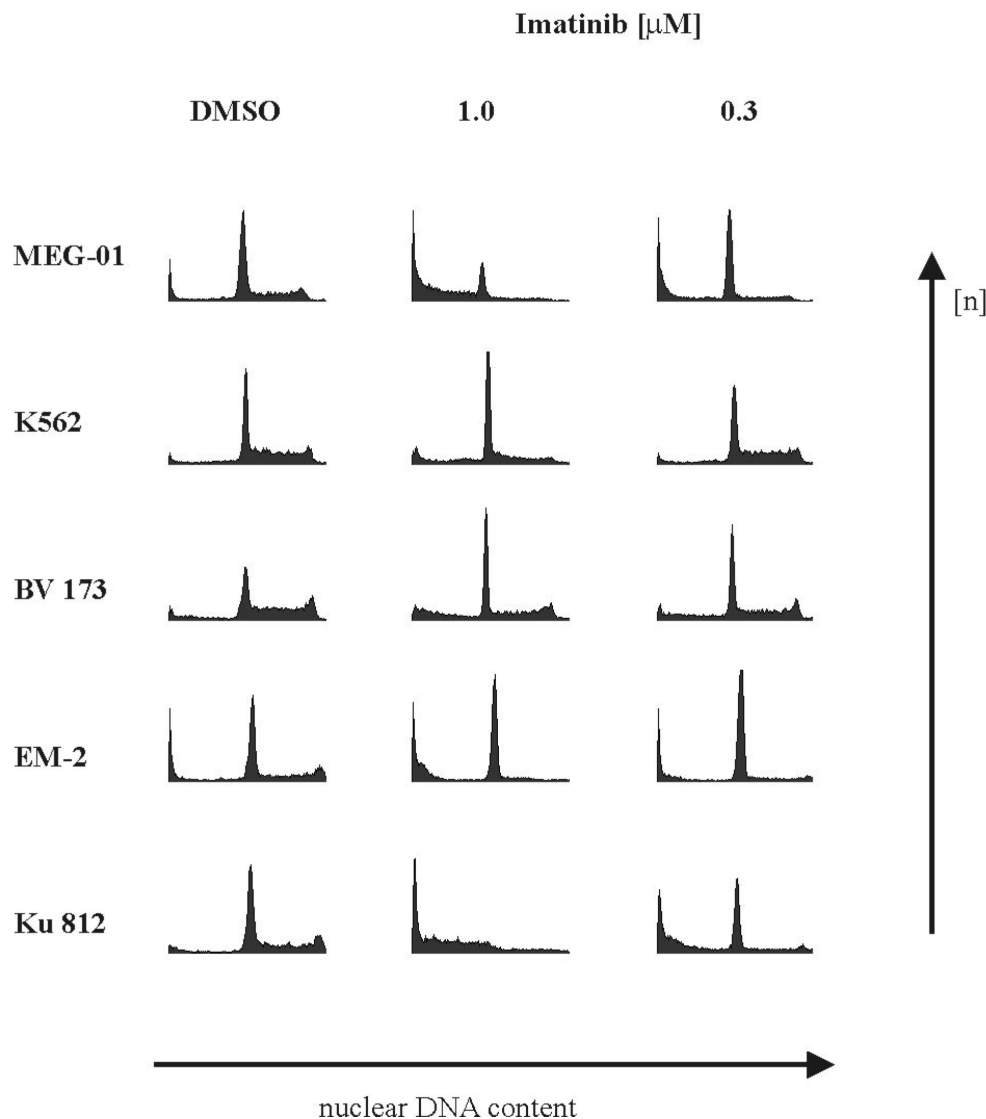

Figure 23: **Imatinib induces cell cycle arrest and apoptosis.** Five Bcr/Abl positive leukaemia cell lines were treated with Imatinib or solvent alone (DMSO) for three days. Cell cycle distribution was monitored by flow cytometric analysis of nuclear DNA content. Graphic settings are DNA content on x-axis and number of cells on y-axis. The central peak represents the population of diploid cells in G1.

To answer the question whether ERK5 is involved in survival pathways of Bcr/Abl positive leukaemia cells, endogenous ERK5 levels of MEG-01 cells were reduced by stable transfection of an ERK5-specific small interfering RNA (siRNA) generating vector (Fig. 24*B*). The phenomenon of RNA interference (RNAi) was first discovered in the nematode *Caenorhabditis elegan*s as a response to double-stranded RNA which results in sequence-specific gene silencing based on directed mRNA degradation (Fire et al., 1998). Nowadays RNAi has also found multiple applications in mammalian cells. The design of vectors using the H1-RNA promoter to generate siRNAs has further broadened the field of application and allows the study of long-term phenotypes (Brummelkamp et al., 2002a; Brummelkamp et al., 2002b).

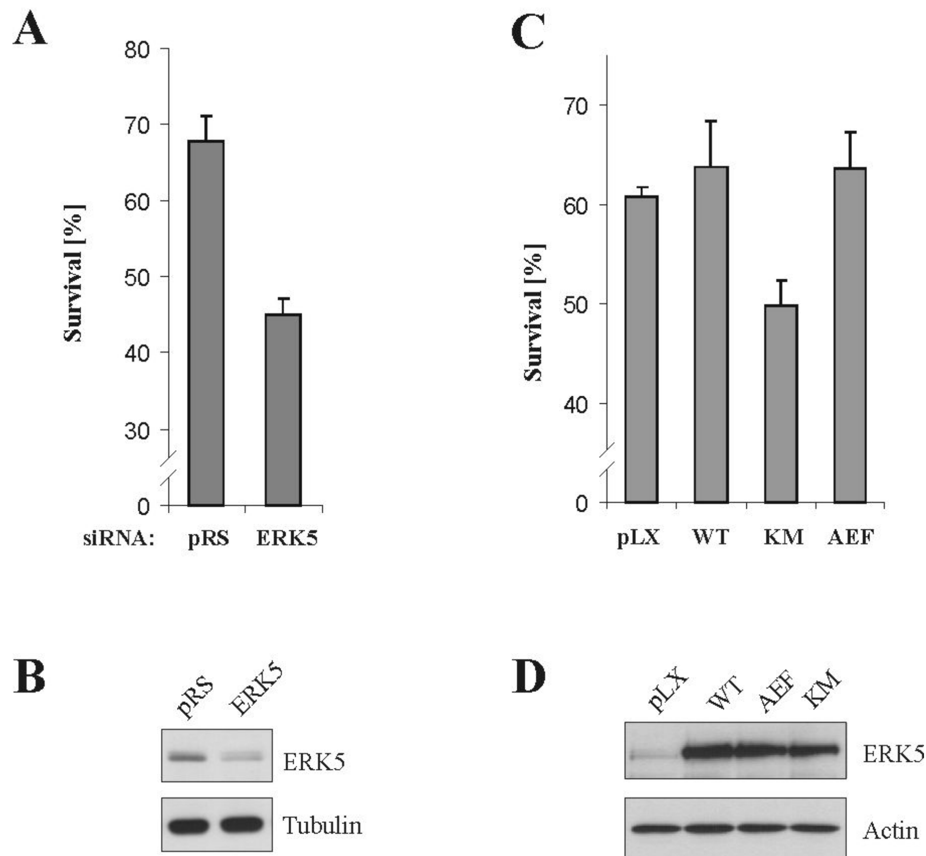

Figure 24: **ERK5 mediates cell survival after transient Imatinib treatment.** *A*, MEG-01 cells were infected with a retroviral pRetroSUPER construct generating ERK5-specific siRNA and selected for three days with puromycin. Polyclonal lines were treated with 0.3 μM Imatinib for two days and cell survival was assessed 24 hours after drug removal by flow cytometric analysis. *B*, expression levels of ERK5 and Tubulin are shown by immunoblot analysis of crude cell lysates. *C*, MEG-01 infected with and selected for retroviral expression vectors for wild-type ERK5, KM, AEF or the empty vector (pLX) were treated and analysed as described in *A*. D, crude lysates were again analysed by anti-ERK5 and anti-Tubulin immunoblot.

Cells were treated with low concentrations of Imatinib for two days and cell survival was analysed 24 hours after agent removal. As shown in Figure 24*A* survival was reduced by about one third in cells with reduced ERK5 expression. Over-expression of the kinase dead KM mutant was also able to negatively interfere with cell survival while wild-type ERK5 and the AEF mutant had virtually no effect (Fig. 24*C* and *D*). Comparably to the observations made in the focus formation study this underlines the difference between non-activatable AEF and kinase dead KM mutant and further indicates that basal activity of ERK5 but not MEK5 dependent activation is required for cell survival.

Contrary to many cellular signalling pathways which are transiently switched on by diverse stimuli, survival pathways should be constitutively active in order to fulfil their anticipated function. Since basal kinase activity of ERK5 seems to be sufficient for cell survival, stabilisation and destabilisation of the ERK5 protein could comprise a way of regulation.

3.3 Patterns of ERK5 Expression, Localization and Activation

3.3.1 Expression and Localisation of ERK5

Diverse tissues were tested positive for ERK5 and MEK5 mRNA transcripts (English et al., 1995; Lee et al., 1995) and the ERK5 protein was further detected in many and diverse cell types including cortical neurons and myoblasts (Cavanaugh et al., 2001; Dinev et al., 2001). In order to analyse ERK5 expression specific antibodies were generated. A stretch of about 100 amino acids taken from the unique C-terminus of ERK5 was fused to glutathione-S-transferase and subsequently used for immunization of rabbits.

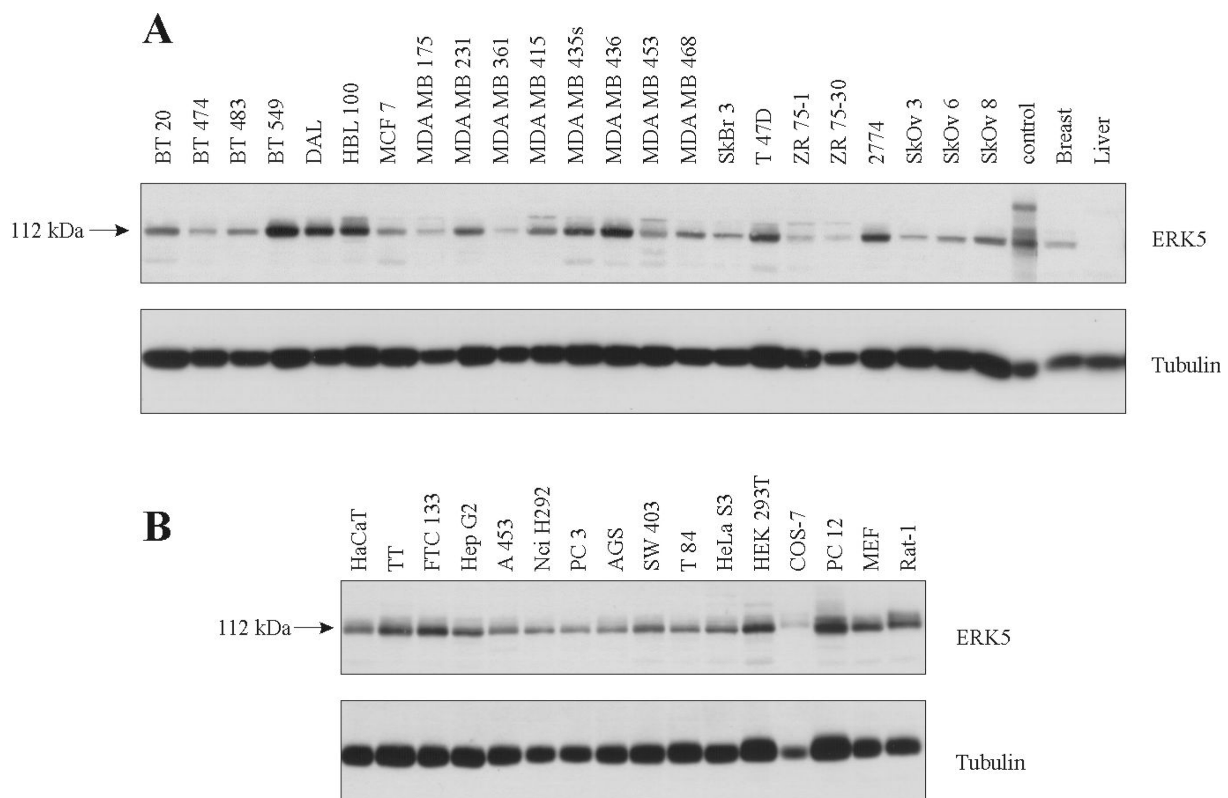

Figure 25: **Expression of ERK5.** Crude cell lysates from different human and non-human cancer and non-cancer cell lines as well as mouse liver and breast tissues were analysed by immunoblot using anti-ERK5 and anti-Tubulin antibodies: *A*, breast cancer cell lines (BT 20 to ZR 75-30) and ovary cancer cells (2774 to SkOv 8) and *B*, various human cell lines (HaCaT to HEK 293T), avian COS-7 and rodent PC 12, MEF and Rat-1 cells.

As shown in Figure 25*A* and *B* the generated antibody readily detected a specific band migrating at approximately 112 kDa in SDS-PAGE. Albeit to different extents, ERK5 was expressed throughout in all cell lines tested.

The detected levels of ERK5 were highest in several breast cancer lines and in rat phaeochromocytoma PC12 cells. Since the domains of ERK5, which were used as antigens,

are highly conserved among mammals, not surprisingly, rat and murine othologues of ERK5 were detectable in three tested rodent cell lines. Contrary to murine breast tissue, ERK5 expression was lowest and hardly detectable in liver. In agreement with earlier reports that implicated ERK5 in tumourigensis of breast cancer significant amounts of protein were found in 19 breast cancer cell lines as displayed in Figure 25*A*. One line of research suggested that ERK5 might mediate mitogenic signalling from neuregulin-activated HER2/HER3 receptors (Esparis-Ogando et al., 2002), others obtained evidence for the involvement of ERK5 in the onset of resistance to the chemotherapeutic drug doxorubicin (Weldon et al., 2002).

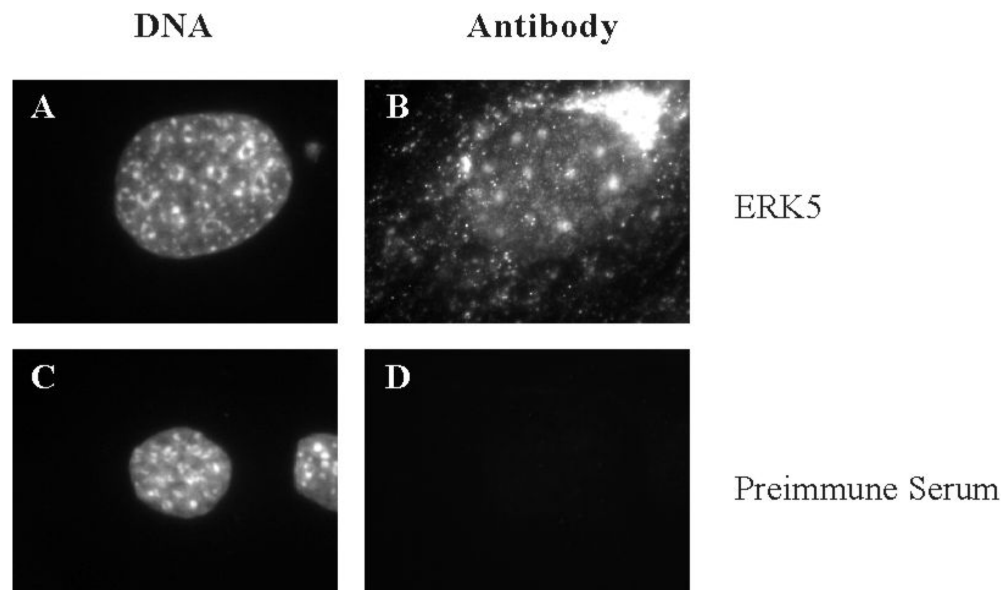

Figure 26: **Immunofluorescence analysis of ERK5 subcellular localisation in Swiss 3T3 fibroblasts**. Cells were fixed with paraformaldehyde, permeabilised with Triton-X100 and incubated with either anti-ERK5 or the corresponding preimmune serum (*B* and *D*, respectively). DNA counterstain of same sections of cells is shown in *A* and *C*, respectively.

Localisation of ERK5 was determined by immunofluorescence analysis. Due to their flat and stretched out shape fibroblasts are commonly used cells for these studies. Permeabilised Swiss 3T3 cells were stained with anti-ERK5 antibodies and the corresponding preimmune serum as control. As shown in Figure 26*B* specific staining for ERK5 was found in cytosol and nucleus, the latter being marked by its DNA stain (Fig. 26A). In particular perinuclear regions were strongly stained. Strikingly, most nuclear ERK5 resided in discrete spots, between 2 and 12 in number per nuclei. This could indicate that ERK5 might be concentrated in distinct subnuclear compartments. The best characterised nuclear bodies namely the nucleoli, however, can be excluded for two reasons: First, the number of observed spots exceeds the usual number of nucleoli and secondly, ERK5 does not colocalise with points of strongest chromatide stain. Nuclear ERK5 was also found in COS-7 and A431 cells (Fig. 14 and data not shown).

3.3.2 Activation of ERK5

In order to study the activation of ERK5 transfected COS-7 cells were treated with different agonists and exogenous HA-tagged ERK5 was immunprecipitated. Its capability to autophosphorylate in immuno complex kinase assays was used as measure for the degree of its activation. Figure 27*A* shows that osmotic as well as oxidative stress (imposed onto cells by addition of 0.4 M Sorbitol and 200 μM H_2O_2, respectively) enhanced ERK5 activity. Mitogenic stimuli like EGF and fetal calf serum were also able to activate ERK5.

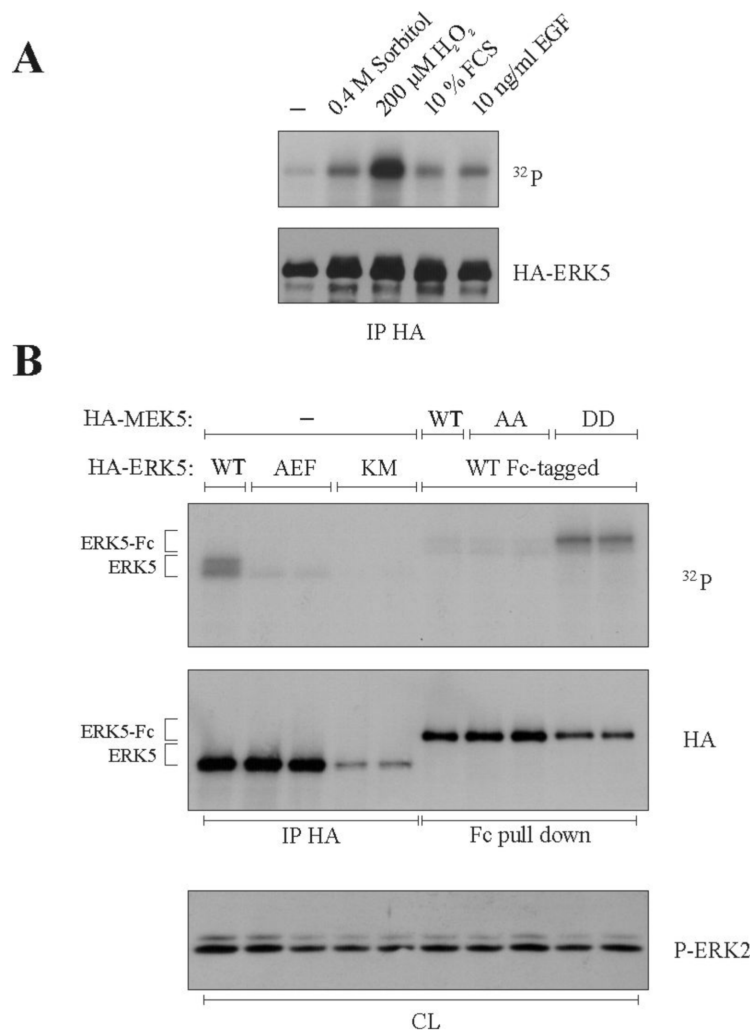

Figure 27: **Activation of ERK5.** *A*, transfected COS-7 cells expressing HA-tagged ERK5 were starved by serum deprivation over night and subsequently stimulated for 20 minutes as indicated. Autophosphorylation activity of anti-HA immunoprecipitates was monitored in a kinase assay (*upper panel*). Precipitated ERK5 was visualised by anti-HA immunoblot. *B*, COS-7 cells were either transfected with constructs for wild-type or mutant forms of HA-ERK5 alone or with Fc-tagged HA-ERK5 in combination with wild-type or mutant MEK5. The mutants used were: AEF – not activatable, KM - kinase dead, AA – not activatable and DD - constitutively active. HA-ERK5 and HA-ERK5-Fc were precipitated by anti-HA immunoprecipitation and by protein A sepharose pull down, respectively. The autophosphorylation activity of ERK5 was assessed as above (*upper panel*). The corresponding anti-HA immunoblot is shown in the *middle panel*. ERK2 activity was analysed by anti-Phospho-ERK2 immunoblot of crude cell lysates (*bottom panel*).

These results together with similar observations made by others (Abe et al., 1996; Kamakura et al., 1999; Kato et al., 1997) positions ERK5 between the mitogenic MAPKs ERK1 and ERK2 on one side and the stress-activated p38 and JNK subfamilies of MAPKs on the other side.

Although most components involved in the signalling cascade that ultimately leads to the activation of ERK5 are unknown, MEK5 has clearly been shown to be the MAPK kinase directly upstream of ERK5 and to specifically phosphorylate and thereby activate ERK5 (Kato et al., 1997; Lee et al., 1995). In order to interfere with the connection of MEK5 and ERK5 and to elucidate the importance of this pathway, mutants of both kinases were used. Since ERK5 is activated in analogy to other MAPKs by phosphorylation of threonine and tyrosine in its TEY activation motif, mutation of these two residues yielded the AEF mutant which cannot be activated.

As shown in Figure 27 *B* in comparison to the wild-type form the activity of ERK5 AEF was strongly reduced to a level that most likely reflected the basal activity of the kinase. The KM mutant of ERK5 bearing an exchange of the catalytically invariant lysine involved in ATP binding to methionine was rendered completely inactive. Similar to MAPKs MEKs are also activated by two phosphorylations, in the case of MEK5 on serine and threonine. Mutation of these residues to negatively charged aspartic acids (DD) mimics the phosphorylated form and results in constitutive activity. On the other hand exchanging these amino acids to alanines (AA) consequently creates a not activatable mutant. When co-expressed with ERK5 only constitutively active MEK5 DD potently activated ERK5 (Fig. 27*B*). A C-terminally Fc-tagged variant of ERK5 was used that allowed direct precipitation with protein A sepharose. The intriguing specificity of MEKs for only one or two MAPKs is a characteristic in MAPK signalling and is here exemplified since overexpression of neither form of MEK5 did influence the activity of ERK2, which is the closest homologue of ERK5 (Fig.27*B*, *bottom panel*).

To extend the results obtained from ectopic expression experiments the activation pattern of ERK5 was further analysed in rat phaeochromocytoma PC12 cells and murine Swiss 3T3 fibroblasts. Almost a decade ago amplitude and duration of MAPK activity were shown to be critical for the determination cell fate. In the very same cell, MAPKs can be involved in the control of different and even opposing functions. In these cases the profile of MAPK activation specifies signal identity (Marshall, 1995). In PC12 cells EGF as well as nerve growth factor (NGF) activate ERK2, however, cells proliferate in response to the first stimulus and differentiate in the presence of the latter (Cowley et al., 1994; Traverse et al.,

1994). These distinct responses were attributed to the ability of NGF but not EGF to cause a sustained activation of MAPK.

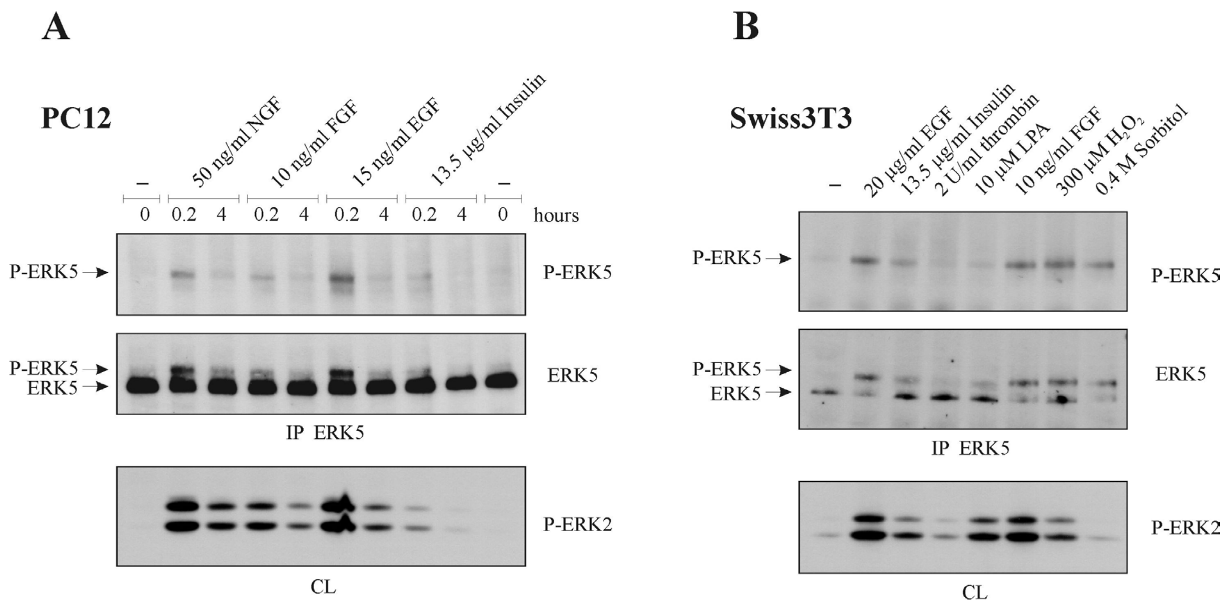

Figure 28: **ERK5 activation in PC 12 and Swiss 3T3 cells.** *A* and *B*, cells were starved over night by serum withdrawal and stimulated as indicated. Swiss 3T3 cells were stimulated for 15 minutes. Anti-ERK5 immunoprecipitates (IP) were analysed by anti-Phospho-ERK5 (P-ERK5) and anti-ERK5 immunoblot. Activation of ERK2 was assessed by anti-Phospho-ERK2 (P-ERK2) immunoblot of crude cell lysates (CL).

Here, the activation of ERK5 and ERK2 in PC12 cells was analysed by Western blot again using the phosphorylation state-specific antibodies that exclusively detect the activated forms. ERK5 was activated by NGF and EGF and to a lower extent by FGF and Insulin. Activation was transient and strongly reduced but still detectable after 4 hours (Fig. 28*A*). Phosphorylation correlated with the mobility shift of a small fraction of ERK5. These shifts had been widely used as measure for MAPK activation before phosphorylation state-specific antibodies became commercially available. The ratio between shifted and unshifted ERK5 indicates that only a minor fraction of all ERK5 was activated. Interestingly, an additional faint band of a faster migrating variant of ERK5 was also detected by anti-Phospho-ERK5 immunoblot but was not resolved in the reblot below (Fig. 28*A*, *top and middle panel*). The pattern of ERK2 activation as shown in the *bottom panel* resembled the one of ERK5.

Contrary in Swiss 3T3 cells, ERK5 was activated and almost completely shifted upon stimulation with various agents ranging from growth factors to osmotic stress (Fig. 28*B*). Further comparison of ERK2 and ERK5 revealed differential activation in that ERK2 but not ERK5 was potently activated by lysophosphatidic acid (LPA) and vice versa upon sorbitol stimulation.

To answer the question whether the observed shift of ERK5 is indeed exclusively caused by phosphorylations, calf intestine alkaline phosphatase (CIAP) was used to quantitatively remove any phosphoryl group. About one half of all ERK5 was shifted when immunoprecipitated from exponentially growing COS-7 cells.

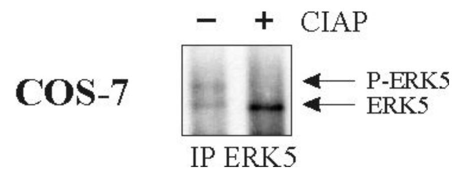

Figure 29: **Phosphorylation induced mobility shift of ERK5.** ERK5 was immunoprecipitated (IP) from COS-7 cell lysates and treated with calf intestine alkaline phosphatase (CIAP) to quantitatively remove phosphorylations. An anti-ERK5 immunoblot is shown.

Treatment with CIAP resulted in the disappearance of the upper band and condensed the total ERK5 protein to an entity migrating as a single band. The observed shift of ERK5 is thus in fact solely caused by phosphorylations. However, it still remains possible that other phosphorylation events beside the activating modifications of the TEY motif might occur and contribute to the mobility shift of ERK5.

3.3.3 Modulators of ERK5 Activation

Various studies have shown that cellular responses to LPA and other ligands of G-protein coupled receptors (GPCRs) require the function of the EGF receptor (EGFR) in several cell systems (Daub et al., 1997; Luttrell et al., 1999; Marinissen and Gutkind, 2001). Further it was shown that this phenomenon termed EGFR signal transactivation involves the shedding of membrane bound EGFR ligand precursors by metalloproteinases (Prenzel et al., 1999), which can be blocked by the hydroxamic acid derivative batimastat (BB94). Depending on the cellular context different members of the metalloprotease-disintegrin (ADAM) family are responsible for EGFR transactivation (Geschwind and Hart, personal communication). Thereby, EGFR has been shown to be an integral component of the pathway linking GPCR activation to the MAPKs ERK1 and ERK2 (Gschwind et al., 2002; Zwick et al., 1997).
In order to test whether ERK5 is also activated by GPCR ligands in an EGFR-dependent manner, transfected and serum deprived COS-7 cells were treated with thrombin and LPA. Both GPCR ligands as well as the control stimulus EGF potently activated ERK5 as again monitored by its capacity to autophosphorylate in a kinase assay (Fig. 30*A*).

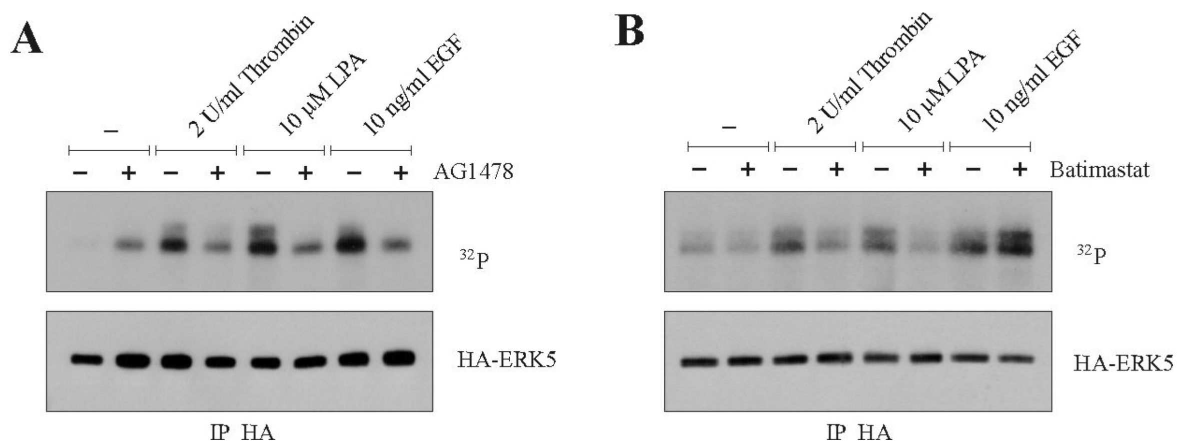

Figure 30: **EGFR mediates ERK5 activation by GPCR ligands.** 16 hours after serum withdrawal, transfected COS-7 cells were stimulated with Thrombin and LPA for 15 minutes or EGF for 5 minutes. Cells were preincubated with 250 nM of EGF Receptor inhibitor AG1478 (*A*) or 10 µM Batimastat (*B*) for 30 minutes. Autophosphorylation activity of HA-ERK5 was assessed in anti-HA immuno complex kinase assays (*upper panels*). *Lower panels* show the corresponding anti-HA immunoblots.

Pre-treatment of cells with the specific EGFR receptor inhibitor AG1478 blocked activation of ERK5 by either ligand. Most notably, the activity of ERK5 in unstimulated control cells was slightly enhanced upon addition of AG1478 which might comprise a stress stimulus itself under these conditions. Alternatively, this result might indicate that negative pathways that render ERK5 inactive, are controlled by basal EGFR activity. The involvement of transactivational events in ERK5 activation by LPA and Thrombin was further exemplified using the metalloprotease blocker batimastat, which impaired ERK5 activation by both GPCR ligands but not by EGF (Fig. 30*B*).

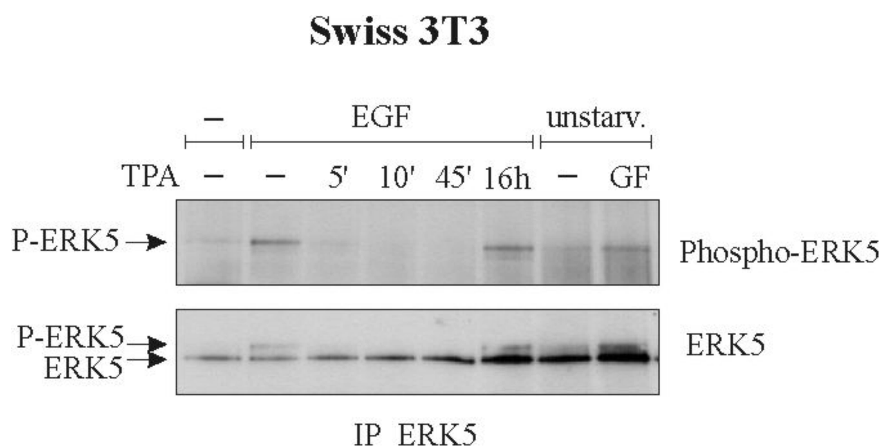

Figure 31: **PKC inhibits ERK5.** Swiss 3T3 cells were serum starved over night, preincubated with 1 µM of the phorbolester TPA for 5 minutes (‘) and up to 16 hours as indicated and then stimulated with 20 ng/ml EGF for 5 minutes. Alternatively, unstarved cells were treated with the PKC inhibitor GF-109203X for 30 minutes. ERK5 immunoprecipitates were analysed by anti-Phospho-ERK5 (P-ERK5) and anti-ERK5 immunoblot (*top* and *bottom panel*s, respectively).

In the search for effectors of ERK5 various commercially available inhibitors were screened for their ability to interfere with the activation of ERK5 (not shown). Treatment of unstarved Swiss 3T3 fibroblasts with the GF109203X inhibitor of the protein kinase C (PKC) family of kinases resulted in the induction of ERK5 activity. As shown in Figure 31 activation of PKC by addition of the phorbol ester TPA completely counteracted activation of ERK5 by EGF. Addition of PKC inhibitor to unstarved cells induced ERK5 activity. In line with these observations it was shown that ERK5 was activated by granulocyte colony-stimulating factor in murine BAF-3 cells and that this activation was also inhibited by TPA and reinforced by co-administration of PKC inhibitor, while ERK2 activation was not affected (Dong et al., 2001). These results indicate that members of the PKC family are involved in the negative regulation of ERK5.

3.3.4 The C-Terminus of ERK5 determines Kinase Activity and Localisation

The most pronounced difference between ERK5 and other members of the MAPK family is certainly its unique C-terminal tail. Two proline-rich domains are the only striking features within this 400 amino acid long extension. To gain some information on its function, various C-terminally truncated mutants were created. Numbers given in Figure 32 indicate the last amino acids of each shortened ERK5 variant. The shortest 409 form reduces ERK5 to its MAPK domain which shares high homology with ERK1 and ERK2. All mutants were readily detected in crude lysates of transfected COS-7 cells by Western blot using antibodies directed against an N-terminal hemagglutinin (HA) tag (Fig. 32*A*). Performance of in vitro kinase assays revealed that each truncated mutant was at least 6-fold more active than the wild-type form (Fig. 32B). Removal of the last 100 amino acids was sufficient to potentiate ERK5 activity. Likewise, it was reported elsewhere that dominant active RasV12 more potently activated a mutant lacking the C-terminal 400 amino acids than full-length ERK5 (English et al., 1998).

Taken together this leads to the hypothesis that the tail of ERK5 might have an autoinhibitory domain. Since the C-terminal tail contains several consensus phosphorylation sites for MAPKs and since ERK5 was shown to autophosphorylate, the incorporation of negatively charged phosphoryl groups into C-terminal domains might be a trigger for conformational changes and subsequent release of autoinhibition. However, validation of this hypothesis will require a more detailed analysis of kinetics underlying kinase activation. Elucidation of the

ERK5 crystal structure will be of great value in order to clarify the exact role of the C-terminal tail in ERK5 kinase action.

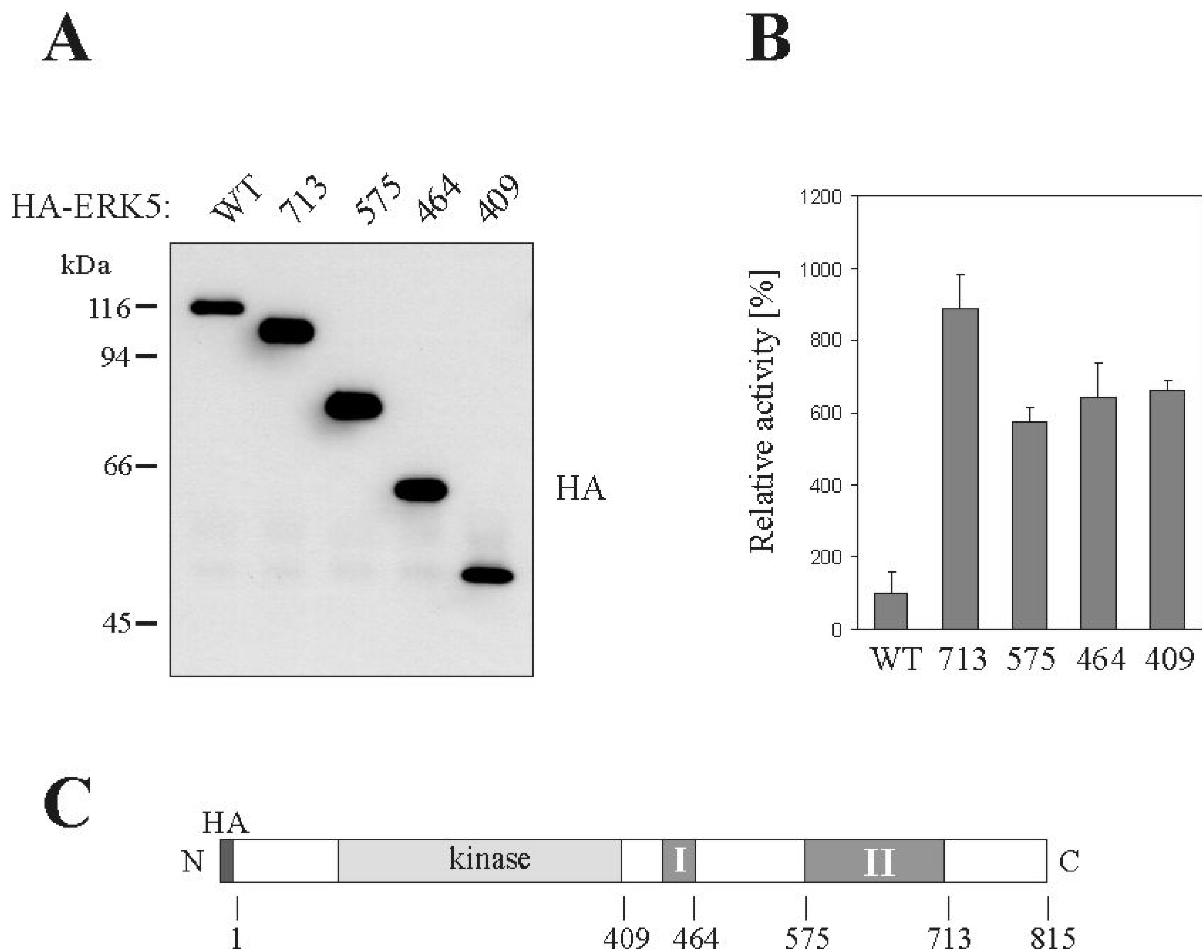

Figure 32: **Autoinhibition of ERK5 by its C-terminus.** COS-7 cells were transfected with contsructs for HA-tagged ERK5 and different truncated mutants (numbers refer to the last amino acid). *A*, expression is shown by anti-HA immunoblot of crude cell lysates. *B*, anti-HA immunoprecipitates were subjected to kinase assays and autophosphorylation activity was assessed. Data represents normalised relative activity ± SD. *C*, schematic presentation of ERK5 with indicated points of truncation: I and II, proline rich domains.

MAPK substrates are located in the cytoplasm as well as in the nucleus. The translocation of MAPKs to the nucleus is a requirement for the phosphorylation of many transcription factors and thus the regulation of transcription (Karin and Hunter, 1995; Whitmarsh and Davis, 2000).

Having shown that ERK5 indeed resides in both major cellular compartments, cytosol and nucleus, the truncated mutants were used to address the question whether the C-terminus does contribute to the subcellular localisation of ERK5. Transfected COS-7 cells were analysed by immunofluorescence using the HA-tag as epitope. Localisation of ectopic wild-type ERK5 was predominantly cytosolic as shown in Figure 33*A*.

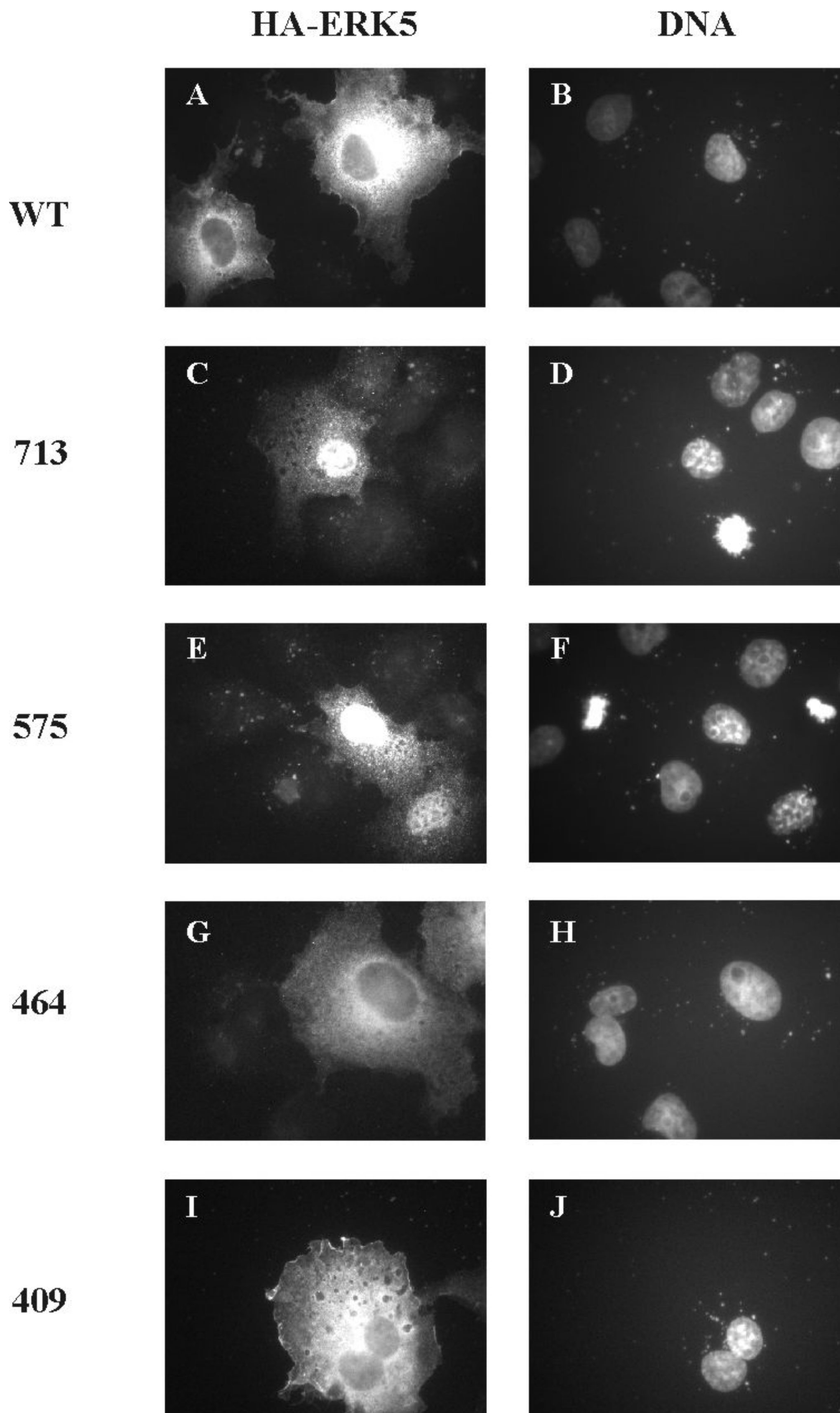

Figure 33: **The C-terminus of ERK5 regulates nuclear localisation.** COS-7 cells were transfected with wild-type ERK5 or different deletion mutants. 24 hours after gene transfer cells were fixed with methanol and incubated with anti-HA antibodies (*A*, *C*, *E*, G and *I*). Right panels show the DNA counterstain of the same sections.

In contrast the 713 mutant that lacked only the last 100 amino acid was primarily found in the nucleus. While similar results were obtained for the 575 mutant, further truncation led to a distribution that strongly resembled the wild-type form. Among the plethora of possibilities the easiest explanation would be the following: Localisation of ERK5 mutants is determined by active import and export sequences within the C-terminus and removal of one or the other directly affects the equilibrium of ERK5 shuttling in and out the nucleus. The recent identification of a putative nuclear localisation in the linker between the two proline rich domains provides some evidence to the credibility of this hypothesis (Yan et al., 2001).

The C-terminus of ERK5 is an unique feature among MAPKs and has been the fundament for the classification of ERK5 as a fourth and self-contained MAPK subgroup besides the families of JNK, p38 and ERK2. Future studies unravelling the unknown functions of this tail can be expected to largely contribute to the understanding of the very particular role of ERK5 in cellular signalling.

4 DISCUSSION

MAPKs are crucial elements in the process converting extracellular signals into distinct transcriptional changes. Although virtually all kinds of extracellular stimuli lead to the activation MAPKs they evoke very distinct cellular responses. The activation profile of MAPKs and the pattern created by the activation of a certain subset of MAPK family members critically determine signal identity and ultimately cell fate. Therefore, understanding the mechanisms of MAPK regulation will elucidate some fundamental aspects of how cells are able to adapt to a constantly changing environment.
The role and the regulation of ERK5 were studied in various cellular systems and diverse contexts. The study highlights two mechanisms of ERK5 regulation: control of activation loop phosphorylation and regulated protein stability. Phosphotyrosine-specific phosphatases (PTPs) and Abl kinases were shown to interact with and consequently to modify the activity of ERK5. On one hand so called KIM containing PTPs were demonstrated to be involved in the negative regulation of ERK5. The non-receptor tyrosine kinase Abl on the other hand and in particular its oncogenic forms activated and stabilised ERK5 which contributed to transformation and leukaemia cell survival.

4.1 Aspects of ERK5 Expression, Activation and Localisation

For along time MAPKs were divided into three subfamilies, ERKs, JNKs and p38 kinases. The cloning of ERK5 in 1995 gave rise for the introduction of a fourth MAPK subgroup. In contrast to the other three types of MAPKs, orthologues of ERK5 do not exist in yeast, the nematode *Caenerohabditis elegans* or *Drosophila melanogaster*. From the evolutionary point of view ERK5 is thus a younger gene product. ERK5 comprises two major domains of comparable size: a MAPK domain sharing high similarity with full-length ERK2 and a unique C-terminal tail. In the following, the characteristics of ERK5 will be discussed in comparison to its closest homologue ERK2.
ERK5 expression was detected in various and virtually all cell types tested. Similar to other MAPK ERK5 thus seems to be ubiquitously expressed. Notably, levels of ERK5 varied more strongly between different cell lines than those of ERK2. Though, the most striking differences between ERK5 and ERK2 lay in the profile and the dynamics of their activation. As indicated by shift and phosphorylation analysis, only a small fraction of all ERK5 was activated, whereas strong mitogens for instance EGF induce the activation of virtually all

cellular ERK2. The low percentage of activated ERK5 might be the reason why many laboratories encountered problems in detecting active and phosphorylated species of ERK5 (M. Burow and J. Barros, personal communication). Furhermore, activation of ERK5 is not only induced by mitogens but also by cellular stress such as high osmolarity and reactive oxygen species. This particular responsiveness to such different external stimuli positions ERK5 between the mitogen-activated ERK family and the stress-activated JNK and p38 kinases.

Finally, ERK5 was found to localise in the cytosol as well as in the nucleus. This was surprising because MAPKs are usually localised in the cytosol and thereby in the same compartment as the components of the activating cascade until they become activated and transiently translocate to the nucleus. In contrast, ERK5 resided in the nucleus even under unstimulated conditions. The identification of a putative nuclear localisation sequence in the C-terminal tail of ERK5 might account for its particular cellular distribution. The use of different C-terminally truncated mutants of ERK5 implied that its unique domains do not only regulate nuclear import but also the export albeit by an unknown mechanism.

The C-terminal tail structurally distinguishes ERK5 from any other MAPK and was thus expected to account at least for some functional differences as well. Further results of study as well as observations reported elsewhere (English et al., 1999b) suggest that the C-terminal tail might be involved in kinase regulation by an autoinhibitory mechanism. Intramolecular binding of domains that lock a kinase in an inactive conformation is a common theme in kinase regulation and has most extensively been studied for the tyrosine kinase Src (Tatosyan and Mizenina, 2000). Whether the C-terminal tail of ERK5 is also responsible for the relatively low percentage of activated ERK5 proteins remains to be elucidated.

In summary, ERK5 differs in its activation pattern and its subcellular localization from other MAPKs and can thus be expected to play a particular and at least partially autonomous role in cellular signalling.

4.2 PTP-SL in the negative Regulation of ERK5

ERK5 is one of the least examined members of the MAPK family. Similar to ERK1 and ERK2, dual phosphorylation of threonine and tyrosine residues in the kinase-activating motif TEY is crucial for ERK5 activation. Since in analogy to ERK1/2 dephosphorylation of either residue is potentially sufficient for inactivation of ERK5 (Canagarajah et al., 1997), the

question was addressed whether protein tyrosine phosphatase PTP-SL might be involved in the regulation of the ERK5 pathway.

PTP-SL did not only bind to ERK5 but also modulated kinase activity *in vitro* and *in vivo*. Overexpression of wild-type PTP-SL in PC12 and COS-7 cells reduced endogenous as well as exogenous ERK5 activity, whereas the catalytically inactive CS mutant of PTP-SL obviously enhanced kinase activity. The positive effect of the inactive phosphatase on kinase activity might be due to the competition between PTP-SL and endogenous phosphatases for ERK5 binding sites which suggests that ERK5 might be under permanent control of phosphatases that presumably belong to the KIM-containing PTP family.

Whereas inactivation of ERK5 by PTP-SL monitored *in vitro* is most probably simply due to the dephosphorylation of tyrosine 220 in the activating TEY motif (Lee et al., 1995; Zhou et al., 1995), the *in vivo* situation might be much more complex. PTP-SL is potentially able to affect the ERK5 pathway in several ways. Overexpression of wild-type as well as catalytically inactive PTP-SL, for instance, reduced the amount of ERK5 in the nucleus. Zuniga et al described a similar influence of PTP-SL on the localisation of ERK2 (Zuniga et al., 1999). Since translocation of MAPKs to the nucleus is essential in order to phosphorylate transcription factors that subsequently regulate gene activity (Karin and Hunter, 1995; Marshall, 1995), retention of ERK5 in the cytoplasm might reflect an additional mechanism by which PTP-SL can modulate the action of ERK5.

A further consequence of the interaction of ERK5 with PTP-SL is the phosphorylation of the phosphatase. The fact that phosphorylation could be localised to the juxtamembrane domain of PTP-SL alludes to threonine 253 as modified amino acid in view of that it is also phosphorylated by ERK2. Not only PTP-SL but also other members of this PTP family were phosphorylated by both ERK2 (Pulido et al., 1998; Saxena et al., 1999a) and ERK5. Even though it has been shown that phosphorylation of a specific serine residue in the KIM of PTP-SL and HePTP by PKA triggers the release of ERK2 (Blanco-Aparicio et al., 1999; Saxena et al., 1999b), it is unlikely that the observed phosphorylation of PTP-SL by ERK5 directly influences the binding properties between these two enzymes since no change in complex formation of purified PTP-SL and ERK5 protein was observed after *in vitro* phosphorylation (data not shown). Interestingly, phosphorylation of PTP-SL by ERK5 had only a minor influence on enzymatic activity; binding of the kinase to the phosphatase, however, enhanced phosphatase activity substantially. A similar effect was shown for the MAP kinase phosphatase (MKP) 3, a dual specificity phosphatase that is readily activated by formation of a stable complex with ERK2 (Camps et al., 1998).

The effects on MAPK signalling exerted by MKPs and by KIM containing PTPs are generally considered to be temporarily and spatially distinct from each other (Keyse, 2000). Saxena and colleagues (Saxena and Mustelin, 2000) have proposed a 'sequential phosphatase model' in which KIM containing PTPs control MAPK activity during their initial cytosolic phase whereas, after translocation to the nucleus, termination of a sustained MAPK signal would be accomplished by MKPs. We found that PTP-SL does not only reduce ERK5 activity but also hampers its translocation to the nucleus. This result adds a new aspect to the regulation of MAPK by KIM-containing PTPs.

Unlike ERK5 which was detected in tissues and cell lines of various origin and thus seems to be ubiquitously expressed (Abe et al., 1996; Cavanaugh et al., 2001; Dinev et al., 2001) PTP-SL expression was shown to be restricted and predominantly found in brain and neuroendocrine cells (Augustine et al., 2000; Hendriks et al., 1995; Ogata et al., 1995; Sharma and Lombroso, 1995; Shiozuka et al., 1995). Since other members of the same PTP family such as HePTP (Zanke et al., 1992) show a similarly restricted expression pattern, it is conceivable that specificity of the ERK5 signal is further determined by different KIM containing PTPs in a cell type dependent manner.

The direct control of MAPK activity by phosphatases is one of the most important regulatory mechanism in MAPK signalling. In analogy to other MAPK family members different types of phosphatases tightly regulate the activity of ERK5. The cytosolic KIM containing PTPs affect ERK5 not only by dephosphorylation but also by interfering with the nuclear translocation of ERK5.

4.3 ERK5 and Abl Tyrosine Kinases

4.3.1 Oncogenic Abl activates and stabilises ERK5

The non-receptor tyrosine kinase (NRTK) c-Abl and its oncogenic forms were shown to interact with ERK5. This association resulted in the activation, tyrosine phosphorylation and stabilisation of ERK5.

Abl kinases and in particular the Bcr/Abl fusion protein were known to activate multiple MAPK pathways. Bcr/Abl dimerises and autophosphorylates and thus mimics an activated receptor tyrosine kinase (RTK). Activation of the ERK1 and ERK2 occurs then in analogy to EGF receptor signalling by recruitment of adaptor proteins, Sos mediated activation of Ras

and subsequently Raf activation. Raf is the first kinase in the three-tethered MAPK cascade and thus consequently leads to the activation of ERK1 and ERK2.

As shown in this work and by others many RTKs also activate ERK5 (Buschbeck et al., 2002; Dong et al., 2001; Kamakura et al., 1999; Karihaloo et al., 2000; Kato et al., 1998). Although some of the components of the pathway activating MEK5 and consequently ERK5 are not known, the whole mechanism is presumably similar to the one described for ERK1 and ERK2. Thus Bcr/Abl also most likely activates ERK5 by its 'RTK mimicry' via the MAPK cascade containing MEK5 (illustrated in Fig. 34).

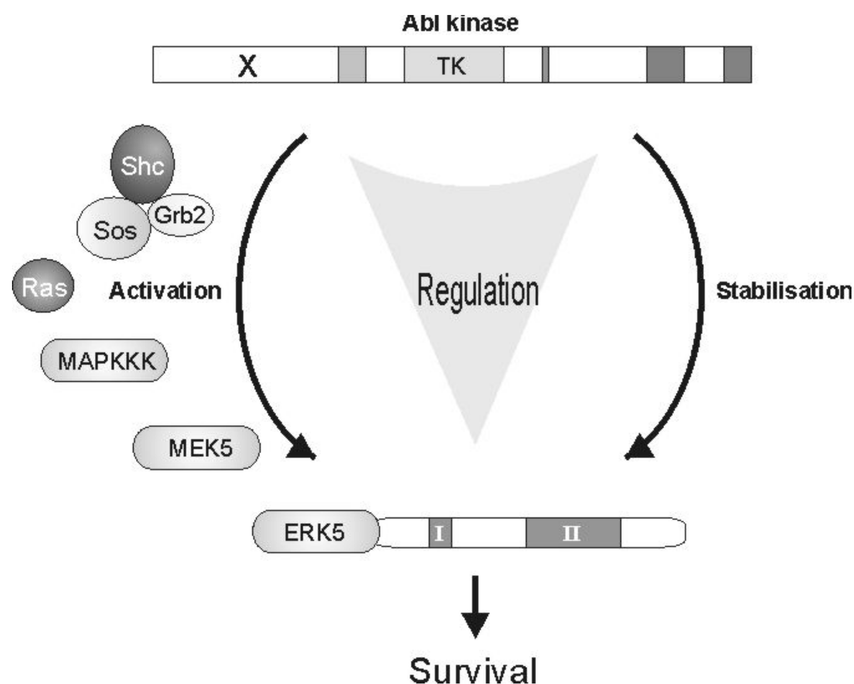

Figure 34: **Abl kinases regulate ERK5 by activation and stabilisation**. Activation of ERK5 by c-Abl or its oncogenic variants most likely occurs in analogy to receptor tyrosine kinase signalling via adaptor proteins, Ras and the MAPK cascade. Stabilisation on the other hand does not involve the MAPK cascade and thus represents an alternative way of regulation. TK, tyrosine kinase domain; X stands for the variable N-terminal domains of various Abl kinases e.g. Bcr portion.

Moreover, the ERK5 protein was shown to be stabilised by Abl kinases. Stabilisation was dependent on the kinase activity of Abl and could be blocked by the addition of the specific inhibitor Imatinib. Overexpression of c-Abl or any oncogenic variant of the kinase potently raised the level of ERK5. Abl kinases do thus not only regulate ERK5 by MAPK cascade mediated activation but also by stabilisation of the protein itself. Both mechanisms were independent of each other as shown by using mutants that were uncoupled from MEK5 mediated activation but still readily stabilised.

Protein domain mapping pointed to the proline rich domain I within the C-terminal tail of ERK5. by The identification of a putative Abl SH3 domain binding site within the proline rich

domain I of ERK5 with the Scansite algorithm (Yaffe et al., 2001) supported this result. Together these observations lead to the hypothesis that the C-terminal tail of ERK5 might be able to modulate kinase action by binding the SH3 domain of Abl which would subsequently stabilise the ERK5 protein.

The MEK5-independence of this mechanism is particularly interesting since stabilisation of ERK5 could still occur in compartments where ERK5 is separated from its activating kinase cascade. ERK5 shuttles in and out the nucleus, whereas MEK5 is exclusively cytosolic. In the nucleus Abl-mediated stabilisation of ERK5 could still occur and hence comprise an important parameter in ERK5 regulation.

Taken together, Abl kinases regulate ERK5 by at least two independent mechanism: first, activation via MEK5 dependent phosphorylation and second, by stabilisation of the ERK5 protein.

Analysis of ERK5 in Bcr/Abl expressing leukaemia cells indicated that basal activity but not MEK5 dependent activation is crucial for cell survival. MAPKs are involved in a multitude of cellular responses elicited by a broad variety of external stimuli. The amplitude and duration of MAPK activation determine signal identity and ultimately cell fate (Marshall, 1995). While strong stimuli such as mitogens are inducing a pulse of MAPK activity, stabilisation of the MAPK protein should be reflected by a persisting activation profile of low amplitude. Contrary to many cellular signalling pathways which are transiently switched on by diverse stimuli, survival pathways should be constitutively active in order to attain their anticipated function. Since basal kinase activity of ERK5 seems to be sufficient for cell survival, stabilisation and destabilisation of the ERK5 protein could represent a way of regulation. The recent observation that ERK2 degradation but not its inactivation by phosphatases is a prerequisite for sorbitol-induced apoptosis in fibroblasts (Lu et al., 2002) again substantiates the importance of regulating MAPK protein levels in the control of cell survival.

4.3.2 Tyrosine Phosphorylation and a potential Mechanism of ERK5 Stabilisation

The mechanism underlying the stabilisation of ERK5 by Abl remains largely elusive. Since stabilisation is dependent on the integrity of the Abl kinase domain, tyrosine phosphorylation is most presumably involved one way or the other. ERK5 itself was found to be tyrosine phosphorylated in Bcr/Abl positive leukaemia cells as well as in cells overexpressing any

other Abl kinase. Whether the phosphorylation of ERK5 contributes to its stabilisation is not known. Mapping and mutation of phosphorylation sites will help to answer this question.
Most cytosolic proteins are ubiquitinoylated by specific E3 ligases and subsequently degraded by the proteasome, a huge multiprotein complex. How could the addition of a phosphoryl group to a tyrosine residue interfere with this machinery? It is conceivable that such a chemical modification might hinder the binding of specific E3 ligases to their substrate. For instance, the immediate early gene product c-Fos has been shown to be indeed stabilised by phosphorylation (Chen et al., 1996; Okazaki and Sagata, 1995).
ERK5 contains seven tyrosine residues with reasonable phosphorylation probability according to the program Netphos (Blom et al., 1999). The phosphorylation of any of these sites of which all are located in the MAPK part and none in the C-terminal tail, could somehow influence protein stability. At least one of these seven tyrosine residues, namely the one in the activation motif, can be excluded from further analysis since the corresponding mutant was readily stabilised. Noteworthy, one of the remaining six tyrosine residues is located in the so-called MAPK docking motif. This motif is conserved among MAPKs from yeast to man and was shown to mediate the binding of ERK2 to regulators including phosphatases and the E3 ubiquitin ligase MEKK1 (Lu et al., 2002; Tanoue et al., 2000; Tarrega et al., 2002). This motif is also present in ERK5 and could mediate the interaction of ERK5 to a protein of the degradation machinery. If so, phosphorylation of the tyrosine within the docking motif might disrupt or at least hamper such an interaction and could thus contribute to a prolonged half-life of ERK5.
C-terminal domains of ERK5 in particular the proline rich domain I were shown to be important determinants for protein stabilisation. Deleting the complete C-terminal tail of ERK5 strongly reduced Abl mediated stabilisation but did not abolish it completely. Likewise, ERK2 not having any comparable C-terminal domains was stabilised to a weak extent that could be amplified by the addition of ERK5's tail. These observations lead to the speculation that although the C-terminal tail of ERK5 seems to mediate the interaction to Abl kinases, the resulting phosphorylation of one or more tyrosine residues within the MAPK domain might be required for stabilisation.

4.3.3 Functional consequences of interacting ERK5 and c-Abl

Although the involvement of ERK5 in signalling by oncogenic v-Abl as well as Bcr/Abl has been illustrated, the question what role a potential interaction of ERK5 and cellular c-Abl

might have in untransformed cells remains unkown. c-Abl is spacially and functionally divided in a cytosolic and a nuclear fraction. The best characterised function of nuclear c-Abl lies within the pathway inducing cell cycle arrest if DNA is severely damaged by ionising radiation or ultraviolet light (Shaul, 2000; Van Etten, 1999). In this context c-Abl was shown to be activated and to stabilise the gatekeeper gene product p73 which in turn induces cell cycle arrest or even apoptosis (Agami et al., 1999; Costanzo et al., 2002; Gong et al., 1999). Two characteristics of ERK5 point to putative involvement in c-Abl signalling. First, a considerable fraction of ERK5 resides in the nucleus. Second, ERK5 was shown to be activated in various stress situations and might thus could be sensitive to ionising radiation as well.

Most notably, p73 was recently shown to be phosphorylated on threonines adjacent to prolines which is the characteristic for MAPK phosphorylation consensus sites. This modification was clearly dependent on c-Abl and contributed to the stabilisation of p73 (Sanchez-Prieto et al., 2002). This phosphorylation has been attributed to p38 kinases and thus indicates that members of the MAPK family can indeed contribute to the regulation of p73. Future studies will have to show whether and how the interaction of ERK5 and c-Abl might contribute to the pathway linking DNA damage to the cell cycle machinery.

It is a long known fact that c-Abl has a DNA binding domain (Van Etten, 1999) and more recently the C-terminus of ERK5 was postulated to possess a transcriptional activation domain (Kasler et al., 2000). Whether a complex of both proteins could directly influence transcriptional processes remains speculative.

Similar to the non-receptor tyrosine kinase Src, the c-Abl kinase is rendered inactive by autoinhibition based on intramolecular binding of domains that lock the kinase in an inactive conformation. In the case of Src this mechanism involves the binding of a C-terminal phosphotyrosine residue to the SH2 domain (Tatosyan and Mizenina, 2000). In contrast Abl kinase activity is largely independent of its phosphorylation state but seems to be blocked by binding of the N-terminal stretch of 81 amino acids to several regions ‘across’ the molecule (Pluk et al., 2002). Since ERK5 most likely binds to the SH3 domain adjacent to this N-terminal cap, it might affect Abl activity by interfering with its autoinhibition. The product of the ataxia-telangiectasia-mutated gene Atm potently activates c-Abl and was shown to bind the SH3 domain (Shafman et al., 1997). However, Pag/Msp23 was also shown to bind to the SH3 domain of Abl but to exert an inhibitory influence on Abl kinase activity (Wen and Van Etten, 1997).

Although it is clear that Abl could affect ERK5 signalling in several ways, whether ERK5 may conversely influence Abl activity remains to be investigated.

4.4 MAPKs as potential Intervention Points in Cancer Therapy

Most anti-tumour agents despite having diverse mechanisms of action, ultimately mediate their effects by inducing apoptosis of tumour cells. The commitment of a cell to apoptosis or the ability to evade apoptosis after any kind of damage involves the integration of survival and death pathways. Under normal conditions survival signals are dominant over pro-apoptotic signals and sustain cells, keeping the death machinery at bay. It is well established that cell death can be triggered by two different mechanisms, either rapidly by activation of strong pro-apoptotic pathways e.g. those downstream of death receptors, or relatively slow by suppressing survival pathways. Therefore, dual therapy activating both mechanisms can be considered to be the most promising approach to the treatment of cancer. Efficacy of many chemotherapeutics that should actively induce apoptosis is severely reduced by primary resistance or the onset of *de novo* resistance to the drug. Particular in these cases combinational therapies additionally targeting survival pathways might proof to be powerful tools in preventing cancer progression caused by expansion of resistant clones.

One major survival signal is mediated by MAPKs ERK1 and ERK2 which are involved in transcriptional regulation of pro-survival factors (Ballif and Blenis, 2001; Fan and Chambers, 2001). Chemical compounds that interfere with the pathway leading to the activation of these MAPKs may have potential as chemotherapeutics themselves or as resensitising agents. Various inhibitors targeting different components of this signalling cascade are currently tested. They include for instance farnesyltransferase inhibitors blocking the formation active Ras and the Hsp90 inhibitor geldanamycin that destabilises the MAPKKK Raf. In leukaemia cells MEK1 inhibitors such as PD98059 and U0126 were shown to synergistically enhance the induction of apoptosis by Abl kinase inhibitor Imatinib (Yu et al., 2002). Noteworthy, the same inhibitors also – but less effectively – inhibited the MEK5-ERK5 pathway (Mody et al., 2001). This finding was the first hint that research on MAPK mediated survival has to be extended to ERK5.

The here reported correlation of ERK5 protein levels with leukaemia cell survival is one but not the only indication for an involvement of ERK5 in pro-survival signals. In neurons ERK5 was shown to mediate retrograde signals from axons to the cell body that also lead to cell survival (Watson et al., 2001). Further attention was drawn to ERK5 signalling by the analysis

of differential expression patterns in doxorubicin resistant and sensitive MCF-7 breast cancer cells. The anthracycline doxorubicin is a widely used chemotherapeutic whose potency in apoptosis induction is mainly attributed to its inhibition of topoisomerase II (Fan and Chambers, 2001). Weldon and colleagues showed that mRNA levels of MEK5 were strongly increased in resistant and thus surviving cells (Weldon et al., 2002). This observation implies that the activation of ERK5 by MEK5 is important for the survival of doxorubicin-treated MCF-7 cells. In contrast, survival of Imatinib-treated leukaemia cells was at least in part MEK5 independent but clearly required the intrinsic basal activity of ERK5. Nonetheless, these results establish a role for ERK5 in pro-survival signalling.

How could the knowledge about ERK5 signalling be applied in the context of cancer? In order to enhance the efficiency of Imatinib in the treatment of Bcr/Abl expressing leukaemia, inhibition of the ERK5 survival pathway could provide a potential way of chemotherapeutic intervention. Since ERK5 mediated survival is at least to some extent MEK5 independent, an effective therapeutic agent should directly impede ERK5 kinase activity rather than just interfere with the activating upstream kinase cascade like currently available compounds.

The inhibition of survival pathways mediated by MAPKs or other signalling molecules is considered as a modality in cancer therapy in general. ERK5 and its stabilisation by Bcr/Abl contribute the survival of leukaemia cells and should therefore be considered as an additional aspect in the therapy of chronic myeloid leukaemia.

4.5 ERK5 is a convergence point of Signal Transduction Pathways

MAPKs are generally considered to be points of convergence and integration of multiple incoming signals. The resulting activation profile of MAPKs as well as the activated subset of MAPKs will determine the cellular response. Not surprisingly, many different signalling pathways thus also affect the action of ERK5. As shown in this study and elsewhere various stimuli ranging from growth factors to cellular stress activate the kinase cascade leading to ERK5 (Kamakura et al., 1999; Karihaloo et al., 2000; Kato et al., 1998; Watson et al., 2001). Although many components of the pathway that link activated receptors to ERK5 are not known, the dual phosphorylation dependent activation of ERK5 is exclusively attributed to MEK5. The remarkable specificity of MEKs for only one or two particular MAPKs is a hallmark in MAPK signalling. So far the two MAPKKKs MEKK2 and MEKK3 were shown to be able to activate MEK5 in a context dependent manner (Chao et al., 1999; Garrington et

al., 2000). The alternate use of different MAPKKKs might couple the MEK5-ERK5 module to various activated receptors. Similar to JNKs and p38 kinases ERK5 is also activated by oxidative stress and hyperosmolarity (Abe et al., 1997). How these environmental stresses feed into the MAPK cascade is not clear. Since the activity of many kinases depends on their phosphorylation state inhibition of phosphatases by oxidation of their catalytical cystein is one possible mechanism for oxidative stress-induced MAPK activation. The molecular basis of osmolarity sensing is completely unknown. Moreover, this study shows that in addition to extracellular stimuli also the products of oncogenes evoke the activation of ERK5. Characterising the interaction of oncogenic Abl variants with ERK5 further revealed that ERK5 signalling can be modulated in more ways than just by MAPK cascade mediated activation. Protein stability for instance is an additional regulatory parameter and might be particularly important in survival pathways that do not require ERK5 activation but depend on its basal activity.

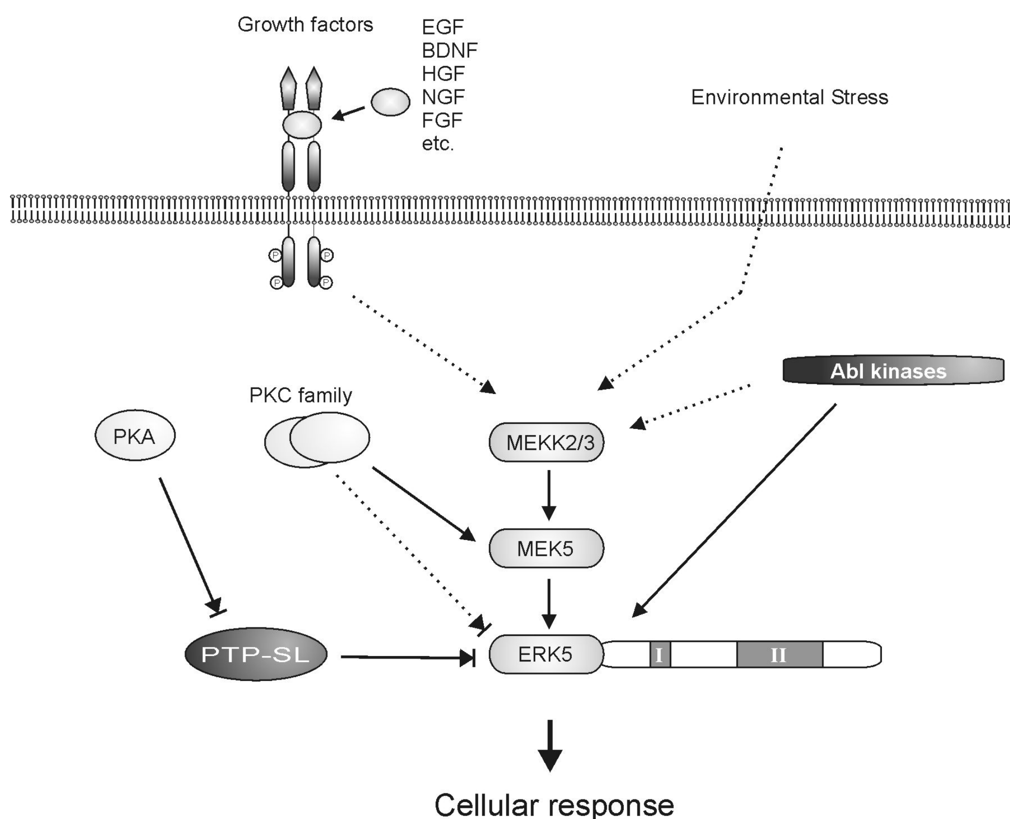

Figure 35: **Multiple pathways affect ERK5 signalling**. Growth factors and stress activate the MAPK cascade leading to ERK5 activation. PTP-SL inactivates ERK5 and interferes with its subcellular localisation. PKA and PKC indirectly affect ERK5 in several ways. Abl kinases feed into MAPK cascade dependent activation but also directly interact with ERK5 resulting in its stabilisation. Full lines indicate direct protein-protein interaction, dotted lines indicate indirect effects.

The finding that PTP-SL does not only dephosphorylate and inactivate ERK5 but also interferes with its translocation to the nucleus further exemplifies the multifaceted character of ERK5 regulation. To add to the complexity protein kinase A (PKA) was shown to phosphorylate PTP-SL and other members of its family on their kinase interacting motif and thereby to release MAPK from PTP dependent constraints (Blanco-Aparicio et al., 1999; Saxena et al., 1999b). Thus, PKA could indirectly contribute to ERK5 activity. Members of the protein kinase C (PKC) family were also implicated in the regulation of ERK5, their role, however, is rather ambivalent. On one hand, atypical PKC isoforms were shown to interact with MEK5 and to be sufficient for its activation (Diaz-Meco and Moscat, 2001). On the other hand as shown here and reported elsewhere (Dong et al., 2001) activation of PKC by phorbolesters inhibited ERK5 activation.

In summary, a large and still increasing number of pathways influence the ERK5 signalling pathway. The activation profile but also the localisation and the stability of ERK5 result from the integration of all these converging signals and ultimately determine the contribution of ERK5 to the cellular response.

4.6 Perspectives

Expression of ERK5 has been found in virtually all kinds of cells and ERK5 signalling has by now been implicated in a large – and with ongoing research increasing – number of cellular functions. ERK5 is shown in this study to be involved in the survival of Bcr/Abl expressing leukaemia cells treated with Imatinib. This raises the question whether the function of ERK5 in cell survival is restricted to this specific cellular context or whether it occurs in other cellular systems as well. Since research of two other groups focusing on neurons and breast cancer cells also attributed survival functions to ERK5 (Watson et al., 2001; Weldon et al., 2002), a rather general role for ERK5 in cell maintenance can be anticipated. Thus, it might be worthwhile to evaluate the potential of ERK5 inhibition as supportive element in the therapy of other kinds of cancer besides chronic myeloid leukaemia.

There is an increasing body of evidence that ERK5 is rather involved in developmental processes and cell differentiation than the regulation of proliferation as supposed previously. While an early report implied a function of ERK5 in cell cycle progression (Kato et al., 1998), the major part of current research points to a role for ERK5 in processes like cell specialization and differentiation. For example in C2C12 myoblasts ERK5 was shown to be essential for differentiation and myotube formation (Dinev et al., 2001). The importance of

ERK5 in development is manifestated by the embryonically lethal phenotype of ERK5 deficient mice. ERK5-/- embryos die between day E9.5 and E11.5 with angiogenic failure and severe cardiovascular defects (Regan et al., 2002; Sohn et al., 2002). This suggests an important role for ERK5 in the regulation of early angiogenesis. Further *in vivo* research applying conditional knock-out models or transgenic animals with inducible ERK5 expression will allow us to study the role of ERK5 in organogenesis and in later developmental stages.

Analysis of ERK5 in leukaemia cells revealed that rather the protein level of ERK5 combined with its basal activity than its activation by MEK5 are critical parameters in mediating cell survival. The question to what extent MAPK cascade-mediated activation of ERK5 on one hand and its protein level on the other contribute to its more complex functions provides future research with an interesting aspect.

5. SUMMARY

Mitogen activated protein kinases (MAPKs) are found in all eukaryotic cells and represent crucial elements in the signal transduction from the plasma membrane to the nucleus. Although a broad variety of extracellular stimuli activate MAPKs, they evoke very distinct cellular responses. The amplitude and duration of MAPK activation determine signal identity and ultimately cell fate. A tight and finely tuned regulation is therefore critical for a specific cellular response.

The role and the regulation of extracellular signal-regulated kinase 5 (ERK5), a MAPK with a large and unique C-terminal tail, were studied in different cellular systems. The study highlights two aspects of ERK5 regulation: control of the phosphorylation state and regulated protein stability.

In analogy to other MAPKs ERK5 is activated by dual phosphorylation of threonine and tyrosine residues in its activation motif. A first part of the study concentrates on whether and how the protein tyrosine phosphatase PTP-SL is involved in the downregulation of the ERK5 signal. The direct interaction of both proteins is shown to result in mutual modulation of their enzymatic activities. PTP-SL is a substrate of ERK5 and, independent of its phosphorylation, binding to the kinase enhances its catalytic phosphatase activity. On the other hand, interaction with PTP-SL does not only downregulate enzymatic ERK5 activity but also effectively impedes its translocation to the nucleus.

The second part of this study focuses on the interaction of ERK5 with c-Abl and its oncogenic variants Bcr/Abl and v-Abl. In this study these tyrosine kinases are demonstrated to regulate ERK5 by two mechanisms: first, by induction of kinase activity and secondly, by stabilisation of the ERK5 protein. Stabilisation involves the direct interaction of unique ERK5 domains with Abl kinases and is independent of MAPK cascade activation. The level of ERK5 and its intrinsic basal activity – rather than its activation – are essential for v-Abl-induced transformation as well as for survival of Bcr/Abl-positive leukaemia cells. Stabilisation of ERK5 thus contributes to cell survival and should therefore be considered as an additional aspect in therapy of chronic myeloid leukaemia.

Taken together, the results obtained in this study demonstrate that diverse pathways regulate ERK5 signalling by affecting kinase activity, localisation and protein stability. While the phosphatase PTP-SL is involved in negative regulation of ERK5, Abl kinases potently activate ERK5 and increase its half-life. Protein stabilisation thus is presented as a novel mechanism in the regulation of MAPKs.

6 ZUSAMMENFASSUNG (German Summary)

Eine wesentliche Grundlage für jede Form von Leben ist die Fähigkeit auf eine sich ständig ändernde Umgebung reagieren zu können. Jede einzelne Zelle muss in der Lage sein, äußere Reize wie zum Beispiel die Präsenz eines Hormons wahrzunehmen und dann angemessen, meist mit einer spezifischen Änderung der Genexpression, zu reagieren.

Auf molekularer Ebene nehmen die sogenannten Mitogen-aktivierten Proteinkinasen (MAPKn) eine zentrale Rolle in der Weiterleitung von Signalen von der Zellmembran zum Zellkern ein. Die Proteinfamilie der MAPKn lässt sich traditionell in drei Gruppen unterteilen, und zwar in die durch extrazelluläre Signale regulierten Kinasen (ERKs), die c-Jun aminoterminalen Kinasen (JNKs) und die durch Stress aktivierbaren p38 Kinasen. Mit der Klonierung der strukturell einzigartigen ERK5 musste eine weitere Untergruppe definiert werden (Lee et al., 1995; Zhou et al., 1995). Als einziges Mitglied der gesamten MAPK-Familie besitzt ERK5 C-terminal der homologen MAPK-Domäne einen etwa 400 Aminosäuren umfassenden Anhang mit bislang unbekannter Funktion. Die vorliegende Arbeit untersucht die Rolle und die Regulierung der ERK5 in verschiedenen Zellsystemen. Im Besonderen werden hier zwei grundlegende Mechanismen der Regulierung hervorgehoben: 1) die Kontrolle der Kinaseaktivität durch Phosphorylierung und Dephosphorylierung und 2) die Modulation der Proteinstabilität.

Analog zu anderen MAPKn wird ERK5 durch gleichzeitige Phosphorilierung eines Tyrosin- und eines Threoninrestes aktiviert (English et al., 1999a). Diese sogenannte duale Phosphorylierung von ERK5 wird ausschließlich der spezifischen Kinase MEK5 zugeschrieben. Um eine MAPKn zu inaktivieren reicht es allerdings aus eine der beiden Phosphatgruppen wieder zu entfernen (Canagarajah et al., 1997). Phosphatasen sind daher die Hauptantagonisten von MAPKn, da sie nicht nur die Phosphorylgruppen von deren Substrate entfernen, sondern auch die MAPKn selbst dephosphorilieren und somit inaktivieren können. Die Anzahl von Phosphatasen, die an der Negativregulation der ERK5 beteiligt sein könnten, ist recht groß und steht damit im Gegensatz zu der einzelnen aktivierenden Kinase.

Ein erster Schwerpunkt dieser Arbeit ist die Frage, ob und wie die phosphotyrosin-spezifische Phosphatase PTP-SL in die Modulation von ERK5-vermittelten Prozessen eingreifen könnte. Die PTP-SL gehört zu einer Familie von Phosphatasen, die ein sogenanntes Kinase-interagierendes Motiv besitzen und bereits als potentielle Regulatoren der klassischen MAPKn ERK1 und ERK2 beschrieben wurden (Pulido et al., 1998; Saxena et al., 1998). In der vorliegenden Arbeit konnte gezeigt werden, dass ERK5 und PTP-SL sowohl in intakten

Zellen als auch *in vitro* miteinander interagieren. Letzteres deutet darauf hin, dass beide Proteine direkt aneinander binden können und keine zusätzlichen Faktoren benötigen. Die beiden Bindungspartner beeinflussen sich gegenseitig in ihrer enzymatischen Aktivität. So konnte einerseits gezeigt werden, dass die katalytische Aktivität der PTP-SL im Komplex mit der Kinase erhöht ist. Obgleich PTP-SL ein Substrat für ERK5 ist, war diese Erhöhung der Phosphataseaktivität unabhängig von etwaigen Phosphorilierungen. Auf der anderen Seite war PTP-SL in der Lage ERK5 *in vitro* sowohl als auch in transfizierten Zellen zu inaktivieren. Die in PC12 Zellen endogen exprimierte ERK5 wird durch Zugabe von Stimulanzien wie zum Beispiel dem epidermalen Wachstumsfaktor aktiviert. Die Überexpression der aktiven PTP-SL unterdrückt diese Aktivierung, während ganz im Gegensatz dazu die Expression einer katalytisch inaktiven Mutante der PTP-SL die Aktivierung der ERK5 zusätzlich verstärkt. Diese positiven Effekte der Mutante, die auch in anderen Zellsystemen beobachtet wurden, lassen sich vermutlich darauf zurückführen, dass die inaktive PTP-SL die zelleigene Inaktivierung der ERK5 beeinträchtigt, indem sie mit endogenen Phosphatasen um die Bindung an ERK5 kompetitiert.

Weiterhin konnte gezeigt werden, dass die PTP-SL auch in der Lage ist, die subzelluläre Verteilung der ERK5 zu beeinflussen. Die gesamte Menge der ERK5 verteilt sich über das Zytosol und den Zellkern. Da eine Hauptaufgabe der MAPKn die Phosphorylierung und die Aktivierung von Transkriptionsfaktoren und somit die gezielte Veränderung der Genexpression ist, stellt die subzelluläre Lokalisation von MAPKn, insbesondere ihre Präsenz im Zellkern, einen ausschlaggebenden Parameter für die Signalweiterleitung dar (Whitmarsh and Davis, 2000). Die Überexpression der PTP-SL führt unabhängig von ihrer katalytischen Aktivität zu einer Umverteilung der ERK5 vom Kern hin zum Zytosol. PTP-SL ist demnach in der Lage in zweierlei Weisen in ERK5-vermittelte Prozesse einzugreifen, und zwar sowohl durch Dephosphorylierung und Inaktivierung der Kinase als auch durch Beeinflussung der Lokalisation von ERK5 innerhalb der Zelle.

Ein weiterer Teil der Arbeit konzentriert sich auf die Wechselwirkung von ERK5 mit der Proteintyrosinkinase Abl und ihren onkogenen Varianten, welche für die Pathogenese von Blutkrebs mitverantwortlich sind. Die chronische myeloide Leukämie (CML) ist durch das abnormale Philadelphia Chromosom gekennzeichnet. Entstanden aus der Translokation der Chromosome 9 und 22 kodiert es für ein Fusionsprotein aus dem Transkriptionsfaktor Bcr und der Abl Kinase. Die Forschung der letzten Jahre konnte zeigen, dass das so entstandene *Bcr/Abl* Onkogen sowohl für die Entstehung als auch für die Progression der CML verantwortlich ist (Daley et al., 1990; Huettner et al., 2000). Im Gegensatz zu dem zellulären

Gegenstück c-Abl ist das Fusionsprodukt rein zytoplasmatisch und besitzt permanente Kinase- und Signalaktivität (Sawyers, 1997). Die Behandlung von CML Patienten mit dem seit 2002 zugelassenen Abl-spezifischen Inhibitor Imatinib führte zu einem Krankheitsrückgang in nahezu allen Fällen (Druker et al., 2001a; Druker et al., 2001b). Allerdings ist die Behandlung von Patienten in vorangeschrittenen Stadien der CML durch die rasche Entwicklung von Resistenzen gegen Imatinib limitiert (Druker et al., 2001a; Ottmann et al., 2002). Da die Wirkungsweise von Imatinib auf der Fähigkeit beruht, den Zellzyklus zu arretieren und das zelleigene Selbstmordprogramm auszulösen, könnte die Wirkungsweise von Imatinib unterstützt werden, indem man Signalwege, die zum Überleben der Zelle beitragen, inhibiert. Ein wichtiges Überlebenssignal wird in vielen Zellarten von MAPKn reguliert (Ballif and Blenis, 2001) und vorläufige Untersuchungen deuteten darauf hin, dass dies auch in Leukämiezellen der Fall sein könnte (Yu et al., 2002). Zusammen mit einer Studie, die ERK5 mit der Chemoresistenz von Brustkrebszellen in Zusammenhang brachte, führte dies zu der Frage, ob ERK5 eine Rolle in von onkogenen Abl-Kinasen vermittelten Prozessen spielen könnte.

Zunächst konnte gezeigt werden, dass ERK5 in Bcr/Abl positiven Zellinien exprimiert wird. Bcr/Abl aber auch andere Abl-Kinasen wie zum Beispiel die virale Form v-Abl waren in der Lage, mit ERK5 zu interagieren. Die Konsequenzen dieser Wechselwirkung waren die Aktivierung, die Tyrosinphosphorylierung und interessanterweise eine Erhöhung der zellulären Proteinmenge von ERK5. Letzteres beruhte auf der posttranslationalen Stabilisierung des ERK5-Proteins, da dieser Effekt durch die Inhibierung der Translations-maschinerie nicht beeinträchtigt wurde. Eine genauere Untersuchung zeigte, dass eine der beiden Prolin reichen Domänen, welche im C-terminalen Bereich von ERK5 liegen, für die Stabilisierung durch Abl-Kinasen benötigt wird. Die Identifizierung einer potentiellen Bindestelle für die sogenannte SH3 Domäne von Abl in eben dieser Region mit Hilfe des Scansite-Suchprogrammes (Yaffe et al., 2001) stützte dieses Ergebnis. Die Relevanz der einzigartigen Sequenzen von ERK5 wurde zusätzlich durch die Beobachtung bestätigt, dass eine Chimäre, welche aus der MAPK ERK2 und dem gesamten C-terminalen Schwanz von ERK5 bestand, in vergleichbaren Maße wie ERK5 stabilisiert wurde.

Basierend auf folgenden zwei Beobachtungen konnte die Schlussfolgerung gezogen werden, dass Stabilisierung und Aktivierung unabhängig voneinander verlaufen. Erstens, eine konstitutiv aktive Mutante von MEK5, die ERK5 maximal zu aktiviert, hatte keinen Einfluss auf die zelluläre Proteinmenge der ERK5. Zweitens, eine AEF-Mutante von ERK5, deren

MEK5 Phosphorylierungsstellen mutiert wurden und die daher nicht aktiviert werden kann, wird ohne weiteres durch Abl-Kinasen stabilisiert.

Ein erster Hinweis darauf, dass die Wechselwirkung von ERK5 mit Abl-Kinasen pathophysiologische Bedeutung besitzen könnte, wurde durch die Beobachtung geliefert, dass die Überexpression von ERK5 zu einer Erhöhung der Transformationsrate von viralem Abl führte. Das onkogene Potential von Abl-Kinasen lässt sich durch ihre Fähigkeit, Bindegewebszellen von Nagern zu transformieren, untersuchen. Transformierte Zellen verlieren die Kontaktinhibition, die sie normalerweise bei Erreichen einer geschlossenen einzelligen Schicht von weiteren Zellteilungen abhält, und sind daher an der Bildung von mehrschichtigen Aggregaten leicht zu erkennen. Während eine katalytische inaktive Mutante von ERK5 keine Auswirkung hatte, war der Effekt der AEF-Mutante vergleichbar mit dem des Wildtyps von ERK5. Die AEF-Mutante ist zwar nicht aktivierbar, besitzt aber basale Kinaseaktivität, welche vermutlich in Verbindung mit der zellulären Proteinmenge für die Verstärkung der Transformation ausschlaggebend ist.

Um zu der anfänglichen Fragestellung, ob ERK5 in der Weiterleitung von Überlebenssignalen beteiligt sein könnte, zurückzukehren, wurde die Überlebensrate von Bcr/Abl positiven Leukämiezellen nach kurzer Imatinibbehandlung untersucht. Zellen, deren Expressionslevel der ERK5 mit Hilfe der RNS-Interferenz reduziert wurde, wiesen eine wesentlich verringerte Überlebensrate auf. Eine vergleichbare Reduzierung konnte durch die Überexpression der inaktiven ERK5-Mutante erzielt werden, welche vermutlich negativ mit dem endogenen ERK5-Signalweg interferiert. Im Gegensatz dazu konnte die Überexpression der inaktivierbaren Form von ERK5 das Zellüberleben nicht beeinflussen. Vergleichbar mit den Effekten in der Transformation sind also auch für die Überlebensrate von Zellen die basale Aktivität und die Proteinmenge der ERK5 und nicht die Aktivierung durch die MEK5 von Bedeutung. Die Stabilisierung der ERK5 durch Bcr/Abl könnte demnach zur Pathogenese der CML beitragen und ein zu berücksichtigender Aspekt in der Therapie sein.

Wie im letzten Abschnitt der vorliegenden Arbeit gezeigt, wird ERK5 in der Präsenz sowohl von Wachstumsfaktoren als auch Stressfaktoren wie Hyperosmolarität aktiviert und sollte aufgrund dieses Aktivierungsprofils zwischen den mitogenen ERKs und den stress-sensitiven JNKs und p38 Kinasen eingeordnet werden. Im starken Gegensatz zu ERK2 wird zum Beispiel in PC12 Zellen nur ein sehr kleiner Prozentsatz der gesamten ERK5 aktiviert. Eine mögliche Erklärung für die reduzierte Aktivierbarkeit von ERK5 konnte durch die Beobachtung geliefert werden, dass der C-terminale Anhang in der Lage war, die Aktivität

der Kinase zu unterdrücken. Neben der Autoinhibierung konnte für die Domänen des C-Terminus eine Rolle in der Regulation des Kernimports und Exports aufgezeigt werden.

Obgleich MAPKn durch eine enorme Zahl verschiedenster Stimuli aktiviert werden, führen sie doch zu spezifischen und angemessen Reaktionen der Zelle. Die molekularen Grundlagen dieser auf den ersten Blick paradoxen Situation konnten bisher nur zum Teil entschlüsselt werden. Zumindest steht fest, dass die Dauer und die Amplitude der induzierten MAPK-Aktivität zwei der Parameter sind, die das Schicksal der Zelle mitbestimmen (Marshall, 1995). Starke Stimuli wie zum Beispiel mitogene Wachstumsfaktoren induzieren eine starke pulsartige Aktivierung von MAPKn. Im Gegensatz dazu ist anzunehmen, dass die Stabilisierung einer Kinase ein andauerndes Aktivierungsprofil mit niedriger Amplitude hervorrufen dürfte. Während viele Signalwege nur vorrübergehend angeschaltet werden müssen, ist es unabdinglich für die normale Zellfunktion, dass Überlebenssignalwege kontinuierlich aktiv sind. Da die basale Aktivität von ERK5 ausschlaggebend und vor allen Dingen auch hinreichend für das Überleben von Leukämiezellen war, könnte in diesem Falle die Stabilisierung und die Destabilisierung einen bedeutenden, wenn nicht sogar den wichtigsten, Regulationsmechanismus darstellen. Auch wenn die hauptsächliche Kontrolle von MAPKn durch ihren Phosphorilierungsstatus unumstritten ist, könnte in all jenen Fällen, die unabhängig von der Aktivierung der MAPKn sind, die Proteinstabilität als regulatorisches Moment dienen. Die kürzlich beschriebene Beobachtung, dass die Degradation und nicht die Dephosphorilierung von ERK2 eine Vorraussetzung für die Initiierung des zelleigenen Selbstmordprogramms ist (Lu et al., 2002), unterstreicht die Bedeutung, die der Kontrolle der Proteinmenge von MAPKn im Zusammenhang mit dem Überleben von Zellen zukommt.

Zusammengenommen zeigen die Daten dieser Arbeit, dass zahlreiche Signalwege durch ERK5 vermittelt werden und dass nicht nur die Kinaseaktivität sondern auch die zelluläre Lokalisation und die Proteinstabilität wichtige Parameter der Regulation sind.

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Poster Presentations

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8 ABBREVIATIONS

‘	Minute
aa	Amino acid
Ab	Antibody
ADAM	A disintegrin and metalloprotease domain
AEF	Not activatable MAPK mutant
AML	Acute myeloid leukaemia
Amp^r	Ampicilline resistence
APC	Adenomatous polyposis coli gene
APS	Ammoniumpersulfate
AR	Amphiregulin
ATP	Adenosintriphosphate
bp	Base pairs
BSA	Bovine serum albumin
BCR	Break point cluster region gene
Bcr/Abl	Leukaemic fusion protein
°C	Degree celsius
c-Abl	Cellular form of v-Abl
cAMP	Cyclic adenosinmonophosphate
Ca^{2+}	Calcium Ions
CaM Kinase	Ca^{2+}-calmodulin-dependent kinase
cDNA	Complementary DNA
cds	Coding sequence
CML	Chronic myeloid leukaemia
CMV	Cytomegalo virus
c-jun	Cellular homologue to v-jun (avian sarcoma virus 17 oncogene)
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonukleic acid
dsDNA	Dooble-stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethlendiamintetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
ERK	Extracellular signal-regulated kinase
FAP	Familial adenomatous polyposis
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Fig	Figure
g	Gramm
GDP	Guanosindiphosphate
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor

Grb2	Growth factor receptor binding protein 2
GST	Glutathion-S-transferase
GTP	Guanosintriphosphate
h	Hour
HA	Hemagglutinin
H_2O_{bidest}	Twice-destilled, deionised Water
HEPES	N-(2-Hydroxyethyl)-piperazin-N‘-2-Ethansulfonic acid
HER	Human EGFR-related
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
HSCT	Hematopoietic stem cell transplantation
HRP	Horseradish peroxidase
IFN-α	Interferon-α
Ig	Immunglobulin
IP	Immunoprecipitation
IPTG	Isopropyl-ß-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
kb	Kilobase
kDa	Kilodalton
KIM	kinase intercating motif
KIM PTP	KIM containing tyrosine-specific phosphatase
l	Liter
LPA	Lysophosphatydic acid
LTR	long terminal repeats
µ	Micro
m	Milli
M	Molar
MAP	Mitogen-activated protein
MAPK	MAP kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MBP	Myelin basic protein
MCS	Multiple cloning site
MEK	MAPK/ERK Kinase
MEKK	MAPK/ERK Kinase Kinase
min	Minute
MMP	Matrix metalloprotease
n	Nano
Neo^r	Neomycine resistance
NGF	Neuronal growth factor
OD	Optical density
p.a.	Per analysis
PAGE	Polyacrylamide gel elektrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylenglycole
PI 3-Kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-diphosphate
PKA	Protein kinase A
PKC	Protein kinase C

PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl-fluoride
pNPP	p-Nitrophenyl-phosphate
pRS	pRetroSUPER vector
PTP	Phosphotyrosine-specific phosphatase
PY	Phosphotyrosine
Raf	Homologue to v-raf (MAPKKK)
Ras	Homologue to v-ras (rat sarcoma viral oncogene)
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
S.D.	Standard deviation
SDS	Natriumdodecylsulfate
SH	Src homology
SHP-2	SH2-containing PTP-2
Sos	Son of sevenless
Src	Homologue to v-src (sarcoma viral oncogene)
SV40	Simian virus
Tel/Abl	Leukaemic fusion protein
TEMED	N, N, N‘, N‘-Tetramethyletylendiamine
TNFα	Tumor necrosis factor alpha
TPA	12-O-Tetradecanoyl-phorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethan
Tween 20	Polyoxyethylensorbitanmonolaureate
U	Enzymatic activity unit
O/N	Overnight
UV	Ultraviolett
V	Volt
VSV	Vesicular stomatitis virus glycoprotein VSV-G
v-Abl	Oncogene of Abelson leukaemia virus
Vol	Volume
WT	Wild type

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