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Identification of novel MARK3 substrates and upstream regulators

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Preface

MARK3, also known as Cdc25c-associated protein kinase (c-tak-1), is a serine/threonine specific kinase that has been shown to be involved in cellular programs such as cell signalling, transcription regulation, cell cycle control and cell adhesion. In this thesis, the identification of two novel MARK3 substrates - KIFC3 and KLC4 - and one novel MARK3 upstream kinase are described. KIFC3 and KLC4 are both involved in transport or transport-dependent functions, which suggests a so far undiscovered role for MARK3 within these cellular programs.

Abstract

MARK3, also known as Cdc25c-associated protein kinase (c-tak-1), is a serine/threonine specific kinase that has been implicated in the regulation of crucial cellular programs such as cell signalling, cell adhesion, transcription regulation and cell cycle control. Since deregulation of these programs is linked to cancer development and progression, MARK3 is assumed to participate in tumour formation. This hypothesis is supported by the fact that upstream regulators of MARK3 comprise tumour suppressor and oncogenic kinases.

This project aimed to characterize the position of MARK3 within the cellular network more precisely by identifying novel substrates and upstream kinases of MARK3. These results should lead to novel insights into the MARK3 function(s) of MARK3 and concurrently shed light on the potential role of MARK3 in cancer development.

Kinesin family member C3 (KIFC3) is a microtubule minus end-directed motor protein, and Kinesin light chain (KLC4) was identified as novel MARK3 substrate. Kinesins are motor proteins that transport membraneous organelles and macromolecules along microtubules. These findings indicate a novel role for MARK3 in cellular transport or cellular transport-dependent functions. Further efforts identified a novel MARK3 phosphorylating kinase of 60 kDa, which is of substantial interest since MARK3 upstream regulators may be used as therapeutical targets.

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LIST OF ABBREVIATIONS

AA	Amino acid
ADP	Adenosine diphosphate
AG	Autoradiogram
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
aPKC	Atypical protein kinase C
AT	Ataxia telangiectasia
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
c.p.m	Counts per minute
CA	Constitutively active
CDC2	Cell division cycle 2
CDC25C	Cell division cycle 25c
CDK	Cyclin dependent kinase
DNA	Deoxy ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
ERK	Extra-cellular signal related kinase
EST	Expressed Sequence Tags
FCS	Foetal calf serum
Fig	Figure
FSBA	5'fluorosulfonylbenzoyladenosine
FSBA	5'-p-fluorosulfonylbenzoyladenosine
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
HDAC	Histone deacetylase
HIS	Histidine
HRP	Horseradish peroxidase
IGKA	In-gel kinase assay
IKB	Nuclear factor of kappa light polypeptide gene enhancer inhibitor
KA1	Kinase assay 1
kb	Kilo base pairs
KHC	Kinesine heavy chain
KIF	Kinesin family member
KIFC3	Kinesin family member C3
KLC	Kinesin light chain
KLC4	Kinesin light chain 4
KSI	Kinase substrate identification
KSR1	Kinase suppressor of RAS 1
LB	Luria-Bertani
LKB1	Liver kinase B1
MAP	Microtubule affinity protein

MAPK	Mitogen activated protein kinase
MAPKKK	Mitogen activated kinase kinase kinase
MAPT	Microtubule associated protein tau
MARK	Microtubule affinity regulating kinase
MARKK	Microtubule affinity regulating kinase kinase
MBP	Maltose binding protein
MEK	Mitogen activated ERK kinase
MITF	Microphthalmia-associated transcription factor
MO25	Mouse embryo scaffolding protein
MRB	MARK reaction buffer
MTOC	Microtubule-organizing center
NCBI	National Center for Biotechnology Information
ODN	Oligonucleotide
OE	Overexpression
p.f.u.	Plaque forming units
PAGE	Polyacrylamide gel electrophoresis
PAR	Partitioning defective
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PIM1	Proviral integration site 1
PKP	Plakophilin
PLEKHA	Pleckstrin homology domain containing, family A
PP	Phosphatase
PTPH1	Protein Tyrosine Phosphatase
RAC	Ras related C
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TAOK1	Thousand and one kinase 1
TCA	Trichloric Acid
TE	Tris-EDTA
TFIIIB	Transcription factor IIIB
TPR	Tetratricopeptide repeat
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
TWB	Triton wash buffer
UBA	Ubiquitin-associated domain
UTC	Untransfected cells
WB	Western blot

Chapter I: Introduction

1.1 Cell signalling

Cells in multicellular organisms need to communicate with each other to retain the integrity of the entire organism. This cellular discourse occurs through both electrical and chemical signals. Communication through chemical signals, for instance neurotransmitters, hormones or growth factors are by far the major form of information transfer between cells (Morgan 1989; Dhanasekaran 1998).

The signalling cell releases a molecule (ligand), which is transported to the target cell. Receptors on the target cell recognize the ligand and then trigger the appropriate intracellular signalling pathways. In the majority of the cases, this leads to the modification of transcription factors and along with these the cellular gene expression pattern changes. Modification of the cellular gene expression initiates the appropriate cellular response according to the stimulus (Marsh, Neiman et al. 1991; Errede and Levin 1993; Bluthgen and Legewie 2008).

Phosphorylation by protein kinases is a significant signal transduction mechanism by which intercellular signals are converted into cellular processes. Protein kinases phosphorylate approximately one third of all mammalian proteins and have been implicated in the regulation of most crucial processes such as hormone responses, cell proliferation, apoptosis, migration, differentiation, transport, motility and learning (Salomon, Ficarro et al. 2003; Johnson and Hunter 2005; Lawrence, Jivan et al. 2008). Disruption of protein kinase function or regulation is implicated in a host of major

diseases, such as cancer, diabetes and rheumatoid arthritis. Defects in genes that encode protein kinases underlie a number of inherited and acquired disorders, which include leukemias, lymphomas and autoimmune diseases (Cohen 2002; Estey 2008; Touitou and Kone-Paut 2008; Pytel, Sliwinski et al. 2009). The key function of this family of proteins in signal transduction makes it a very attractive target for therapeutic interventions in many diseases (Blume-Jensen and Hunter 2001; Cohen 2001; Manning, Plowman et al. 2002; Manning, Whyte et al. 2002; Shchemelinin I 2006; Zhang, Yang et al. 2009)

1.2 Protein Kinases

In the early part of the 20th century Levene and Lipmann identified phosphate in the protein vitellin and by 1933 had mapped the phosphate to a serine residue of this protein (Levene 1906; Lipman 1932). However, it took another 20 years before the first "enzymatic phosphorylation of proteins" could be demonstrated by Eugene P. Kennedy (Burnett and Kennedy 1954). Edmond H. Fischer and Edwin G. Krebs were awarded the 1992 Nobel Prize for Physiology and Medicine (Fischer and Krebs 1955) for discovering reversible protein phosphorylation as a biological regulatory mechanism. Reversible phosphorylation of proteins occurs in both prokaryotic and eukaryotic organisms and is probably the most important post-translational modification in the process of signal transduction (Cozzone 1988; Hunter 2000; Sefton and Shenolikar 2001; Manning, Whyte et al. 2002). The process results in conformational changes within the structure of many proteins, causing them to change

their activity (Johnson and Barford 1993). In eukaryotic and prokaryotic organisms phosphorylation occurs on serine, threonine, tyrosine, histidine, arginine and lysine residues (Kowluru 2008). Addition of a negatively charged hydrophilic phosphate molecule (PO_4^{3-}) to a polar R group of an amino acid residue can for example outbalance adjacent hydrophobic residues or attract positively charged side chains respectively. This can lead to conformational changes in the structure of the protein elsewhere, which can modify the activity and/or interaction characteristics of the protein. (Fischer and Krebs 1955; Johnson and Barford 1993).

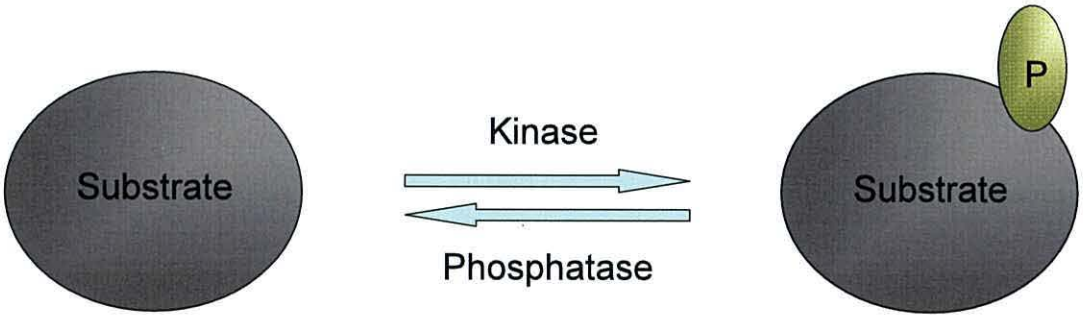


Figure 1: Protein kinase and Protein phosphatase
Protein kinases phosphorylate target proteins, whereas protein phosphatases dephosphorylate their target proteins.

Enzymes called protein kinases catalyze the phosphorylation reaction, while protein phosphatases act as their antagonists and are responsible for dephosphorylation of proteins (Barford 1996; Zhang 2002). The human genome comprises ~518 putative kinase genes and the entirety of these genes is referred to as the human kinome, which is ~1.7% of all human genes (Manning et al. 2002). Most of the eukaryotic protein kinases belong to a single super family (~480) containing a similar catalytic domain. This super family is separated into a hierarchy of groups (9), families (134) and sub

families (201) (Manning, Plowman et al. 2002). The "classification" is primarily based on the sequence of the catalytic domain, knowledge of sequence similarities or non-catalytic domain structures, biological function and classifications in other organisms (Manning, Plowman et al. 2002). The catalytic domain comprises ~250-300 amino acids and fullfils three separate tasks. With the help of divalent cations such as Mg^{2+} and Mn^{2+} the domain binds and orientates the phosphate donor, ATP or GTP. Furthermore, the domain associates and adjusts the protein substrate and finally it catalyzes the γ-phosphate transfer from ATP or GTP to the acceptor hydroxyl residue of the target protein (Knighton, Zheng et al. 1991; Taylor, Radzio-Andzelm et al. 1993; Hanks and Hunter 1995). ´

The catalytic domain can be divided into 12 smaller sub domains, which contain characteristic patterns of conserved residues. Twelve amino acids within the kinase domain have been demonstrated as invariant and are considered essential. The conserved structures of the kinase domain implies that the domain of most protein kinases are folded into a topologically similar 3-dimensional core structure, which makes it likely that phosphor transfer is subject to a common mechanism (Knighton, Zheng et al. 1991; Taylor, Radzio-Andzelm et al. 1993).

The catalytic domain usually folds into a small and a large lobe. The small lobe (N-terminal lobe) is responsible for ATP (GTP) binding and orientation, whereas the larger lobe (C-terminal lobe) is mainly involved in substrate recognition and initiation of the phosphotransfer (Fig.: 2). The secondary structure of the N-terminal lobe predominately shows anti-parallel β-sheet structures. In contrast to this, the secondary

structure of the C-terminal lobe shows mainly α-helical structures. The two lobes form a deep cleft, which is recognized as the site of catalysis (Knighton, Zheng et al. 1991; Taylor, Radzio-Andzelm et al. 1993).

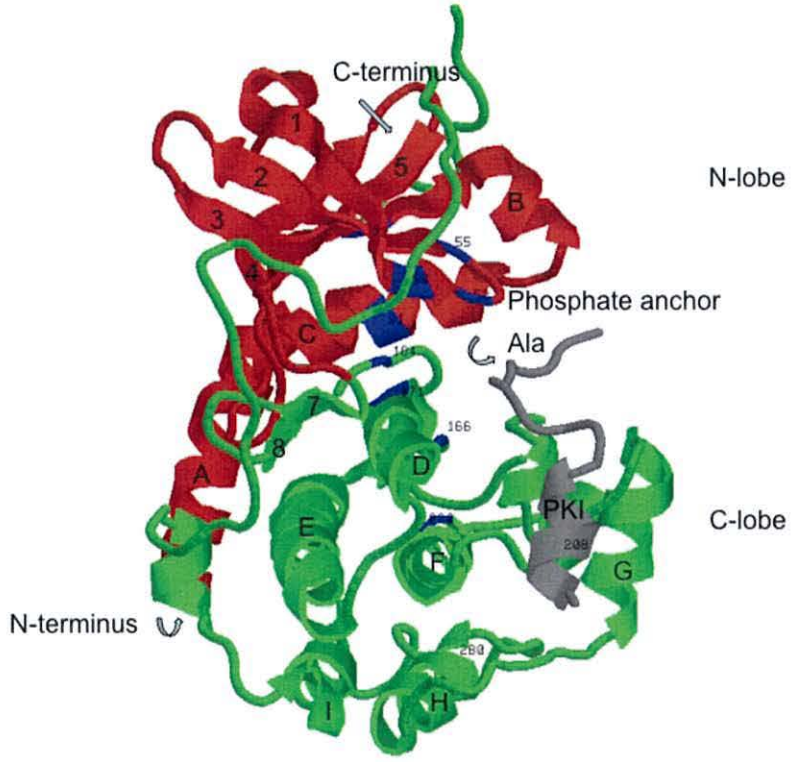


Figure 2: Ribbon diagram of the cAMP-dependent protein kinase
Ribbon diagram of the binary complex of the catalytic subunit of the cAMP-dependent kinase and a pseudosubstrate peptide inhibitor. Residues 15-126 are indicated in red and constitute to the N-lobe. The C-lobe is indicated in green (AA126-350). The inhibitor peptide is indicated in grey (PKI 5-24). β-strands and α-helices are indicated by flat arrows and helices, respectively and are numbered according to Knighton et al. (Knighton, Zheng et al. 1991). Conserved residues are marked purple (Gly50, Glu55, Lys72, Glu91, Asp166, Asn171, Asp184, Arg280, Glu208). Aspartate 166 near the side of phosphotransfer is positioned to serve as the catalytic base. The alanine side chain at the P-site in the inhibitor is also indicated with an arrow showing where phosphotransfer would take place if a serine/threonine were located at this site. The NH_2- and COOH-terminus is labelled. The figure is based on PDB entry number: 1ATP (Taylor, Radzio-Andzelm et al. 1993).

This catalytic side is often presented in an inactive state blocked by a segment of amino acids, called the phosphorylation lip or T-loop. Phosphorylation lips are divided into two functional groups. Gated activation loops synchronously modulate substrate binding and the phosphotransfer step, whereas non-gated activation loops modulate solely the phosphotransfer step (Adams 2003).

Many kinases require phosphorylation within the phosphorylation loop for catalytic activity. Most of these are mono-phosphorylated, however, some are phosphorylated on two or three residues. Phosphorylation can occur by other regulating kinases or autocatalytically and leads to relocalisation of the phosphorylation lip. The relocalisation initiates conformational changes within the kinase so that substrate binding and phosphotransfer can take place (Hanks and Hunter 1995; Adams 2003)

Kinases, as major players in signal transduction, are a very attractive target class for therapeutic interventions in many disease states such as cancer, diabetes, inflammation, and arthritis (Cohen 2001; Shchemelinin I 2006). Based on the discovery that many different types of cancers show deregulation of kinase signalling, which contributes to aberrant cell growth, invasion, tumour derived angiogenesis and metastasis, inhibition of kinase activity became a valuable target for cancer treatment (Blume-Jensen and Hunter 2001; Cohen 2002). In this regard, protein kinases represent as much as thirty percent of all protein targets under investigation by pharmaceutical companies (Cohen 2002). Research groups have developed several ways to target these enzymes therapeutically, such as with antibodies or small molecules that block kinase-substrate interaction, or that block the adenosine triphosphate (ATP) binding site of the enzyme (Novak 2004). Recent successful

launches of drugs with kinase inhibition as the mode of action demonstrate the ability to deliver kinase inhibitors as drugs with the appropriate selectivity, potency, and pharmacokinetic properties (Blume-Jensen and Hunter 2001; Cohen 2001; Deremer, Ustun et al. 2008; Herbst and Sandler 2008; Jeanes, Gottardi et al. 2008; Ocio, Mateos et al. 2008). Many other drugs are currently being tested in clinical trials for their ability to target kinases (Milella, Kornblau et al. 2001; Ivanenkov, Balakin et al. 2008).

Even with the enormous amount of research and clinical success the majority of kinase enzymes remain largely uncharacterized. Therefore, characterizing kinases that might be involved in tumour formation and progression would be desirable to identify new potential cancer therapy targets and additionally to estimate the likelihood of unintentional side effects of drugs against a specific kinase.

MARK3 (c-tak-1), a kinase of MAP/Microtubule Affinity Regulating Kinase (MARK) group, has been implicated in cell cycle regulation, growth factor signalling, transcription silencing and cell adhesion (Tassan and Le Goff 2004). These are all processes that are deregulated in most tumours (Dreesen and Brivanlou 2007; Jeanes, Gottardi et al. 2008; van der Maarel 2008; Malumbres and Barbacid 2009). In addtion, the group of MARK3 upstream regulators comprises tumour suppressor kinases as well as oncogenic kinases that can be clearly linked to cancer development in various tissues (Fields, Frederick et al. 2007; Shah, Pang et al. 2008).

1.3 MAP/Microtubule Affinity Regulating Kinase (MARK) group

Members of the MARK group are conserved from yeast to mammals and belong to the superfamily of the Ca2+/calmodulin-dependent protein kinases (Manning, Plowman et al. 2002; Tassan and Le Goff 2004) Within this superfamily, the MARK kinases belong to the subfamily of the AMP-activated protein kinases (AMPK) related kinases (Lizcano, Göransson et al. 2004). AMPK and AMPK related kinases are involved in the regulation of cell metabolism and cell polarity (Lizcano, Göransson et al. 2004; Koh et al. 2007; Williams and Brenman 2008). In this regard MARK kinases have been implicated in various cellular functions including cell polarity, cell cycle control, metabolism regulation, intracellular signalling, transcription control, microtubule stability and protein stability (Drewes, Ebneth et al. 1997; Peng, Graves et al. 1998; Muller, Ory et al. 2001; Tassan and Le Goff 2004; Dequiedt, Martin et al. 2006; Timm, Matenia et al. 2006; Hurov and Piwnica-Worms 2007; Hurov, Huang et al. 2007). Deregulation of the MARK kinases can be observed in diseases such as autism and Alzheimer's (Churcher 2006; Timm, Matenia et al. 2006; de Leng, Jansen et al. 2007; Maussion, Carayol et al. 2008; Timm, Marx et al. 2008). The human genome contains four MARK kinases (MARK 1-4) that all share a similar primary structural organisation. The NH_2-terminal half contains a conserved catalytic domain followed by an ubiquitin binding domain (UBA) implicated in regulation of the catalytic domain (Tassan and Le Goff 2004; Marx, Nugoor et al. 2006; Panneerselvam, Marx et al. 2006; Murphy, Korzhnev et al. 2007). The last 80 amino acids comprise a kinase associated domain 1 (KA1), which is assumed to be involved

in localisation of the kinase (Fig.: 3) (Hurov, Watkins et al. 2004).

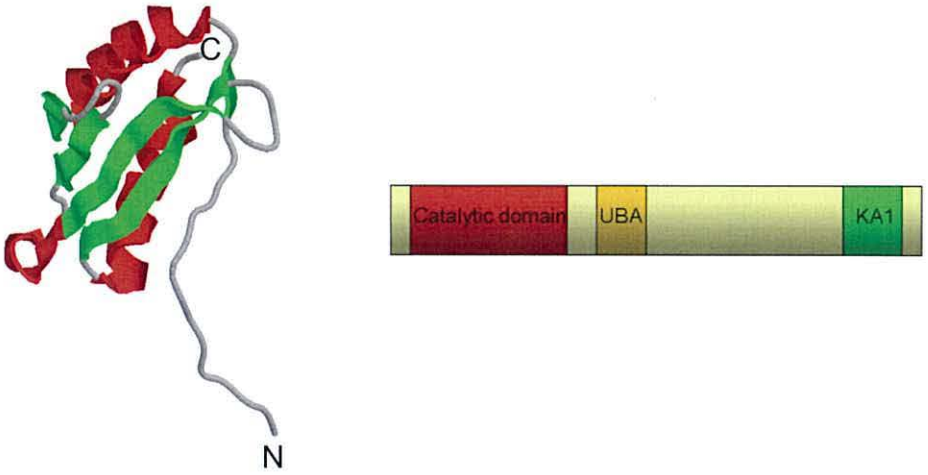


Figure 3: Ribbon diagram of mouse MARK3 KA1 domain (674-753)
The left side shows a ribbon diagram of the mouse KA1 domain. β-strands and α-helices are coloured green and red, respectively. The NH_2- and COOH-terminus is labelled. The figure is based on PDB entry number: 1ULZ (Tochio, Koshiba et al. 2006). The right side shows the organization of the MARK3 protein. The NH_2-terminal located catalytic domain is followed by an UBA domain and a COOH-terminal located KA1 domain

The kinase domain shows a characteristic bilobal fold with a linker sequence running up the face of the catalytic domain. The linker sequence opposes the active site toward the N-lobe of the catalytic domain. The C-lobe of the kinase domain is associated *via* the linker sequence to the UBA domain, which binds by means of hydrophobic residues the catalytic site. The atypical folding of the UBA domain in which the α3 helix is inverted compared to the canonical UBA topology is responsible for this interaction. An open conformation of the N- and C-terminal lobes of the catalytic domain seems to be stabilized by the UBA domain, which is crucial for phosphorylation of the kinase domain by upstream kinases (Fig.: 4) (Marx, Nugoor et al. 2006; Panneerselvam, Marx et al. 2006; Murphy, Korzhnev et al. 2007).

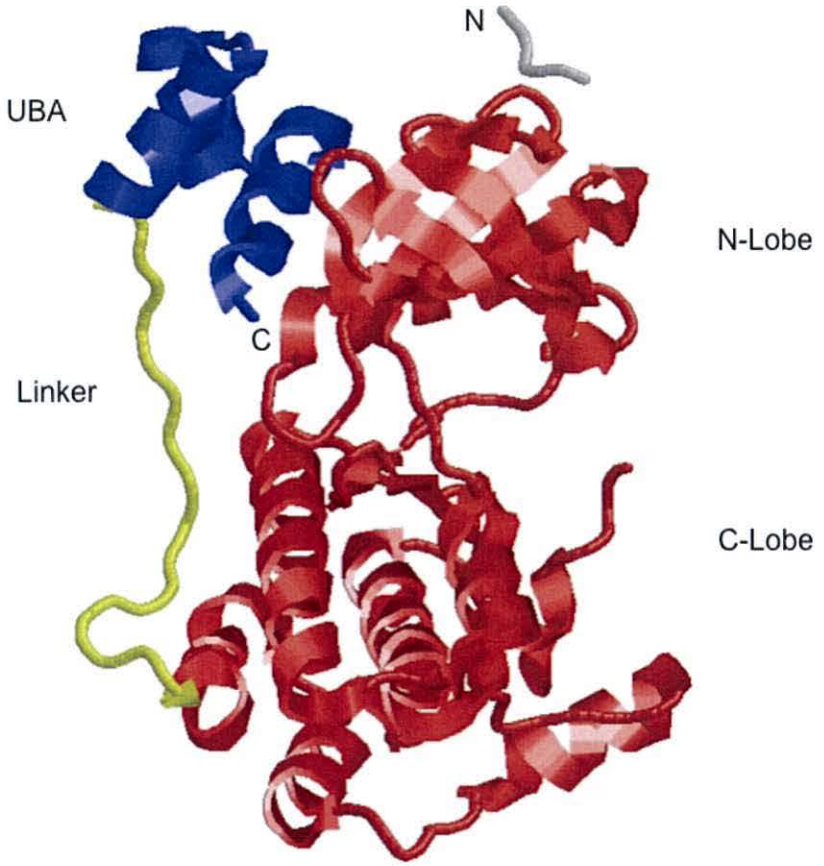


Figure 4: Ribbon diagram of the catalytic and UBA domain of human MARK3 (AA48-370).
The catalytic domain (D48–N308) is coloured red; the linker (A309–D328) is indicated in yellow; and the UBA domain (Q329–R366) is indicated in blue. The NH_2- and COOH-terminus is labelled. The figure is based on PDB entry: 2QNJ (Murphy, Korzhnev et al. 2007).

MARK1, 2 and 4 are closely related kinases that have been shown to phosphorylate microtubule associated proteins (MAPs) thereby regulating microtubule stability (Ebneth, Drewes et al. 1999; Drewes 2004; Trinczek, Brajenovic et al. 2004; Timm, Marx et al. 2008). Microtubules with actin filaments and intermediate filaments organize the cytoplasm of eukaryotic cells. Such filaments have been shown, *inter*

alia, to connect protein complexes, serve as intracellular routes for transport of cargoe vesicles, are involved in organelle positioning and provide mechanical stability of the cell (Braga 2002; Wiesner, Legate et al. 2005; Goldman, Grin et al. 2008). Microtubules show a polar structure and are composed of tubulin proteins. The plus pole rapidly grows by the addition of tubulin monomers, whereas the minus pole (if not stabilized) tends to lose tubulin monomers. In most cells the minus ends are imbedded in the Microtubule Organizing Centre (MTOC), which generally lies near the nucleus. Microtubule associated proteins serve to stabilize microtubules against disassembly and thus speed up their polymerisation (Wade 2007). MARK1, 2 and 4 are efficient at detaching MAPs from microtubules by phosphorylating a KXGS motif in the repeat domains of the MAPs, MAP2, MAP4 and tau (Ebneth, Drewes et al. 1999; Schneider, Biernat et al. 1999; Trinczek, Brajenovic et al. 2004; Timm, Marx et al. 2008). Microtubules are involved in induction or maintenance of anterior-posterior (e.g. migration, asymmetric cell division), apical-basal (as found in epithelial layers) and planar (or tissue) cell polarity (e.g. *Drosophila melanogaster* wing) (Dow and Humbert 2007). The generation of cell polarity in all cases requires active remodelling of microtubules and the actin cytoskeleton and is highly dependent upon polarized vesicle trafficking to different cellular domains (Dow and Humbert 2007). Most studies concerning involvement of the MARK group in cell polarity have been performed in *D. melanogaster* and *Caenorhabditis elegans* (Goldstein and Macara 2007). However, these results are difficult to transfer into the mammalian system since there is as yet no clear evidence that *C. elegans* Polarity defective 1 (PAR-1) functions as a MARK (Labbe, Maddox et al. 2003). Moreover, although fruit fly

PAR-1 seems to affect microtubule stability, the proteins are unlikely to function biochemically in the same way as the mammalian MARK proteins since it regulates microtubule stability positively rather than negatively (Doerflinger, Benton et al. 2003). However, a substantial amount of studies could link human MARK 1 and 2 to neuronal and epithelial cell polarization *via* their function to regulate microtubule stability (Cohen, Tian et al. 2007; Saadat, Higashi et al. 2007; Terabayashi, Itoh et al. 2007; Sapir, Sapoznik et al. 2008). Additionally, studies with MARK2 null mouse lines implicated MARK2 in a diverse subset of physiological functions such as fertility, immune system homeostasis, learning and memory and growth and metabolism (Bessone, Vidal et al. 1999; Hurov, Huang et al. 2007; Segu, Pascaud et al. 2008).

In contrast to MARK1, 2 and 4, MARK3 is assumed to adopt a special position within the MARK group since so far neither involvement in microtubule stability nor in cell polarity has been shown. MARK3 has been implicated in the regulation of crucial cellular programs such as cell cycle progression, MAPK signalling, transcription regulation and cell adhesion. (Peng, Graves et al. 1998; Muller, Ory et al. 2001; Dequiedt, Kasler et al. 2003; Muller, Ritt et al. 2003).

1.4 MARK3/c-tak-1

MARK3 also known as Cdc25c-associated protein kinase (c-tak-1) is a ubiquitously present basophilic serine/threonine kinase of the MARK group (Muller, Ritt et al. 2003; Tassan and Le Goff 2004). The present data indicates a role for MARK3 in

cancer development. This assumption is based on the fact that all identified substrates of MARK3 either participate in or control cell programs that are deregulated in tumour cells (Stark and Taylor 2006; Roberts and Der 2007; Al-Janadi, Chandana et al. 2008; Stemmler 2008). Furthermore, the known upstream regulators of MARK3 comprise oncogenes and tumour suppressor genes that have been demonstrated to be deregulated in various malignant neoplasms (Wang, Bhattacharya et al. 2001; Fields and Regala 2007; Sanchez-Cespedes 2007).

1.4.1 Substrates of MARK3

The substrates of MARK3 form a heterogeneous group of proteins regarding their cellular function, although the regulatory mechanism by which MARK3 affects them seems to be conserved. Mitogen activated protein kinase (MAPK) cascade scaffolding protein kinase suppressor of RAS-1 (KSR-1), cell cycle regulating phosphatase cell division cycle 25 homolog (Cdc25c), protein tyrosine phosphatase H1 (PTPH1), desmosomal protein plakophilin 2 (PKP2) and class IIa Histone deacetylases (HDAC) are all phosphorylated at a specific serine residue which subsequently leads to 14-3-3 protein dimer binding. The 14-3-3-dimer masks a cellular localisation motif and prevents the translocation to the cellular compartment in which these proteins normally exert their function (Fig.: 5) (Zhang, Kobayashi et al. 1997; Peng, Graves et al. 1998; Muller, Ory et al. 2001; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006).

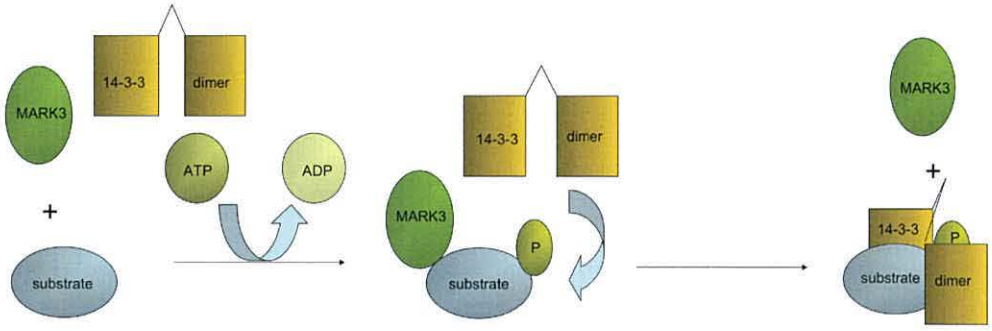


Figure 5: MARK3 regulatory mechanism
The regulatory mechanism by which MARK3 affects its substrates seems to be conserved. MARK3 phosphorylates its substrates at one specific serine residue, which subsequently leads to 14-3-3 binding and relocalization of the substrate.

1.4.1.1 Kinase suppressor of Ras 1(KSR1)

KSR1 is a scaffold protein of the MAPK pathway that binds MEK1/2 and ERK1/2. After cells are exposed to growth factors, KSR1 and the bound proteins translocate from the cytosol to the membrane. KSR1 facilitates and accelerates the transduction of the signal from the activated Ras/Raf-1 to the MAPK by bringing the individual compounds closer together (Muller, Ory et al. 2001).

KSR1 is localized in the cytoplasm of quiescent cells with the MAPKK, MEK1/2, bound to its kinase-like domain. MARK3 constitutively associates with the NH_2-terminal region of KSR1 to maintain the phosphorylation of S392 and consequently the binding of 14-3-3 to this site (Muller, Ory et al. 2001). In response to input signals, such as growth factor treatment, ERK1/2 binds to the complex and the S392 site of KSR1 is dephosphorylated by protein phosphatase 2A (PP2A). This leads to translocation of the KSR1 complex to the membrane (Ory, Zhou et al. 2003). So far the temporal order of these events has not been determined. The complex associates with the membrane bound MAPKKK RAF, which triggers the MAPK cascade by

phosphorylating the MAPKK, MEK1/2, which in turn activates the MAPK, ERK1/2 (Fig.: 6) (Muller, Ory et al. 2001).

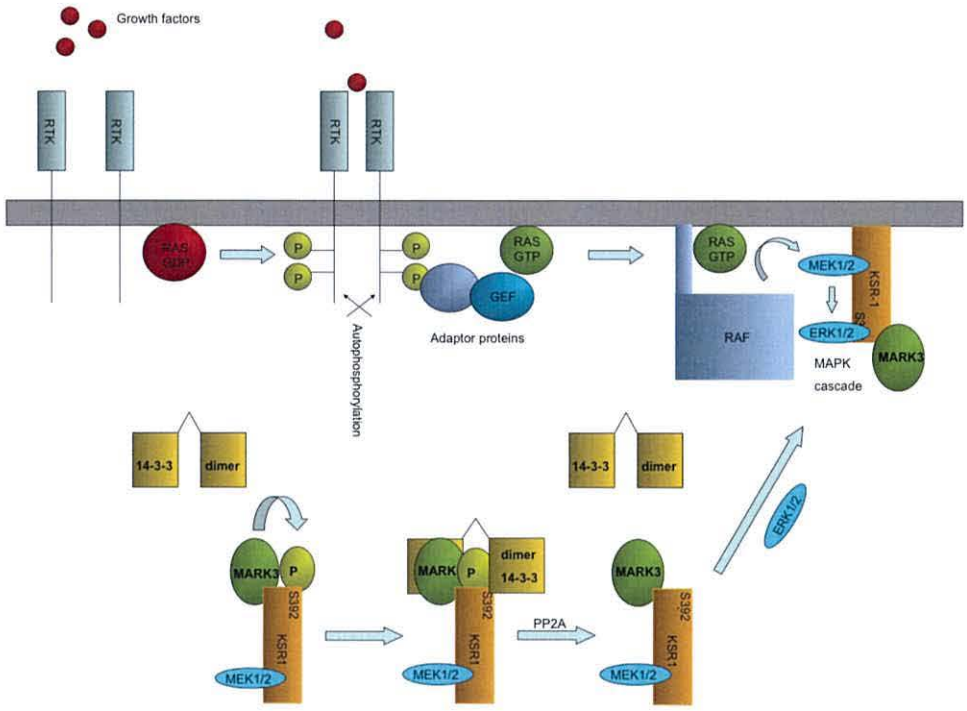


Figure 6: Model for KSR1/MARK3 interaction
MARK3 phosphorylation ensures that, in quiescent cells, KSR1 is localized in the cytoplasm. Upon stimulation the MARK3 phosphorylated serine is dephosphorylated by PP2A and the KSR1/MEK1/2 complex relocalizes to the plasma membrane where the MAPK cascade is triggered. The temporal order of KSR complex formation and translocation has not yet been determined.

1.4.1.2 Cell division cycle 25 homolog C (CDC25c)

The cell cycle consists of four distinct phases: G_1 phase, S phase (DNA replication), G_2 phase and M phase (mitosis). M phase is itself composed of two tightly coupled processes: mitosis, in which the chromosomes of a cell are divided and segregated into the two daughter nuclei, and cytokinesis in which the cell's cytoplasm is split into

two units forming distinct cells. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G_0 phase (Wolgemuth 2008, Malumbres et al. 2009).

Progression through the cell cycle is associated with sequential activation of cyclin dependent kinases (CDKs) (Pines 1993; Jackman and Pines 1997; Vermeulen, Van Bockstaele et al. 2003). Activation of these kinases is dependent on regulatory subunits called cyclins as well as phosphorylation and dephosphorylation of the cyclin-CDK complexes (Atherton-Fessler, Hannig et al. 1993). This ensures stringent regulation and proper timing of the cell cycle.

Cdc2-cyclin B complexes regulate mitosis entry. Cyclin B synthesis and its association with Cdc2, takes place during the S- and G_2 -phases. These are phosphorylated on three different regulatory sites immediately after the assembly of the complexes. Two of the three sites (T14 and Y15) have an inhibiting effect whereas the third site (T161) has an activating effect on cdc2. The two inhibiting phosphorylations outbalance the activating phosphorylation so that the complexes are held in an inactive state. The final step of activation is the dephosphorylation of the inhibiting phosphorylation sites which is accomplished by Cdc25 phosphatases (Lorca 1993). The human genome encodes for three partly redundant Cdc25 phosphatases denoted Cdc25 A, B and C. (Moreno and Nurse 1991; Galaktionov, Lee et al. 1995). Cdc25c is constitutively expressed throughout the entire cell cycle and its activity is regulated by MARK3. MARK3 phosphorylates Cdc25c on serine 216 within the cytosol, which leads to 14-3-3 binding and prevents the translocation of Cdc25c into

the nucleus. Therefore, MARK3 prevents the activation of Cdc2-Cyclin B complexes in the nuclear and premature entry into mitosis during the cell cycle (Peng, Graves et al. 1998).

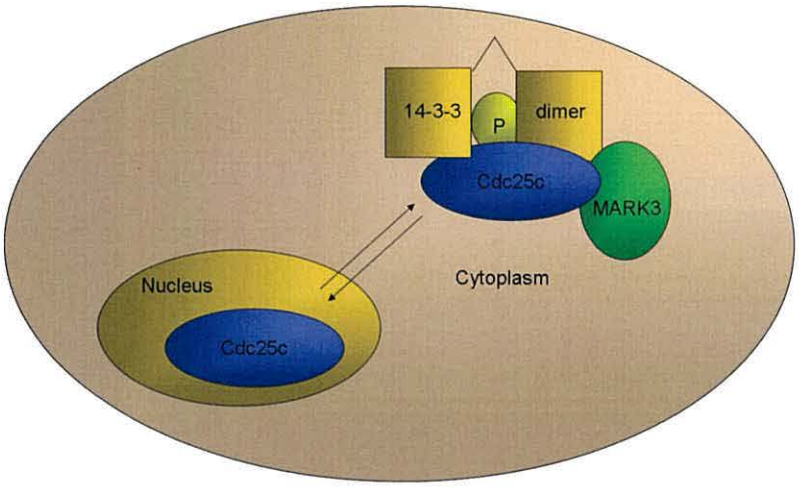


Figure 7: Model for Cdc25c MARK3 interaction
MARK3 phosphorylates Cdc25c on serine 216, prevents Cdc25c from entering the nucleus.

1.4.1.3 Protein tyrosine phosphatase H1 (PTPH1)

Protein phosphatases play a crucial role in cell signalling as antagonists of protein kinases. The protein tyrosine phosphatase H1, a member of the ubiquitous protein tyrosine phosphatase (PTP) family has been described as an *in vitro* substrate of MARK3. *In vitro* phosphorylation of Serine 359 by MARK3 leads to enhanced 14-3-3β PTPH1 association (Zhang, Kobayashi et al. 1997). PTPH1 is involved in T-Cell-Receptor signalling, cardiac ion channel regulation and has recently been linked to papilloma viruses derived tumour formation (Sozio, Mathis et al. 2004; Jespersen, Gavillet et al. 2006; Topffer, Muller-Schiffmann et al. 2007). However, since the MARK3 derived PTPH1 phosphorylation was not analysed *in vivo* no final

conclusions can be drawn considering the involvment of MARK3 in PTPH1 dependent signalling (Zhang, Kobayashi et al. 1997).

1.4.1.4 Plakophilin 2 (PKP2)

Cells are mostly organized into cooperative assemblies called tissues, where they are in contact with a complex network of secreted macromolecules called the extracellular matrix. The matrix helps to hold cells and tissues together and provides an organised lattice within which the cells can interact with each other. Furthermore, cells are often held in place by cell-cell adhesion. Cell junctions can be categorized in three groups: occluding junctions, which seal cells together; anchoring junctions, which mechanically attach the cytoskeleton of one cell with the extracellular matrix; or another cell; communication junctions, which mediate the passage of signals. The junctions introduced in the following paragraph – desmosomes and cell-cell adherence junctions - belong to the group of adherence junctions (Caplan, Seo-Mayer et al. 2008; Garrod and Chidgey 2008).

Desmosomes

Desmosomes connect adjacent cells; they are predominantly found in epithelial tissues but also in meninges, dendritic reticulum cells of lymph node follicles and the myocardium (McGrath 2005). Apart from the task of adhesion, they also play a role as signalling intermediates composed of a network of tissue-specific membrane and membrane-cytoskeletal linker molecules (Garrod and Chidgey 2008). Desmosomes consist of proteins of three gene superfamilies: the desmosomal cadherins, the

armadillo family of nuclear and junctional proteins and the plakins (North, Bardsley et al. 1999).

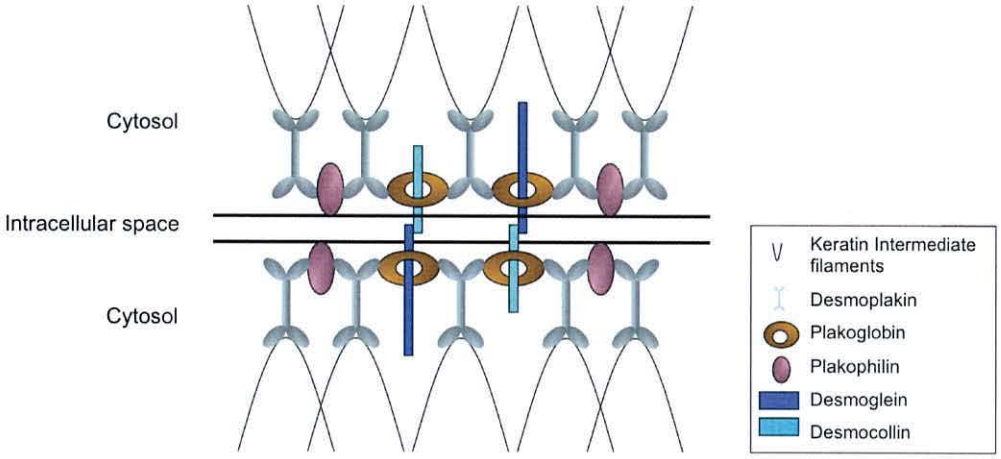


Figure 8: Molecular composition of a desmosome in human skin.
Desmosomes are molecular complexes of cell adhesion proteins and linking proteins. The cell adhesion proteins desmoglein and desmocollin, bridge the space between adjacent cells by way of homophilic binding of their extracellular domains to the desmosomal adhesion proteins of the adjacent cell. This extracellular region of binding is called Desmoglea. On the cytoplasmic side of the plasma membrane the intracellular regions of the desmosomal adhesion proteins are connected to intermediate filaments *via* the linking proteins plakophilin and plakoglobin.

The heterophilic associated transmembrane linker proteins desmoglein and desmocollin belong to the protein family of the cadherins. The plakin family comprise desmoplakins I and II, plectin and the cell envelope proteins, envoplakin and periplakin. Plakoglobin, plakophilin (PKP) and p0071 are the major representatives of the armadillo proteins, which have an adaptor function (Calkins, Hoepner et al. 2003; Papagerakis, Shabana et al. 2003).

There are three different PKPs (denoted PKP1, 2, 3). PKP2, which is a substrate of MARK3, is constitutively expressed in desmosomes. Moreover, it is also produced by

many cell types lacking desmosomes in which case a nuclear accumulation of PKP2 can be observed (Mertens, Kuhn et al. 1996; Schmidt, Langbein et al. 1999). Additionally, PKP2 has been identified to interact with β-catenin, a major participant of the Wnt-signalling pathway, the largest RNA polymerase III subunit and the transcription factor TFIIIB (Mertens, Hofmann et al. 2001)

MARK3 phosphorylation *in vivo* of PKP2 on serine 82 causes 14-3-3 binding and leads to a increased cytosolic location of PKP2 whereas the mutant form (PKP2 S82A) shows a stronger nuclear localisation (Muller, Ritt et al. 2003).

Cell-cell adherence junctions

Cell-cell adherence junctions occur in many non-epithelial tissues but are prominent in epithelial tissues. In non-epithelial tissues they take the form of small punctuate or streak=like attachments whereas in epithelial sheets they often form a continous adhesion belt called zonula adherence (Perez-Moreno, Jamora et al. 2003; Meng, Michaud et al. 2008). The adhesion belt is located around each interacting cell near the apex and continuous filaments in the cortical cytoplasm of adjacent cells. The adhesion belts of neighbouring cells are directly apposed and the interacting plasma membranes are connected *via* transmembrane linker proteins of the cadherin family (Wallez and Huber 2008; Stemmler 2008). Cadherins are responsible for Ca^{2+}-dependent cell junctions in vertebrates and are named after the tissue they are predominately expressed in. The mostly characterized cadherin is E-cadherin (epithelial tissues), which is usually concentrated in the adhesion belt (Stemmler 2008). The adhesion belt is connected *via* a set of intracellular attachment proteins

that include α-, β-, γ-catenin, vinculin, α-actin and plakoglobin to contractile bundle of actin filaments (Weber, Fraemohs et al. 2007; Nyqvist, Giampietro et al. 2008; Wallez and Huber 2008).

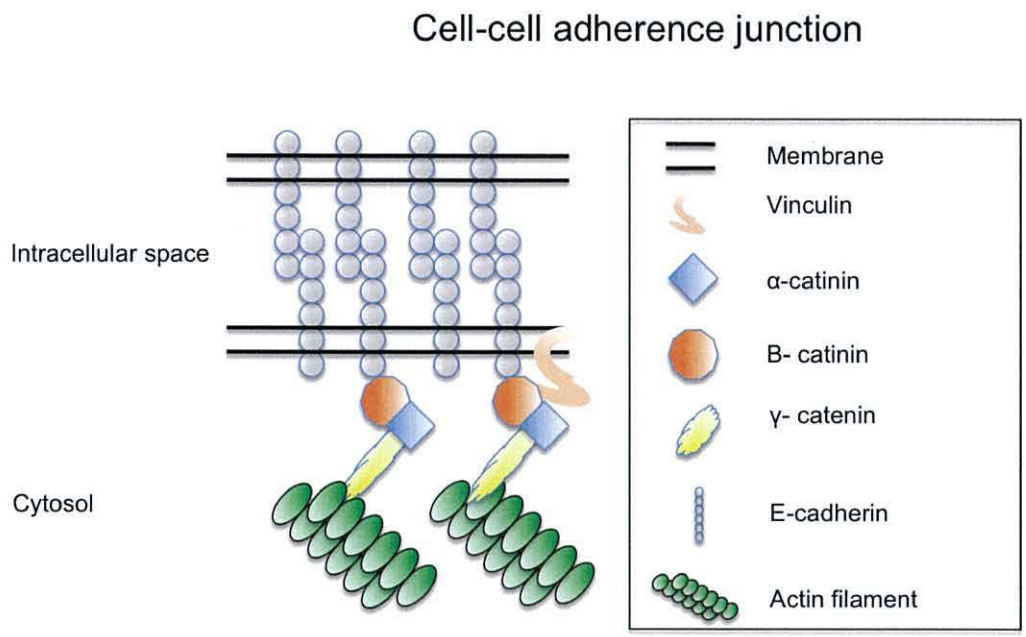


Figure 9: Molecular composition of a cell adherence junction
A network of proteins spans the intracellular space and provides attachment to actin filaments within the cell.

1.4.1.5 Histone deacetylases class IIa (HDACs)

Deacetylation of histones by histone deacetylases (HDACs) leads to a compact chromatin structure that restrains the transcriptional machinery from accessing DNA thereby functioning as transcription repressors. (Ng and Bird 2000; Thiel, Lietz et al. 2004). There are two protein families with HDAC activity: the recently discovered silent information regulator 2 (SIR2) family of NAD+-dependent HDACs (this family will not be the subject of discussion), and the classical HDAC family (Schwer and Verdin 2008). Members of the classical HDAC family fall into two different

phylogenetic classes (Class I and II). The class I HDACs (HDAC1, 2, 3 and 8) are most closely related to the yeast, *Saccharomyces cerevisiae,* transcriptional regulator RPD3 (Taunton, Hassig et al. 1996). Class II HDACs (HDAC4, 5, 6, 7, 9 and 10) share domains with similarity to histone deacetylase 1 (HDA1), another deacetylase found in yeast (Yang and Seto 2008).

Class II HDACs are further divided into two subgroups: HDAC4, -5, -7, and -9 form class IIa, whereas the remaining two constitute class IIb. Besides their catalytic domains, class IIa mammalian members have sequence similarity in the extended long NH_2-terminal domains and the COOH-terminal tails which class IIb HDACs lack (Yang and Gregoire 2005).

The COOH-terminal domain of Class IIa HDACs bears the catalytic domain whereas the NH_2-terminal domain, which is involved in regulation, serves as the docking platform for transcription factors and is the target of various post-translational modifications such as proteolytic cleavage (Bakin and Jung 2004; Liu, Dowling et al. 2004; Paroni, Mizzau et al. 2004), ubiquitination (Li, Song et al. 2004), sumoylation (Kirsh, Seeler et al. 2002; Petrie, Guidez et al. 2003) and phosphorylation (Miska, Karlsson et al. 1999; Wang, Bertos et al. 1999; Zhang, McKinsey et al. 2002; Dequiedt, Kasler et al. 2003; Yang and Gregoire 2005). Upon phosphorylation of serine residues within the NH_2-terminal domain, class IIa HDACs have been shown to bind several 14-3-3 proteins, which leads to nuclear export of the HDACs and activation of their target promoters (Grozinger and Schreiber 2000; Kao, Verdel et al. 2001; Dequiedt, Martin et al. 2006). This export mechanism has been implicated in developmental programs such as muscle differentiation (McKinsey, Zhang et al.

2000) and activity (Mejat, Ramond et al. 2005), cardiac hypertrophy (Zhang, McKinsey et al. 2002), T cell apoptosis (Dequiedt, Kasler et al. 2003), bone development (Vega, Matsuda et al. 2004), and neuron survival (Bolger and Yao 2005). Different signalling pathways converge on the signal responsive serine residues of class IIa HDACs. Ca^{2+}/Calmodulin-dependent protein kinases, PKC, MARK2 and MARK3 promote nuclear export of class IIa HDACs (Kao, Verdel et al. 2001; Chawla, Vanhoutte et al. 2003; Linseman, Bartley et al. 2003; Dequiedt, Martin et al. 2006). MARK2 and MARK3 target a unique serine residue, which subsequently leads to phosphorylation of further serine residues suggesting a hierarchical model for the nuclear export mechanism of class IIa HDACs. (Dequiedt, Martin et al. 2006)

1.4.2 Upstream regulators of MARK3

MARK3 contains 145 possible phosphorylation sites (serine, threonine and tyrosine residues), of which 21 sites have been identified to be phosphorylated *in vivo* (Göransson, Deak et al. 2006). However, only 4 of these could be matched to upstream regulators of MARK3. Both the tumour suppressor master kinase of the AMPK family, Liver kinase B1 (LKB-1) and microtubule affinity regulating kinase kinase (MARKK, a Ste20-like kinase) have been described to activate MARK3 by targeting T211 within the phosphorylation loop (Timm, Li et al. 2003; Lizcano, Göansson et al. 2004). Oncogenic serine/threonine kinase proviral integration site 1 (PIM-1), functions as a negative regulator of MARK3, phosphorylating S90 as well as S95 or S96 within the kinase domain (Bachmann, Hennemann et al. 2004). Moreover,

atypical kinase C (aPKC) is assumed to regulate MARK3 localisation by phosphorylation of T564 within the KA1 domain (Hurov, Watkins et al. 2004).

1.4.2.1 Liver kinase B1 (LKB-1)

The tumour suppressor masterkinase of the AMPK family, LKB1 (responsible for Peutz-Jeghers syndrome) has been described to activate MARK3 (and all other MARKs) by targeting T211 within the phosphorylation loop (Rowan, Bataille et al. 1999; Sanchez-Cespedes 2002; Lizcano, Göransson et al. 2004; Velez, Gaitan et al. 2009). The LKB-1 complex is composed of LKB-1, the pseudokinase STE20-related adaptor protein (STRAD) and the scaffolding protein mouse protein 25 (MO25). The complex positively regulates the activity of at least 14 downstream kinases - related to the AMP-activated protein kinase (AMPK) – which have been implicated in the regulation of cellular responses to energy stress and the establishment of polarity (Boudeau, Baas et al. 2003; Lizcano, Göransson et al. 2004; Koh and Chung 2007; Williams and Brenman 2008). Multiple members of this group are likely to affect both polarity control and cell metabolism. Two examples are the MARK group and AMPK. In general MARKs are assumed to promote epithelial polarisation through regulation of microtubule stability but recently MARK2 has also been shown to have a role in growth and fat metabolism (Hurov and Piwnica-Worms 2007). AMPK, which is often referred to as an "energy sensor", regulates the ATP/AMP ratio within the cell, but has also recently been shown to be involved in cell polarity reguation (Hurov and Piwnica-Worms 2007; Lee, Koh et al. 2007; Sag, Carling et al. 2008).

As an established regulator of cell polarity LKB-1, has been implicated in formation of the apical brush border, positioning of junctional proteins surrounding the brush boarder, and the correct sorting of apical and basolateral plasma membrane markers (Jang, Lee et al. 2008; Baas, Kuipers et al. 2004; Zhang, Schafer-Hales et al. 2008). Additionally, it has been demonstrated that LKB-1 is involved in neuronal polarization in the mammalian cortex and hippocampus. (Baas, Kuipers et al. 2004; Barnes, Lilley et al. 2007; Winckler 2007). Moreover, LKB1 has been implicated in the regulation of polarisation events important for mitosis and neuronal cell migration (Asada, Sanada et al. 2007; Lee, Koh et al. 2007).

LKB1 has been described as a tumour suppressor kinase and has been associated with the Peutz-Jeghers-Syndrome (PJS), which is a rare autosomal dominant syndrome characterized by benign gastrointestinal polyps and an enhanced risk of malignancy (McGarrity and Amos 2006). Furthermore, loss of function of LKB1 has been described for a variety of neoplasms such as non-small cell lung carcinomas, pancreatic cancer and melanomas (Avizienyte, Roth et al. 1998; Guldberg, thor Straten et al. 1999; Rowan, Bataille et al. 1999; Sanchez-Cespedes, Parrella et al. 2002; Ikediobi, Davies et al. 2006; Koivunen, Kim et al. 2008). The tumour-suppressor role of LKB1 is indicated by its ability to suppress the growth of tumour cells and promote cell-cycle arrest and apoptosis (Tiainen, Ylikorkala et al. 1999; Karuman, Gozani et al. 2001; Tiainen, Vaahtomeri et al. 2002). Furthermore, it could be implicated in angiogenesis and promotes β-catenin stability *via* inhibition of GSK3β (Ylikorkala, Rossi et al. 2001; Lin-Marq, Borel et al. 2005). Recent studies implicate cell polarity defects in neoplasmic changes. In these studies early alterations

in epithelial cell polarity is a hallmark of epithelial cell cancers or carcinomas and contribute to their development as carcinomas *in situ* or their progression to invasive adenocarcinomas (Humbert, Russell et al. 2003; Bilder 2004; Humbert, Grzeschik et al. 2008). Polarity alterations are difficult to detect in carcinomas *in situ*, which are often fully polarized, but they are obvious in invasive carcinomas. These are usually characterized by low expression levels or loss of function mutations of E-cadherin or its associated proteins, the catenins. These changes often promote a transformation of the epithelial phenotype into that of a migratory mesenchymal cell (epithelial-mesenchymal transition, EMT) (Jeanes, Gottardi et al. 2008). Additionally, the disassembly of the junctional complex triggers a variety of oncogenic pathways such as the Wnt-pathway, which can promote proliferation (Gloushankova 2008). Noteworthy in this regard might be the observation that deletion of LKB1 leads to polarity defects before the onset of tumourigenesis and that LKB-1 is assumed to be a critical mediator of the polarity program in non-small cell lung cancer (Hezel, Gurumurthy et al. 2008).

1.4.2.2 Microtubule affinity regulating kinase kinase (MARKK)

MARKK is a serine/threonine kinase that belongs to the Ste20 kinase family, which was identified as the activating kinase of the MARK family in porcine brain (Timm et *al.*, 2003). MARKK activates MARK3 by targeting T211 within the phosphorylation loop. MARKK is highly homologous to the human thousand and one amino acid kinase (TAO-1) (Hutchison et *al.*, 1998). The target proteins MARK1, 2 and 4 phosphorylate microtubule associated proteins (MAPs) such as MAP2, MAP4 and tau

at the KXGS motifs in their microtubules binding domain (Ebneth, Drewes et al. 1999; Schneider, Biernat et al. 1999). This phosphorylation leads to detachment of the MAPs from microtubules, resulting in microtubules breakdown (Drewes et *al.*, 1997). An aberrant activity of the MARKK–MARK–MAP pathway can lead to changes in the cytoskeleton and impaired cellular functions (Thies and Mandelkow, 2007; Ebneth et *al.*, 1998). One example, which should be noted in this context is the hyperphosphorylation and aggregation of tau proteins in degenerating neurons of Alzheimer's disease patients. (Augustinack, Schneider et al. 2002; Timm, Matenia et al. 2006). Besides its function in microtubule dynamics, MARKK has been described as a regulator of mitotic progression through interaction with an essential spindle checkpoint component, the kinase BubR1 (Draviam et *al.*, 2007). The spindle checkpoint prevents aneuploidy, a hallmark of most malignant aggressive tumors by delaying the metaphase-anaphase transition until all chromosomes have been attached to microtubules (Decordier, Cundari et al. 2008). Moreover, MARKK has been implicated in the stress-induced DNA damage response, and apoptosis *via* mitogen-activated protein kinase (MAPK) signalling (Raman, Earnest et al. 2007; Wu et al. 2008). Deregulation of MAPK signalling, apoptosis and DNA damage response have been shown to be key events within the development and progression of tumours (Downs 2007; Hui, Bakiri et al. 2007). In this regard it might be noteworthy that in cells of ataxia telangiectasia (AT) patients, which have a higher risk of tumour formation, MARKK activation is compromised (Raman, Earnest et al. 2007). In these cells MARKK activation by DNA damage is reduced, as is p38 MAPK activation.

MARKK RNAi experiments showed that knockdown of MARKK impairs the DNA damage-activated G2/M cell cycle checkpoint (Raman, Earnest et al. 2007).

1.4.2.3 Proviral integration site 1 (PIM-1)

The functions of the oncogenic serine/threonine kinase PIM-1 are varied and fundamental in the biology of the cell. PIM-1 has been implicated in signal transduction, cell cycle regulation, transcription regulation and survival (Wang, Bhattacharya et al. 2001; Bachmann, Kosan et al. 2006). Activity of MARK3 has been shown to be down regulated by PIM-1 phosphorylation of S90 as well as S95 or S96 within the kinase domain (Bachmann, Hennemann et al. 2004). Regarding carcinogenesis, PIM-1 plays multiple roles in the transformation of haematopoietic and prostate cells and is a marker of poor prognosis since expression is correlated with tumour aggressiveness (de Vos, Krug et al. 2003; Hoefnagel, Dijkman et al. 2005; Deutsch, Fruhwirth et al. 2009). PIM-1 seems to promote early transformation, cell proliferation and cell survival (Liang, Hittelman et al. 1996; Nieborowska-Skorska, Hoser et al. 2002; Kim, Baird et al. 2005; Qian, Niu et al. 2005; Rainio, Ahlfors et al. 2005). In addition, it is considered that PIM-1 might participate in tumour-derived angiogenesis (Zippo, De Robertis et al. 2004). Recent studies analysing the potential of PIM-1 inhibitors to suppress tumour growth support the idea that PIM-1 is a potential target for cancer therapies (Shah, Pang et al. 2008). For instance an atypical kinase β inhibitor used in trials to treat diabetic complications has been shown to inhibit PIM-1 and *in vitro* experiments suggest its application in acute myeloid leukaemia (Fedorov, Marsden et al. 2007).

1.4.2.4 Atypical protein kinase C (aPKC)

The fourth known MARK3 upstream regulator, aPKC, belongs to the protein kinase C family. The protein kinase C family is formed by a large number of isoenzymes that are classed in three categories - classical, novel and atypical - depending on their structure and function. All isoforms have a relatively conserved kinase domain at their COOH-terminus and a clearly divergent regulatory domain at their NH_2-terminus. The topology of the regulatory domain serves to ascribe a given PKC to a particular category. Classical PKC have a phospholipid and Ca^{2+} binding domain and a double zinc finger domain, which have been shown to be responsible for their regulation by Ca^{2+} and lipid second messengers, such as diacyglycerol. PKCs, which belong to the novel-PKC subfamily have been shown to be insensitive to Ca^{2+} but can still be activated by phorbol esters and stable analogues of diacylglycerol. In contrast to this, atypical PKCs are insensitive to Ca^{2+} and diacylglycerol (Steinberg 2008). In vertebrates the aPKC family has two isoforms aPKCι/λ and aPKCζ which are highly related but encoded by separate genes (Suzuki, Akimoto et al. 2003). At present it is unclear if both aPKCs are functionally redundant and thus can mediate the various aPKC functions, or if different roles are performed by either one of the aPKCs. The aPKCs are key players in the formation of almost all forms of polarity (Suzuki and Ohno 2006). They form a complex with Polarity Defective 6 (PAR-6) and Polarity Defective 3 (PAR-3), which belong to the glycine-leucine-glycine-phenylalanine (GLGF) domain family of adaptor proteins. PAR-6 seems necessary for aPKC activation by connecting aPKC to the GTP bound form of the small GTPases RAS-related C3 botulinum toxin substrate (RACs) and Cell division cycle 42 (Cdc42). This

results in conformational changes in PAR6 allowing phosphorylation and subsequent activation of aPKC (Joberty, Petersen et al. 2000; Lin, Edwards et al. 2000). PAR-3 functions as a scaffolding protein, which ensures the proper recruitment of the PAR6/aPKC to sites where activity is desired (Ebnet, Suzuki et al. 2001).

Together with the Crumbs complex, consisting of Crumbs, Stardust and dPatJ, and the Scribble complex, consisting of Scribble, Discs Large and Lethal Giant Larvae the PAR-3/PAR-6/aPKC complex belongs to the main regulators of cellular polarity in general and apical-basal cell polarity in epithelial cells in particular (Dow and Humbert 2007). The aPKC complex controls the establishment of the basolateral domain by excluding components of the lateral membrane from the apical surface (Dow and Humbert 2007). For instance, aPKC phosphorylates Lethal Giant Larvae to exclude it from the apical membrane and restricts it to the lateral membrane. In turn, Lethal Giant Larvae protein maintains aPKC in an inactive state at the basolateral region (Betschinger, Mechtler et al. 2003). Furthermore, the PAR complex has been involved in polarized cell migration *via* spatial control of actin myosin fiber activity and of GTPase activity (Etienne-Manneville and Hall 2001; Ludford-Menting, Oliaro et al. 2005; Sordella and Van Aelst 2008). Next to its role in polarity, aPKC may also play a role in apoptosis, inflammatory responses and insulin and c-Jun NH_2-terminal kinase pathways (Wooten 1999; Diaz-Meco and Moscat 2001; Moeschel, Beck et al. 2004; Farese, Sajan et al. 2005; Ludford-Menting, Oliaro et al. 2005; Kim, Datta et al. 2007; Win and Acevedo-Duncan 2008).

aPKC deregulation, intrinsic or by other oncogenes, could be involved in breast, ovarian, head-and-neck, lung, liver and colon cancer formation (Eder, Sui et al. 2005;

Regala, Weems et al. 2005; Cohen, Lingen et al. 2006; Zhang, Huang et al. 2006; Kojima, Akimoto et al. 2008). Hyper activation or delocalisation of the kinase affects tumour growth, motility and proliferation, which suggest that releasing aPKC from its normal polarisation tasks unleashes the pro-oncogenic potential of the kinase (Donson, Banerjee et al. 2000; Fields, Murray et al. 2003; Murray, Jamieson et al. 2004; Sun, Gao et al. 2005; Cohen, Lingen et al. 2006; Kuribayashi, Nakamura et al. 2007). The PAR-3/aPKC complex is suggested to change from a complex that promotes normal cell polarity to one that promotes transformation by changing the interaction partners. Besides aPKC, other polarity regulators belonging to the Scribble and the Crumbs complex could be involved in tumourigenesis, which seems to demonstrate that maintenance of cell polarity functions as a tumour suppressor factor (Etienne-Manneville 2008). In this context, deregulation of the PAR complex seems to be oncogenic, which probably results from a combination of organisation loss and diversion of aPKC activity towards other signalling pathways (Tong, Hussain et al. 2000; Etienne-Manneville and Hall 2002; Etienne-Manneville and Hall 2003; Etienne-Manneville, Manneville et al. 2005; Ozdamar, Bose et al. 2005; Bose and Wrana 2006; Farooqui, Zhu et al. 2006; Martin-Belmonte and Mostov 2007). In conclusion, disruption of cell polarity has been shown as an important event in initiation and progression of cancer. Therefore, deregulation of the PAR-3/aPKC complex can have a crucial function on the development as well as progression of a cancer. Targeting aPKC is considered a novel approach to stopping cancer progression. Application of an aPKC inhibitor has shown promising results in various

carcinomas (Stallings-Mann, Jamieson et al. 2006; Jin, Ying et al. 2008; Regala, Thompson et al. 2008).

The MARK kinases, involved in cell polarity *via* their function to regulate microtubules stability, seem to play a role in aPKC's function to promote cellular polarisation. Phosphorylation of T595 within the KA-1 domain of MARK2 by aPKC has been shown to be important in establishing and maintaining epithelial cell polarity (Hurov, Watkins et al. 2004; Chen, Wang et al. 2006). MARK3 is phosphorylated *in vitro* by aPKC on this conserved residue T564, which, is assumed to regulate MARK3 localisation (Hurov, Watkins et al. 2004). At present no further data is available, which could shed light on the physiological function of the MARK3/aPKC interaction.

To conclude, all MARK3 upstream regulators are involved in tumourigenesis. It also appears that tumour suppressor proteins activate MARK3 while oncogenes inactivate it or regulate the localisation of MARK3. This implies an important role for MARK3 in tumour development and progression.

1.5 Aims of the project

Taking the known substrates into consideration, MARK3 is thought to contribute to the silencing of mitogenic pathways in the absence of growth stimuli, to inhibit premature cell cycle progression and to contribute to cellular adhesion. Deregulation of these cellular programs have been shown to be crucial for tumour formation and

progression, which indicates a role for MARK3 in these processes. This model is supported by the observation that tumour suppressor proteins activate, and oncogenes inactivate or define the localisation of MARK3.

This project aims at revealing further cellular functions of MARK3 by finding new substrates as well as upstream regulators of MARK3. These findings should deepen the understanding considering the position of MARK3 within the cellular signalling network. Furthermore, identified substrates or upstream regulating partners might support the hypothesis that MARK3 plays a role in tumour formation and/or progression, which could reveal MARK3 and the upstream regulators as attractive therapeutic targets respectively.

Chapter II: Materials and Methods

2.1 Materials

Table 1: Materials

Plastic ware	
Plastic ware	Greiner Bio-one (Frickenhausen, Germany) VWR (Darmstadt, Germany)
Chemicals	Melford Laboratories Ltd. (Suffolk, U.K.) Sigma-Aldrich Family (Mo, U.S.A) BD Bioscience (NY, U.S.A) Merck (Darmstadt, Germany)
Cell culture solutions	
D-MEM Glutamax	Cambrex (NJ U.S.A)
Trypsin/EDTA,	Cambrex (NJ U.S.A)
Fetal calf serum (FCS),	Cambrex (NJ U.S.A)
Phosphate buffered saline (PBS)	Cambrex (NJ U.S.A)
Kits	
Plasmid Mini. Midi, Maxi kit	Qiagen Ltd. (west Sussex, U.K.)
Gel extraction kit	Qiagen Ltd. (west Sussex, U.K.)
MBP Protein Fusion and Purification system	New England Biolabs (MA, U.S.A.)
Bradford protein Assay kit	Sigma-Aldrich Family (Mo, U.S.A)
Silver stain kit	Bio-rad (CA, U.S.A)
Super Signal West pico kit	Pierce (IL, U.S.A)
Proto-array human protein Microarray KSI	Invitrogen (CA, U.S.A.)
Bacterial strains	
TOP10	Merck (Darmstadt, Germany)
BL21 (de3) pRIPL	Merck (Darmstadt, Germany)
BLT5403	Merck (Darmstadt, Germany)
Enzymes/Ladders	
Restriction enzymes	Promega Corporation Ltd. (Mannheim, Germany)
DNA ladder 1kb	Promega Corporation Ltd. (Mannheim, Germany)
DNA ladder 100bp	Promega Corporation Ltd. (Mannheim, Germany)
Alkaline phosphatase	Promega Corporation Ltd. (Mannheim, Germany)
Klenow enzyme kit	Promega Corporation Ltd. (Mannheim, Germany)
Pfu Polymerase	Promega Corporation Ltd. (Mannheim, Germany)
Lambda phosphatase	New England Biolabs (MA, U.S.A.)
NEB broad range protein ladder	New England Biolabs (MA, U.S.A.)
MARK3	Upstate (Dundee, U.K.)
MELK	Upstate (Dundee, U.K.)
LR clonase enzyme mix	Invitrogen (CA, U.S.A.)

Protein kinase K The Trypsin Singles™	Invitrogen (CA, U.S.A.) Sigma-Aldrich Family (Mo, U.S.A)
Oligonucleotides/Sequencing Oligonucleotides Sequencing	 MWG (Ebersberg, Germany) MWG (Ebersberg, Germany)
Phage cDNA library T7 brain cDNA phage library	 Merck (Darmstadt, Germany)
Antibodies α-MARK3 α-MBP α-MYC α-FLAG α-Phospho-MARK Activation loop goat α-rabbit goat α-mouse	 Upstate (Dundee, U.K.) New England Biolabs (MA, U.S.A.) Serotec (Oxford U.K.) Serotec (Oxford U.K.) Cell signalling (MA, U.S.A.) Pierce (IL, U.S.A) Pierce (IL, U.S.A)
Vectors pmal c2x pmal HISMBP pEntr11 pcDNA3 pDEST3 pcDNA3 Pyo MARK3 pCMV Sport 6 KLC4 pCMV Sport 6 KIFC3	 New England Biolabs (MA, U.S.A.) Addgene (MA, U.S.A.) Invitrogen (CA, U.S.A.) Invitrogen (CA, U.S.A.) Provided by Dr. J. Müller Provided by Dr. J. Müller Open Biosystems (Huntsville, U.S.A.) Open Biosystems (Huntsville, U.S.A.)
Radioactive Isotopes [γ-32P] Phosphate [γ-33P] Phosphate	 Amersham (Bucks, U.K.) Amersham (Bucks, U.K.)
Software ProtoArray® Prospector software version 4.0.	 Invitrogene (CA, U.S.A.)

2.2 Standard molecular biological methodology

2.2.1 DNA analysis and manipulation

2.2.1.1 Agarose gel electrophoresis

DNA fragments were separated on agarose gels (0.7-2% agarose, 1 µg/ml ethidium bromide) in TAE buffer with 10 V/cm. Samples were mixed with 0.2 volumes 6 x DNA loading buffer. Sizes of DNA fragments were determined by comparison with appropriate DNA ladders.

10x TAE Buffer

Tris-base 48.4 g
Glacial Acetic Acid 10.9 g
EDTA 2.92 g
ddH_2O 1.0 L

6x DNA loading buffer

12% FICOLL 400
60 mM Na_2EDTA, pH 8
0.6% SDS
0.003% bromphenol blue

2.2.1.2 Preparation of DNA

Depending on the desired amount of plasmid DNA needed Qiagen Plasmid Mini, Midi or Maxi Prep-kits were used for plasmid purification. The preparation was performed according to the manufacture's protocol.

2.2.1.3 Purification of DNA

DNA purification out of agarose gels or cleaning of PCR products were performed with Qiagen MiniElute PCR Purification Kit according to the manufacture's protocol.

2.2.1.4 Precipitation of DNA

DNA was precipitated by addition of 2.5 volume ethanol and 0.1 volume 3 M sodium acetate (pH 4.8) and subsequent centrifugation at 13,000g for 10 minutes at 4°C. The DNA pellet was washed once with 70% ethanol, allowed to dry and resuspended in ddH_2O and Tris-EDTA buffer respectively.

10x Tris-EDTA buffer

100 ml 1 M Tris-HCl pH 7.5
20 ml 500 mM EDTA pH 8.0
880 ml ultrapure water

2.2.1.5 Quantification of DNA

The purity and concentration of nucleic acids were determined by measuring the absorption at 260 nm and 280 nm. An OD_{260} of 1 is equivalent to 50 µg/ml double

stranded DNA. The ratio of OD_{260}/OD_{280} indicates the purity of the preparation and is ideally 1.8-2.0.

2.2.1.6 Transformation of plasmid DNA

Bacteria (TOP10) were grown in 5 ml LB medium overnight at 37°C. 250 ml LB medium were inoculated with 2.5 ml of the overnight culture and shaken at 37°C to an O.D. of 0.4 (595 nm). The cells were then pelleted at 3500 g for 10 minutes at 4°C. All further step were carried out at 4°C: resuspension of the cells in half of the original volume of pre-cooled 50 mM $CaCl_2$, 20 minutes incubation, centrifugation of the cells as described above, resuspension in one tenth of the original volume of 50 mM $CaCl_2$ plus 20% glycerol, and aliquoting of 500 µl into sterile tubes. The aliquots of competent bacteria were immediately frozen in liquid nitrogen and stored at –70°C. For transformation, 100 ng of DNA were mixed with 100µl of competent bacteria, incubated for 30 minutes on ice, heat-shocked for 45 seconds at 42°C and left on ice again for 2 minutes. Then, 1 ml of LB medium was added and the samples were incubated under agitation for 1 hour at 37°C. 100 µl of the sample were spread onto LB agar plates containing the appropriate antibiotic for selection of transformed bacteria. Transformed colonies appeared after an overnight incubation at 37°C.

2.2.1.7 Transfection of DNA into HEK293(T) cells / Calcium phosphate transfection

3 hours prior to transfection 60-70% confluent HEK-293 cells were exposed to fresh cell culture media to stimulate cell division. After 3 hours 20 µg DNA were

transfected by using a modified calcium phosphate precipitation method as described by Chen and Okayama (1988). 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. The calcium phosphate-DNA complexes were formed in water by mixing the components as follows:

Table 2: Calcium phosphate transfection

Petri dish	100 mm
$CaCl_2$	50 µl
2x HEPES Buffer	500 µl
Plasmid DNA	20 µg
ddH_2O	Add 1 ml

Formation of precipitates was induced by addition of the DNA and $CaCl_2$ mixture to 2x HEPES buffer. After thoroughly mixing the reactions these were incubated for 20 minutes at room temperature and then added to the cells. Expression was analysed 48 hours after transfection.

Calcium phosphate solution

2.5 M $CaCl_2$
Filter sterilize through 0.22 µm nitrocellulose filter

2x HEPES buffer

16.4 g NaCl
11.9 g Hepes
0.21 g $Na_2HPO_47H_20$
800 ml ddH_2O
pH 7.05-7.12
add ddH_2O to 1 litre
Filter sterilize through 0.22 µm nitrocellulose filter

2.2.1.8 Modification of DNA

The following methods were performed according to the manufacturer's protocol of the respective enzyme (Promega):

- DNA digestion with restriction endonucleases
- Ligation of DNA fragments
- Dephosphorylation of DNA with alkaline phosphatase
- Phosphorylation of DNA with T4 Polynucleotide kinase
- Klenow enzyme reactions

2.2.1.9 Site directed Mutagenesis of KLC4 and MARK3

Site directed mutagenesis was performed according to the Quick Change Site Directed Mutagenesis Kit II protocol (Stratagene) with the exception that a different Pfu polymerase (Promega) was used. Briefly, the Quick Change site-directed mutagenesis method is performed using Pfu DNA polymerase, which replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure utilizes a supercoiled double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by Pfu DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *DpnI*. The *Dpn*I endonuclease (target sequence: 5′-Gm^{6}ATC-3′) is specific for methylated and hemi-

methylated DNA and is used to digest the parental DNA template and does not affect mutation-containing synthesized DNA. After the *DpnI* digestion step the DNA is transformed into *E. coli* and positve clones are verfied by sequencing.

Table 3: Mutagenesis PCR conditions:

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	16	95°C	30 seconds
		55°C	1 minute
		72°C	2 minutes/kb of plasmid length

KLC4 Mutagenesis:

Vector template: pDEST3 MYC KLC4 (7.3 kb) Mutants

Primer combinations for mutagenesis of potential KLC4 MARK3 phosphorylation sites:

S519A: GAG TGA TGG TAG AAG GAC TGC GCA GGA GGG CCC TGG AG
S519A: CTC CAG GGC CCT CCT GCG CAG TCC TTC TAC CAT CAC TC

S609A: CTC CAG GTC TCC CGG CGC CTC GCC GCC AGC ACC ATG GAC
S609A: GTC CAT GGT GCT GGC GGC GAG GCG CCG GGA GAC CTG GAG

T498A: GTC CCG GAG ACA GGG CGC CGA CCC TAT CAG CCA G
T498A: CTG GCT GAT AGG GTC GGC GCC CTG TCT CCG GGA C

S566A: GAT GTG CTC CGC AGA AGC GCT GAA CTC TTG GTG AGG
S566A: CCT CAC CAA GAG TTC AGC GCT TCT GCG GAG CAC ATC

S460A: GGC CTG CAA AGT GAG CGC TCC CAC AGT GAA CAC TAC
S460A: GTA GTG TTC ACT GTG GGA GCG CTC ACT TTG CAG GCC

S582A: GAG CCT CGG CCC TCC GCT AGC AAC ATG AAG CGA G
S582A: CTC GCT TCA TGT TGC TAG CGG AGG GCC GAG GCT C

S554A: CTG CAG AGG AGT GGC GCC CTT GGC AAG ATC CG
S554A: CGG ATC TTG CCA AGG GCG CCA CTC CTC TGC AG

S590A: CCA GCA GCA ACA TGA AGC GAG CGG CCG CCT TGA ACT ATC TGA AC
S590A: GTT CAG ATA GTT CAA GGC GGC CGC TCG CTT CAT GTT GCT GCT GG

MARK3 Mutagenesis:

Vector template: pDEST HISMBP MARK3 KD (10.5 kb)

Primer combination to generate kinase inactive MARK3:

K85A 1: GTT TTG TCA ATT ATC GCG ATT GCA ACC TCT CTG
K85A 2: CAG AGA GGT TGC AAT CGC GAT AAT TGA CAA AAC

2.2.1.10 Gateway cloning

The Gateway® Technology is a universal cloning method based on the site-specific recombination properties of bacteriophage lambda (Landy et al., 1989). The Gateway® technology provides a rapid and highly efficient way to move DNA sequences into vectors (Hartley 2001). For this purpose a gene of interest was cloned into an Entry vector (pEntr11). 50-150 ng/µl of the Entry vector were mixed with 150 ng/µl of Destination vector (pDEST pcDNA3). TE buffer and 2µl LR clonase enzyme mix (containing the recombinase) were added to a final volume of 10 µl and incubated for 1 hour at 25°C. Then, the mixture was exposed to 1 µl of Proteinase K solution for 10 minutes at 37°C to terminate the reaction. Subsequently the reaction was transformed into TOP10 bacteria.

2.2.1.11 Amplification of DNA by polymerase chain reaction

DNA was amplified by polymerase chain reaction with Pfu DNA polymerase (Promega) according to the manufacturer's protocol.

2.2.1.12 Vectors

pEntr11 MYC

A double stranded oligonucleotide encoding for two MYC tags – EQKLISEEDL EQKLISEEDL – was cloned into the *NcoI* restriction site of pEntr11 without disrupting the reading frame. For this purpose the original *NcoI* restriction site was destroyed and a new *NcoI* site was introduced downstream of the two MYC tags.

Oligo 1
5`-CAT GCT GGA GCA GAA GCT GAT CAG CGA GGA GGA CCT GGA GCA GAA GCT GAT CAG CGA GGA GGA CCT GGC-3`

Oligo2
5`-CAT GGC CAG GTC CTC CTC GCT GAT CAG CTT CTG CTC CAG GTC CTC CTC GCT GAT CAG CTT CTG CTC CAG-3`

pEntr11 FLAG

A double stranded oligonucleotide comprising three FLAG tags - DYKDDDDK – was cloned into the *NcoI* restriction site of pEntr11 without disrupting the reading frame. For this purpose the original *NcoI* restriction site was destroyed and a new *NcoI* site was introduced downstream of the FLAG tags.

Oligo 1

5`-CAT GCT GGA CTA CAA GGA CCA CGA CGG CGA CTA CAA GGA CCA CGA CAT CGA CTA CAA GGA CGA CGA CGA CAA GGC-3`

Oligo 2
5`-CAT GGC CTT GTC GTC GTC GTC CTT GTA GTC GAT GTC GTG GTC CTT GTA GTC GCC GTC GTG GTC CTT GTA GTC CAG-3`

pEntr11 MARK3 His

Full length MARK3 was amplified by PCR and cloned into the *NcoI* and *XhoI* restriction sites of pEntr11. The applied 5` primer encodes for 6 histidines that were inserted upstream of the MARK3 gene.

5`Primer
5`-ATA TCC ATG GGG CAC CAC CAT CAC CAC CAC ATG TCC ACT AGG ACC CCA TTG CCA AC-3`

3`Primer
5`-A TAT CTC GAG TTA CAG CTT TAG CTC ATT GGC AAT TTT G-3`

Table 4: PCR conditions MARK3 HIS

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	35	95°C	30 seconds
		50°C	30 seconds
		72°C	5 minutes

pEntr11 MYC MARK3 KD

MARK3 KD (AA1-393) was amplified by PCR and cloned into the *NcoI* and *XhoI* restriction sites of pEntr11 MYC

Template: pcDNA3 Pyo MARK3

5`Primer
5`-TCC ATG GGG ATG TCC ACT AGG ACC CCA TTG CC-3`

3`Primer
5`-ACTCGAGTTAGAGATCACTGCTCGGCCTAAC-3`

Table 5: PCR conditions MARK3 KD

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	35	95°C	30 seconds
		47°C	30 seconds
		72°C	4 minutes

pDEST HISMBP MARK3 KD IA

The kinase domain of MARK3 within the vector pDEST HISMBP MARK3 KD was inactivated by mutatgenesis (K85A) of the ATP binding pocket.

pEntr11 MYC KLC4

KLC4 (AA1-620) was amplified by PCR and cloned into the *SalI* and *NotI* restriction sites of pEntr11 MYC

Template: pCMV Sport 6 KLC4

5` Primer
5`-TAT GTC GAC ATG TCA GGC CTG GTG TTG G-3`

3` Primer
5`-TAT AGC GGC CGC TCA GCT GCT TGA AGA GAG GTC-3`

Table 6: PCR conditions KLC4

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	35	95°C	30 seconds
		50°C	30 seconds
		72°C	4 minutes

pEntr11 MYC KIFC3

KIFC3 (AA1-688) was amplified by PCR and cloned into the *SalI* and *Not1* restriction sites of pEntr11 MYC

Template: pCMV Sport 6 KIFC3

5`Primer
5`-GGT GTC GAC ATG GGT GGA GAA TGA GCG ACT G-3`

3`Primer
5`-TAT AGC GGC CGC TCA GGC CGA GGG CTG CAG-3`

Table 7: PCR conditions KIFC3

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	35	95°C	30 seconds
		50°C	30 seconds
		72°C	5 minutes

pEntr11 FLAG KLC4 451

KLC4 (AA1-451) was amplified by PCR and cloned into the *SalI* and *Not1* restriction sites of pEntr11 FLAG

Template: pEntr11 MYC KLC4

5`Primer
5`-TAT GTC GAC ATG TCA GGC CTG GTG TTG G-3`

3`Primer
5`-TAT AGC GGC CGC TTA GCC TCC ATA CTC AGC ATA GGG TGT-3`

Table 8: PCR conditions KLC4 451

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	35	95°C	30 seconds
		50°C	30 seconds
		72°C	3 minutes

pEntr11 FLAG KLC4 451-620

KLC4 (AA451-620) was amplified by PCR and cloned into the *SalI* and *NotI* restriction site of pEntr11 FLAG

Template: pEntr11 MYC KLC4

5`Primer
5`-TAT GTC GAC ATG GGC TGG TAC AAG GCC TGC AAA GTG-3`

3`Primer
5`-TAT AGC GGC CGC TCA GCT GCT TGA AGA GAG GTC-3`

Table 9: PCR conditions KLC4 451-620

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	35	95°C	30 seconds
		50°C	30 seconds
		72°C	2 minutes

pEntr11 FLAG KIFC3 515

KIFC3 (AA1-515) was amplified by PCR and cloned into the *SalI* and *NotI* restriction sites of pEntr11 FLAG

Template: pEntr11 MYC KIFC3

5`Primer
5`-GGT GTC GAC ATG GGT GGA GAA TGA GCG ACT G-3`

3`Primer
5`-TAT AGC GGC CGC TTA CGT CAC GAT GAG CAG TGC GTG-3`

Table 10: PCR conditions KIFC3 515

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	35	95°C	30 seconds
		50°C	30 seconds
		72°C	5 minutes

pEntr11 FLAG KIFC3 515-688

KIFC3 (AA515-688) was amplified by PCR and cloned into the *SalI* and *NotI* restriction sites of pEntr11 FLAG

Template: pEntr11 MYC KIFC3

5`Primer
5`-GGT GTC GAC ATG ACG GTG CGA GGC GTG GAC TG-3`

3`Primer
5`-TAT AGC GGC CGC TCA GGC CGA GGG CTG CAG-3`

Table 11: PCR conditions KIFC3 515-688

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	35	95°C	30 seconds
		50°C	30 seconds
		72°C	2 minutes

pDEST3 MYC KLC4

Destination vector: pDEST3

Entry vector: pEntr11 MYC KLC4

Gateway cloning

pDEST3 MYC KIFC3

Destination vector: pDEST3

Entry vector: pEntr11 MYC KIFC3

Gateway cloning

pDEST3 FLAG KLC4 451

Destination vector: pDEST3

Entry vector: pEntr11 FLAG KLC4 451

Gateway cloning

pDEST3 FLAG KLC4 451-620

Destination vector: pDEST3

Entry vector: pEntr11 FLAG KLC4 451-620

Gateway cloning

pDEST3 FLAG KIFC3 515

Destination vector: pDEST3

Entry vector: pEntr11 FLAG KIFC3 1-515

Gateway cloning

pmal c2x KSR AA320-424

The KSR fragment comprising AA320-424 was cut out of pcDNA3 Pyo KSR with *BamHI* and *XbaI* and cloned into the *BamHI* and *XbaI* restricton sites of pmal c2x.

pmal c2x KSR AA320-424 S392A

The KSR fragment comprising AA320-424 was cut out of pcDNA3 Pyo KSR S392A with *BamHI* and *XbaI* and cloned into the *BamHI* and *XbaI* restricton sites of pmal c2x.

pmal c2x KSRODN AA385-399

A double stranded oligonucleotide encoding for AA385-399 of KSR was cloned into the multiple cloning site of (*EcoRI* x *SalI*) of pmal c2x.

Oligo 1

5`-AAT TCG CCA GGC TTC GGC GGA CAG AGT CTG TCC CGT CAG ATA TCA ACA ACT GAG-3`

Oligo 2

5'-GCG GTC CGA AGC CGC CTG TCT CAG ACA GGG CAG TCT ATA GTT GTT GAC TCA GCT-3`

2.2.2 Purification, analysis and modification of proteins

2.2.2.1 Column chromatography: Maltose binding protein (MBP)

In the pMAL™ Protein Fusion and Purification System, the gene of interest is inserted downstream from the *malE* gene into the pmal c2x vector or into the pmal HISMBP vector respectively. Introduction of the gene results in a plasmid capable of expressing proteins fused COOH-terminally to MBP. Large amounts of the fusion protein are expressed by usage of the strong Ptac promoter and the translation initiation signals of MBP (di Guan, Li et al. 1988; Maina, Riggs et al. 1988; Riggs 2001). The fusion proteins were purified by one-step affinity purification as described by the manufacturer (NEB). Briefly, the MBP-fusion proteins were released out of the bacteria by short pulse sonication. Subsequently the bacterial lysates were centrifugated to pellet all insoluble constituents (10000g for 30 minutes at 4°C). Then the soluble proteins were diluted with column buffer and loaded onto the amylose resin (polymer of glucose). After several washing steps with MBP column buffer the proteins were eluted with MBP column buffer containing 10 mM of the disaccharid maltose. MBP has a higher affinity to maltose than to the amylose resin and therefore the fusion protein elutes. The eluted proteins were dialysed against column buffer containing 20% glycerol and frozen in liquid nitrogen until further usage.

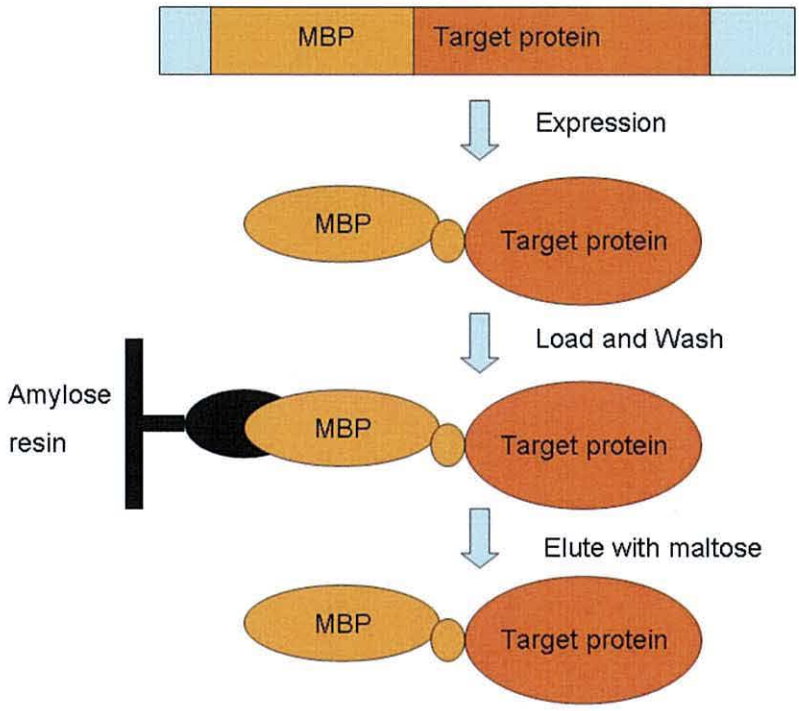


Figure 10: Purification of MBP fusion proteins
The MBP tagged fusion proteins were expressed in *Escherichia coli* and loaded onto a MBP affinity column. After several rounds of washing with MBP column buffer the proteins were eluted with MBP column buffer containing 10 mM maltose.

MBP column buffer

200 mM	NaCl
1 mM	DTT
20 mM	Tris-HCl pH 7.5

MBP elution buffer

200 mM	NaCl
1 mM	DTT
20 mM	Tris-HCl pH 7.5
10 mM	Maltose

Bacterial expression Media

Luria Bertani (LB) Media supplemented with 2% Glucose

2.2.2.2 *Expression conditions of MBP fusion proteins*

Kinase domain MARK3 AA1-393 (MARK KD-MBP), kinase inactive kinase domain MARK3 AA1-393 (MARK3 KD-MBP IA), MBP, mouse KSR1 fragment AA320-424 (KSR WT), mouse KSR1 fragment AA320-424 S392A (KSR MT) and mouse KSR1 ODN S392 ±7AA (KSR ODN) were expressed in BL21 (de3) pRIPL. The bacteria containing the respective plasmid were grown in bacterial expression medium at 37°C until the OD_{600} reached 0.6. Then, the bacteria were cooled down to 26°C and expression was induced with 1 mM IPTG. After 4 hours of expression the purification was performed according to NEB's pMAL™ Protein Fusion and purification system protocol. The purification was performed successfully for all MBP-fusion proteins except for KSR ODN. Lysate of the KSR ODN expressing bacteria were centrifuged (10000g for 30 minutes at 4°C) and the supernatant was used for further experiment

2.2.2.3 Two-dimensional phosphopeptide mapping of KLC4

For two-dimensional phosphopeptide mapping wild type KLC4 and 8 single mutants of KLC4 were expressed in 293T cells and affinity tag purified. To ensure that no protein complexes were purified cell lysis and affinity tag purifcation was performed with RIPA lysis buffer. Then, the proteins were [γ-^{32}P]-labelled *via* phosphorylation by MARK3, resolved with SDS-PAGE and visualised by autoradiography. Protein bands of interest were excised and the gel fragments were dried using a vacuum centrifuge. The proteins were in-gel digested – 100 µl trypsin reaction buffer containing 1 µg of trypsin was added to the dried gel fragment - at 37°C overnight. After incubation the supernatant was removed and lyophilised. Then, the pellet was

resuspended in deionized water and lyophilised at least four times to remove the salt of the trypin reaction buffer. After the fourth lyophilisation step the tryptic digest was resuspended in electrophoresis buffer (pH 1.9) centrifuged at full speed to clear the peptide mix of all particular matter. The supernatant was transferred to a new tube and lyophilised again. The amount of [γ-^{32}P]-radioactivity was determined by Cerenkov-counting and the digest was resuspended in 10µl electrophoresis buffer. The trypsin digest (~2000 c.p.m.) was spotted onto thin layer chromatography plates and the electrophoresis (first dimension electrophoresis buffer pH 1.9) was performed for 30 minutes at 1 kV. After completing the run the plates were dried and the chromatography (second dimension phosphochromatography buffer) was performed. Phosphorylated peptides were visualized by autoradiography. The method was performed as described extensively by Meisenhelder and Hunter et al. (Meisenhelder, Hunter et al.).

Phosphochromatography buffer

750 ml	n-butanol
500 ml	Pyridine
150 ml	Glacial acidic acid
600 ml	ddH_2O

Electrophoresis buffer pH1.9

50 ml	Formic acid (88% w/v)
156 ml	Glacial acidic acid
1794 ml	ddH_2O

Trypsin reaction buffer

40 mM	Ammonium bicarbonate
9%	Acetonitrile

Trypsin solubilisation reagent

1 mM	HCl

2.2.2.4 Immunoprecipitation (IP)

The immunoprecipitation procedure was carried out at 4°C. Solutions that contained protein G sepharose beads were generally pipetted with a cut pipette tip, to avoid shearing of the beads. Cells were lysed in the appropriate buffer - protein complex analysis: NP40 lysis buffer and Triton x-100 lysis buffer // Disruption of protein complexes and nuclear lysis: RIPA lysis buffer - and non-soluble cell components were pelleted by centrifugation (13,000g for 10 minutes at 4°C). The supernatant extract was transferred to a fresh tube and mixed with the antibody and 20 µl protein G sepharose beads, which had been previously equilibrated in the appropriate buffer. After a 3 hour incubation period at 4°C the beads were washed 3 times (1900g for 1 minute at 4°C) with lysis buffer and treated depending on further experiments. For kinase assays, the protein-loaded beads were washed additionally once in kinase buffer before the kinase assay was performed. Samples for Western blots were prepared by heating an aliquot of the immunoprecipitate and the supernatant respectively in an equal volume of 2 x Laemmli buffer at 95°C for 5 minutes and processed for SDS-PAGE electrophoresis.

NP40 Lysis buffer

50 mM	Tris-HCl pH 8.0
150 mM	NaCl
1%	Igepal CA-630

Triton x-100 Lysis buffer

25 mM	Tris-HCl pH 7.4,
150 mM	NaCl
1%	Triton-X-100

RIPA Lysis buffer

50 mM	Tris-HCl pH 8.0
150 mM	NaCl
1%	Igepal CA-630
0.5%	Sodium deoxycholate
0.1%	SDS

2.2.2.5 Sodium dodecyl sulfate-Polyacrylamide gel (SDS-PAGE) electrophoresis

The separation of proteins by their differences in molecular weight was carried out by one dimensional discontinuous SDS-Polyacrylamide gel electrophoresis as described by Laemmli (Laemmli 1970).

2.2.2.6 Staining of proteins with Coomassie blue

Coomassie dyes are a family of dyes commonly used to stain proteins in SDS PAGE. SDS-PAGEs were stained in Coomassie blue staining solution for 10 minutes to 12 hours and destained with Coomassie destain solution. Gels were packed in cellophane paper avoiding air bubbles, wetted with 10% glycerol and dried over night.

Coomassie staining solution

0.25g	Coomassie blue R-250
100ml	Ethanol
25ml	Acetic acid
125ml	ddH_2O

Coomassie destaining solution

100ml	Ethanol
25ml	Acetic acid
125ml	ddH_2O

2.2.2.7 Staining of proteins with silver nitrate

Bio-Rad's original silver stain kit is derived from the method of Merril et al. for staining proteins in SDS-PAGEs (Merril, Dunau et al. 1981). The kit was used according to the manufacturer´s protocol.

2.2.2.8 Determination of protein concentrations

Use of Coomassie G-250 dye in a colorimetric reagent for the detection and quantification of total protein was first described by Dr. Marion Bradford in 1976 (Bradford et al. 1976). Protein concentrations were determined by using the Bradford Protein Assay Kit (Sigma-Aldrich Family, U.S.A) according to the manufacturer's protocol.

2.2.2.9 Western blot

Western blotting is the transfer of separated proteins in a gel to the surface of a thin support membrane matrix where the proteins are bound and immobilized (Gershoni and Palade 1982; Gershoni and Palade 1983). For this purpose the gel is layered next

to a membrane and placed in a voltage gradient perpendicular to the gel to perform the transfer. The negatively charged proteins (SDS) in the gel migrate out of the gel, move towards the anode, and get deposited on the membrane.

Primary antibodies directed towards a protein specific sequence detected the transferred proteins. A secondary antibody labeled with horseradish peroxidas (HRP) and directed towards the primary antibody was utilized to visualize the proteins by chemiluminescence. The Pierce SuperSignal West Pico Chemiluminescent Substrate Kit was used according to the manufacturer´s protocol.

2.2.2.10 Dephosphorylation of proteins

Two large families of enzymes, the protein kinases and the protein phosphatases modulate the level of protein phosphorylation in cells. Lambda phosphatase is a Mn^{2+}-dependent dual specificity phosphatase that is useful for dephosphorylating proteins phosphorylated on serine, threonine or tyrosine residues. The enzyme can be used to determine whether a protein of interest is phosphorylated by plus and minus phosphatase treatment. The enzyme was used according to the manufacturer's protocol.

MARK3 in vitro kinase assay

Kinase assays analyse the ability of kinases to phosphorylate specific substrates. [γ-^{32}P]-ATP serves as a substrate donor, which enables the accumulation of the label in the protein acceptor substrate to be detected by autoradiography. MARK3 and the substrate were incubated in MARK3 kinase buffer for 15-30 minutes at room

temperature. The reaction was stopped by adding 0.2 volumes of 6xSDS-sample buffer and subsequent boiling of the sample for 5 minutes.

MARK3 kinase buffer

variable	Kinase
variable	Substrate
150 mM	NaCl
5 mM	$MnCl_2$
1 mM	DTT
50 mM	Tris-HCl pH 7.4
250 μCi/ml	[γ-^{32}P]-ATP

2.2.2.12 Phosphorimaging

This technique has several advantages over standard autoradiography, but the most important is that it is much more accurate in quantifying the amount The radioactive filter membrane is placed in contact a phosphorimager plate, which absorbs beta-rays. These rays excite molecules on the plate, and these molecules remain in an excited state until the phosphoimager scans the plate with a laser. At that point the trapped energy in the plate is released and monitored by a detector that is connected to a computer. The computer converts the energy to an image.

2.2.3 Bacteria and cell culture techniques

2.2.3.1 Cell Culture Techniques

All cell lines were grown in a humidified 95% air and 5% CO_2 incubator at 37°C. Cells were split every 2–4 days and were counted with a Neubauer chamber before seeding. Adherent cells were split by trypsinization and reseeded. Generally, 10 cm

dishes were used for maintenance culture. HELA, COS-1, HEK293 and HEK293T (constitutively express the SV40 large T-Antigen) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 50 units/ml penicilline and 50 µg/ml streptomycine.

2.2.3.2 Bacterial culture media/plates

Luria Bertani (LB) Media

10 g	Bacto Tryptone
5 g	NaCl
5 g	Yeast extract
1 ml	1M NaOH
per litre	

LB plates

10 g	Tryptone
5 g	NaCl
5 g	Yeast extract
15 g	Agar
1 ml	1M NaOH
per litre	

Media & plates contained the following antibiotics:

Ampicillin	final concentration	100 µg/ml
Kanamycin	final concentration	30 µg/ml
Chloramphenicol	final concentration	34 µg/ml

2.3 Project specific methodology

2.3.1 Expression screening with solid phase phosphorylation, MARK3

In 1997 screening with solid phase phosphorylation was first established by Fukunaga et al. to identify substrates for the MAPK ERK1 (Fukunaga and Hunter 1997; Fukunaga and Hunter 2004). The method uses a protein-expressing phage cDNA library and is based on the fact that cellular proteins immobilized on a nitrocellulose filter can be phosphorylated by a soluble protein kinase with specificity similar to that obtained in conventional liquid-phase phosphorylation (Young and Davis 1983; Valtorta et *al.*, 1986).

The bacterially expressed kinase domain (AA1-393) of MARK3 (MARK3 KD-MBP) was used to screen $1x10^6$ clones of the T7 phage human brain cDNA expression library (Novagen). The protein fragments expressed by Novagen´s T7 phage human brain cDNA expression library are 100 to 1000 amino acids long and fused to the capsid proteins of the phages.

For the primary screening of the library $2x10^4$ plaque forming units (p.f.u.) per 150 mm plate were screened. The phages were diluted in M9TB media, mixed with 600 µl of BLT5403 (OD 0.6-0.7) and 8ml of top agarose. The mixture was poured on LB plates, which were incubated at 37°C until the plaques reached approximately 0.5 mm diameter. Then, the plates were overlayed with nitrocellulose filters for 1 minute. During the overlay procedure the nitrocellulose filters were pierced asymmetrically

with a needle. These markings helped to align the autoradiogram, nitrocellulose filter and plate to isolate phages of interest after the screening procedure.

The nitrocellulose filters were blocked with blocking solution (5% BSA) for 60 minutes to prevent non-specific signals for instance caused by autophosphorylated MARK3 KD-MBP. After blocking, the nitrocellulose filters were washed three times in Triton Washing Buffer (TWB) and then equilibrated in MARK3 Reaction Buffer (MRB) for 10 minutes. ATP binding proteins and proteins, which could undergo autophosphorylation were blocked by incubation of the nitrocellulose filters in MRB containing 0.1 mM 5'-fluorosulfonylbenzoyladenosine (5'-FSBA), which binds covalently to ATP binding sites (Vereb, Balla et al. 2001). After inhibition of ATP binding proteins the nitrocellulose filters were washed several times thoroughly with MRB containing 5 mM dithiothreitol (DTT) which inactivated surplus FSBA (Menzel, Chari et al. 2007). Then, for the phosphorylation reaction the nitrocellulose filters were incubated for 15 minutes in 6 ml MRB containing MARK3 KD-MBP (2 μg/ml), 1 mM DTT, 50 μM sodium orthovanadate, 5 mM Sodium fluoride (NaF), 5 mM β-glycerophosphate (β-g), 0.1% Triton x-100 and 6.5 μCi/ml [γ-^{32}P]-ATP. Finally the nitrocellulose filters were washed six times for at least 5 minutes in MARK3 wash buffer (MWB) and once in MWB without Triton x-100. The nitrocellulose filters were air dried and exposed to x-ray films for 12-72 hours at -80°C. Phages containing cDNA encoding for a potential MARK3 substrate were isolated and taken for a second round of screening to authenticate the phosphorylation. For this purpose the phages were eluted out of agarose pieces over night at 4°C. Then different dilutions of the extracted phages were treated as described

for the primary screening. The different § and their phosphorylation signals can be differentiated.

Media for screening with solid phase phosphorylation:

TB Media

12 g	Bacto tryptone
24 g	Yeast Extract
4 ml	Glycerol
900 ml	ddH_2O

After autoclaving add 100ml sterile K phosphate.

K phosphate

23.1 g	KH_2PO_4
125.4 g	K_2HPO_4

per litre

20x M9 salts

20 g	NH_4Cl
60 g	KH_2PO_4
120 g	$Na_2HPO_4 \cdot 7H_20$

per liter

M9TB Media

5 ml	20x M9 Salts
2 ml	20% Glucose
0.1 ml	1M $MgSO_4$

per 100 ml TB

Plates for screening with solid phase phosphorylation:

LB plates were overlayed with top agarose

Top agarose

per liter:

10 g	Bacto tryptone
5 g	Yeast extract
5 g	NaCl
6 g	Agarose

Buffers for the screening with solid phase phosphorylation:

Triton washing buffer (TWB)

20 mM	Tris-HCl pH 7.5
150 mM	NaCl
10 mM	EDTA
1 mM	EGTA
0.5%	Triton x-100
0.2 mM	PMSF
1 mM	DTT

MARK3 reaction buffer (MRB)

20 mM	HEPES-NaOH pH 7.5
10 mM	Mn_2Cl
50 μM	Na_3VO_4
5 mM	NaF
5 mM	β-Glycerophosphate
2 mM	DTT
0,1%	Triton x-100

MARK3 washing buffer (MWB)

20 mM	Tris-HCl pH 7.5
150 mM	NaCl
10 mM	EDTA
1 mM	EGTA
20 mM	NaF

Blocking solution

20 mM	Tris-HCl pH 8.0
150 mM	NaCl
5%	BSA
1%	Triton x-100

Phage extraction buffer

100 mM	NaCl
20 mM	Tris-HCl, pH 8.0
6 mM	$MgSO_4$

2.3.2 MARK3 complex analysis

Analysis of kinase complexes is a standard method used to identify novel kinase/substrate pairs. For this purpose the bacterially expressed kinase domain of MARK3 (MARK3 KD-MBP) was purified from 25 ml culture but not eluted from the MBP binding resin (100 μl). The MARK3/resin comlex was incubated with HEK293 cell lysates for 3 hours at 4°C to form MARK3/substrate complexes. The resulting protein complexes were washed 10 times with 5 volumes MARK3 kinase buffer for complex purification without [γ-^{32}P]-ATP to reduce the amount of non-specifically bound proteins (1900 g for 2 minutes at 4°C). Subsequently, a [γ-^{32}P]-kinase assay was performed to distinguish between MARK3 substrates in the complex and binding partners of MARK3, which are not MARK3 phosphorylation targets. Boiling for 5 minutes in 0.2 volumes of 6xSDS sample buffer denatured the protein complexes. Subsequently, the proteins were resolved by SDS-PAGE and the gel was silver stained. To visualise MARK3 substrate phosphorylation an autoradiogram was performed. Protein gels of different bisacrylamide concentrations - 7%, and 12% - were used to increase the resolution for proteins of a high or a low molecular weight respectively. The inactive kinase domain of MARK3 (MARK3 KD-MBP IA) and the MBP tag served as controls to confirm that a phosphorylation event was due to the kinase domain of MARK3 and not derived by extrinsic kinases. Additionally, the experiment was performed for all three proteins – MARK KD-MBP, MARK3 KD-MBP IA and MBP – without exposing them to HEK293 lysates to ensure that phosphorylation bands were not derived by degraded autophosphorylated MARK3.

Preparation of HEK293 lysates

Since detergents antagonize complex formation the lysates were prepared by means of a Dounce homogenizer. For this purpose the cells (10 p100 plates grown to confluency) were harvested and washed (150 g for 5 minutes at 4°C) with phosphate buffered saline (PBS) and the packed cell volume was measured after centrifugation. Then the cells were washed with hypotonic lysis buffer and the cell pellet was resuspended in twice the packed cell volume of hypotonic lysis buffer. The cells were allowed to swell for ten minutes on ice and were disrupted in a Kontes all glass Dounce homogenizer by several strokes of the B pestle. The extent of cell lysis is 90-95% as monitored by light microscopy. Nuclear lysis was minimal. The nuclei were removed by centrifugation (500 g for 10 minutes at 4°C). One tenths volume of a 10x MARK3 Kinase buffer solution without [γ-^{32}P]-ATP was added to the supernatant.

Kinase assay for substrate identification

Briefly, all proteins (MARK KD-MBP, MARK3 KD MBP IA and MBP) were expressed as described and after several rounds of washing the bead-coupled proteins were incubated with the cell lysates for three hours to form complexes. Prior to the kinase assay the complexes were washed in kinase buffer without [γ-^{32}P]-ATP to deplete non-specifically bound proteins. For the kinase assays the bead bound protein complexes (30 μl for each reaction) were incubated in 500 μl kinase buffer containing 250 μCi/ml [γ-^{32}P]-ATP and phosphatase inhibitors for 15 minutes at room temperature. After the kinase assay the proteins were denatured and size separated by

SDS-PAGE electrophoresis. The SDS-PAGE was silver stained and an autoradiogram was performed and analysed for substrate phosphorylation.

MARK3 kinase buffer for complex purification

150 Mm	NaCl
5 mM	$MnCl_2$
1 mM	DTT
50 mM	Tris-HCl pH 7.4
250 μCi/ml	[γ-^{32}P]-ATP
50 μM	Na_3VO_4
5 mM	NaF
5 mM	β-Glycerophosphate

Hypotonic lysis buffer

10 mM	Tris-HCl pH 7.9
10 mM	NaCl
1.5 mM	$MnCl_2$
0.5 mM	DTT

2.3.3 ProtoArray® Human Protein Kit

The ProtoArray® Human Protein Kit for Kinase Substrate Identification (KSI) allows the rapid and efficient identification of potential human kinase substrates using a protein kinase of interest. This protein microarray technology is based on the protein microarray technology developed by Zhu et al. to detect molecular interactions with proteins (Zhu, Bilgin et al. 2001).

The assay was performed accordingly to the manufacturer's protocol. Briefly, the ProtoArray® Human Protein Kit consists out of four microarrays two ProtoArray® control Protein Microarrays (further referred to as control microarrays) and two ProtoArray® Human Protein Microarrays for substrate identification (further referred to as human protein microarrays). Firstly, the control protein microarrays were probed with and without a control kinase respectively in the presence of labelled [γ-^{33}P]-ATP to verify the probing and detection protocol. Then, the human protein microarrays were probed with MARK3 (His-tagged full-length MARK3, Upstate, Dundee U.K) and with maternal embryonic leucine zipper kinase (MELK) respectively in the presence of [γ-^{33}P]-ATP. The MARK3 array served to identify novel MARK3 substrates, while the MELK array served as negative control. This approach was chosen out of economic reasons, aware of the fact that the MELK array is not an appropriate negative control for the MARK3 array. Substrates shared by both kinases cannot be detected with this experimental setup. After incubating the microarrays with 150 ng of kinase for 60 minutes at 30°C, these were washed with a denaturing buffer to reduce the amount of non-specifically bound [γ-^{33}P]-ATP, air-dried and an

autoradiogram was performed (Fig.: 10). The obtained data was analysed by the ProtoArray® Prospector software version 4.0. Potential substrates were further investigated.

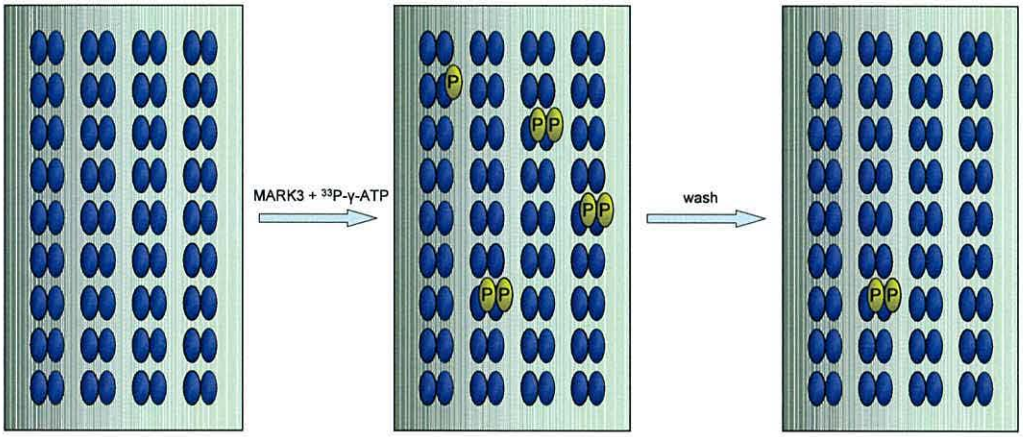


Figure 11: Protein microarray
The ProtoArray® Human Protein Microarray was probed with MARK3 in the presence of γ-^{33}P-ATP. After washing the human protein microarray to reduce the amount of non-specifically bound γ-^{33}P-ATP potential *in vitro* substrates were identified by autoradiography.

2.3.4 In-gel kinase assay

The in-gel kinase assay (IGKA) was applied to identify protein kinases, which phosphorylate the kinase domain of MARK3. For this purpose the mutationally inactivated kinase domain of MARK3 was expressed as a MBP fusion protein in *E. coli*, purified and copolymerized (0.25 mg/ml) within a SDS-PAGE matrix. The copolymerized proteins served as substrates for samples containing putative MARK3 upstream kinases. Freshly prepared HELA and COS-1 cell lysates were loaded on the MARK3-polymerized gel and separated electrophoretically as described for SDS-PAGE. Following the electrophoresis the gel was washed twice in buffer C and subsequently in buffer A for 30 minutes at room temperature to remove SDS from the gel. To denature all proteins, the gel was incubated twice for 30 minutes in 6 M guanidine-HCL. The proteins were renatured slowly overnight at 4°C with several changes of buffer A containing 0.04% Tween. On the next day the gel was washed with buffer B for 30 minutes to remove buffer A. Subsequently the kinase assay was performed for 60 minutes in 25 ml buffer B containing 0.2 μM cold ATP and 50 mCi radioactive [γ-^{32}P]-ATP. Then the gel was washed with buffer D until the radioactivity from around the edges of the gel reached background levels and analysed by autoradiography. A SDS-PAGE in which MBP was copolymerized served as control to distinguish between phosphorylation of the MARK3 kinase domain and the MBP tag. The assay was performed as described in more detail by Wooten et al. (Wooten 2002).

Buffer A

50 mM	Tris-HCl pH 8.0
5 mM	b-mercaptoethanol

Buffer B

40 mM	HEPES pH 7.4
7 mM	$MgCl_2$
0.1 mM	EGTA
2 mM	DTT

Buffer C

20%	Isopropanol
50 mM	Tris-HCl pH 8.0

Buffer D

5%	Trichloroacetic acid
1%	(w/v) sodium pyrophosphate

Chapter III: Identification of novel MARK3 substrates

3.1 Introduction

Post-translational modifications of proteins may be responsible for much of the complexity of higher organisms (Dancey 2003). At present, phosphorylation seems to be one of the most abundant types of post-translational modification. Its regulation affects many cellular process including apoptosis, differentiation, growth, metabolism, transport, motility and learning. The fact that eukaryotes devote almost two percent of all proteins encoded in the genome to kinases and that at least thirty percent of all proteins have been estimated to be phosphorylated highlights the importance of protein phosphorylation in cellular processes (Johnson and Hunter 2005). Their central position in the cellular signalling network explains why aberrant kinase activity could directly be involved in the aetiology of a wide spectrum of diseases and their treatments (Cohen 2002). Several kinase drugs such as Gleevec, Herceptin, and Iressa have been approved to treat diseases. Currently, more than 50 protein kinase drug candidates are currently in clinical trials to treat a wide range of disorders such as cancer, neurodegenerative diseases, chronic inflammation and metabolic disorders (Borsello and Forloni 2007; Budhiraja and Singh 2008; Rokosz, Beasley et al. 2008; Pytel, Sliwinski et al. 2009). Despite their central role in health and disease, the discovery of kinase/substrate pairs remains a significant challenge and has arguably become the limiting step in advancing knowledge on cell signalling. Therefore, unravelling the kinase substrate network is considered as one of the major

tasks of the post genomic area. It has become obvious that a combination of powerful methods is often needed to identify new kinase substrates. Most commonly, lysate proteins phosphorylated by a kinase of interest are isolated from gel, by immunoprecipitation or by affinity chromatography and the identity of the proteins is determined by mass spectrometry. Furthermore, enzyme inhibitors, functional knockouts, *in situ* cDNA library screenings and analogue sensitive kinase alleles combined with mass spectroscopy have been applied to identify kinase substrate interactions (Johnson and Hunter 2005). The most recently developed methods to unravel substrates kinase relationships are peptide and protein microarrays (Schutkowski, Reineke et al. 2005; Meng, Michaud et al. 2008). Each of these methods has limitations such as functional redundancy, poor characterisation of inhibitor specificity, low protein abundance, lack of post-translational modifications of the applied kinase and no *in vivo* relevance of found substrates. A variety of these methods often has to be applied to identify *bona fide* substrates for kinases. So far five substrates have been described for MARK3: protein tyrosine phosphatase H1, cell division cycle 25 homolog c, kinase suppressor of RAS 1, plakophilin 2 and most recently class IIa histone deacetylases (Peng, Graves et al. 1998; Muller, Ory et al. 2001; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006). Since the identification of novel kinase/substrate pairs is a challenging task, three different approaches - screening with solid phase phophorylation, MARK3 complex purification and the ProtoArray® Human Protein Microarray Kit - were applied to accomplish this task.

3.2 Expression screening with solid phase phosphorylation

Expression screening with solid phase phosphorylation was established to identify new substrates for the MAPK, ERK1(Fukunaga and Hunter 1997). However, the method could be successfully adapted for other kinases such as $Ca2^{+}$/calmodulin-dependent protein kinase I and protein kinase B the method could be successfully adapted (Obata, Yaffe et al. 2000; Qin et al. 2003). To identify novel MARK3 substrates a lambda phage human brain cDNA expression library was screened with the bacterially expressed kinase domain of MARK3 in the presence of [γ-^{32}P]-ATP. A human brain tissue-specific library was chosen based on EST analysis which indicates an elevated expression of MARK3 in the brain (Web reference 1).

The advantage of screening with solid phase phosphorylation compared to methods such as kinase complex analysis is that, besides stable kinase substrate interactions, transient interactions can be detected as well. Furthermore, large numbers of potential target proteins can be screened with moderate effort. Providing sufficient quantities of highly active and specific kinase is considered as the main difficulty of this method.

3.2.1 Purification of MBP fusion proteins

Kinase domain of MARK3 (MARK3 KD-MBP)

Large amounts of active kinase are required for screening with solid phase phosphorylation. Therefore, the kinase domain and the UBA domain of MARK3 (AA1-393) was expressed as a MBP fusion protein (~87 kDa) in *E. coli* BL21 (de3)

pRIPL cells and purified *via* MBP column chromatography as described in the Methodology chapter (Fig.: 12, lanes 1-3) (Murphy, Korzhnev et al. 2007). MBP functions as a chaperone and thus positively influenced the folding process of MARK3. Since MBP is extremely stable, NH_2-terminal degradation was also minimized (Bach et al. 2001; Lichty, Malecki et al. 2005). In Figure 12 the lanes 1-3 show three different concentrations - 5 μg, 3 μg and 1 μg - of MARK3 KD-MBP that were loaded and resolved by SDS-PAGE to analyse the purity and degradation of purified MARK3 KD-MBP. The strongest protein band at approximately 87 kDa (80-90% of the total protein in each lane) shows the undegraded MARK3 KD-MBP fusion protein. The amount of degradation and purity of the MARK3 KD-MBP preparation seemed to be acceptable for the screening with solid phase phosphorylation.

Kinase supressor of RAS 1 fragments (KSR WT ~55 kDa *and KSR MT* ~55 kDa*)*

A fragment (AA320-424) of the known MARK3 substrate KSR-1 (KSR WT) containing the MARK3 phosphorylation site (S392) as well as the same fragment (KSR MT) in which the phosphorylation site was destroyed by mutagenesis (S392A), were expressed in *E. coli* as MBP fusion proteins and purified *via* column chromatography (Muller, Ory et al. 2001). Figure 12 shows different concentrations - 5 μg, 3 μg and 1 μg - of the purified fusion proteins KSR WT (Fig.: 12, lane 4-6) and KSR MT (Fig.: 12, lane 7-9) respectively. The undegraded proteins at approximately 55 kDa account for 80-90% of the total protein amounts in each lane. Degradation and purity of the fusion proteins was considered acceptable for further downstream applications.

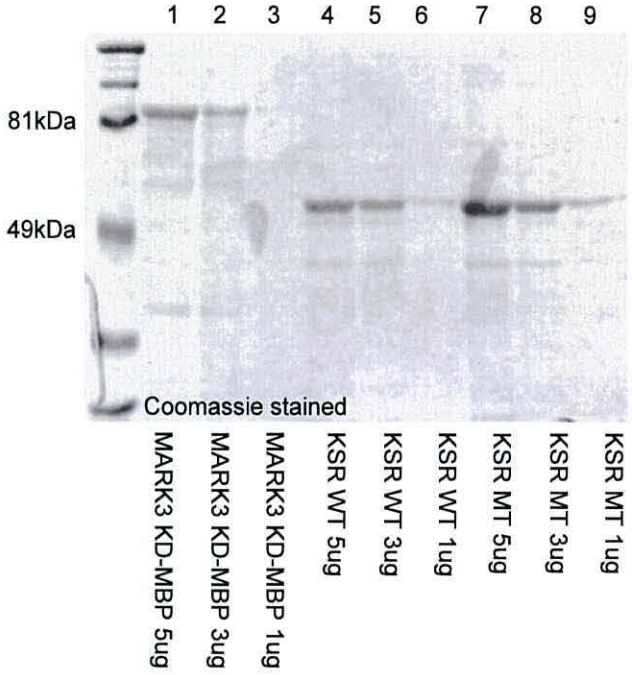


Figure 12: Purification of MARK3 KD-MBP, KSR WT and KSR MT
MARK3 KD-MBP, KSR WT and KSR MT were expressed as MBP tagged fusion proteins in *E. coli* and purified by means of MBP column chromatography. The proteins were resolved by SDS-PAGE. The gel was stained using Coomassie blue and subsequently dried. Identities of the samples are shown in the figure.

3.2.2 Optimization of phosphorylation conditions using liquid phase *in vitro* kinase assays

Activity and specificity of the bacterially expressed kinase domain (MARK3 KD-MBP) was determined by *in vitro* kinase assays. For this purpose, the ability of MARK3 KD-MBP to phosphorylate KSR WT (KSR-1 AA320-424) and KSR MT

(KSR-1 AA320-424 S392A) was analysed. KSR WT was phosphorylated strongly, whereas KSR MT, in which the MARK3 phosphorylation site was disrupted, remained unphosphorylated (compare KSR WT Fig.: 13, lane 3A/B to KSR MT Fig.: 13, lane 2A/B) In addition, it was shown that the bacterially expressed kinase domain underwent autophosphorylation (Fig.: 13 lane 1A/B weak band indicated by MARK3 KB-MBP compare as well with Fig.: 21, lane 4A/B). The autophosphorylation was more prominent when the kinase domain was incubated with a substrat. Therefore, substrate binding might lead to an increase of catalytic activity of the kinase domain (Fig.: 13, lane 3 and 5). Further *in vitro* kinase assays were performed to characterize activity of MARK3 under conditions used for the solid phase phosphorylation protocol.

Kinases depend on divalent cations – magnesium and/or manganese - to orientate the ATP (Taylor, Radzio-Andzelm et al. 1993). Some kinases show a preference for one of these cations. Therefore, the capacity of MARK3 to phosphorylate KSR WT was evaluated using kinase buffers containing either one of the divalent cations or both. Comparison of levels of KSR WT phosphorylation under manganese and magnesium-only conditions led to the conclusion that MARK3 depends rather on manganese than on magnesium to phosphorylate its substrates (compare manganese: Fig.: 13, lane 3A/B to magnesium: Fig.: 13, lane 4A/B). Furthermore, use of both cations together did not result in enhanced substrate phosphorylation, but restored the reduced substrate phosphorylation levels previously shown for the kinase buffer containing only magnesium. (compare magnesium: Fig.: 13, lane 3A/B to magnesium + manganese: Fig.: 13, lane 5A/B). Since MARK3 showed a preference for manganese

in the phosphorylation reaction, magnesium was excluded from the kinase buffer in the screening procedure, which had the advantage that any kinase, either of bacterial or cDNA library origin, dependet on magnesium, was inhibited and could thus not contribute to background phosphorylation signals.

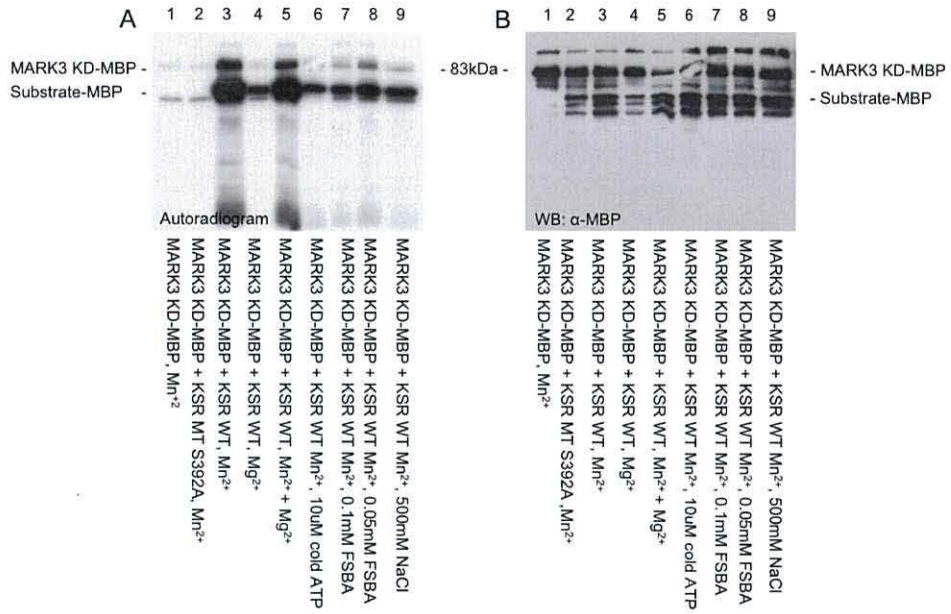


Figure 13: Optimization of phosphorylation conditons using *in vitro* kinase assays
MARK3 KD-MBP´s activity and specificity was analysed by in-vitro kinase assays. Fragments of KSR-1 – KSR WT and KSR MT - served as substrates for MARK3 KD-MBP. KSR MT comprised the same amino acids as KSR WT, but the MARK3 phosphorylation site (S392) was destroyed by mutagenesis (S392A). Phosphorylation of the substrates was analysed by autoradiography (Fig.: 13A). The presence of the relevant proteins was authenticated by Western blot (WB) with antibodies directed against the MBP tag of the proteins (Fig.: 13B). Identities of the samples are indicated in the figure.

In an enzymatic reaction, raising the concentration of substrate can improve the kinetics of the reaction resulting in a higher yield of the product. Therefore, adding 10

μM non-radioactive ATP to the kinase buffer – containing 250μCi/ml radioactive [γ-^{32}P]-ATP – can lead to enhanced incorporation of [γ-^{32}P] into the substrate (Fukunaga and Hunter 1997, Stryker et al 2008).

For MARK3, this observation could not be confirmed; 250μCi/ml of radioactive [γ-^{32}P]-ATP seemed to be sufficient for a high kinase activity, which indicates a low dissociation constant for the MARK3/ATP complex. In addition, exposing the kinase to 10 μM non-radioactive ATP led to a significant reduction of the substrate's phosphorylation signal. A decrease of the phosphorylation signal was probably due to a competitive effect between radioactive and non-radioactive ATP (compare 10 μM cold ATP: Fig.: 13, lane 6A/B to 0 μM cold ATP: Fig.: 13, lane 3A/B). Therefore, only radioactive ATP was added to the kinase buffer during the screening with solid phase phosphorylation procedure

Prior to the phosphorylation reaction the filters were exposed to FSBA, which covalently binds to ATP binding sites on target proteins to reduce false positive phosphorylation signals derived by filter bound non-substrates. (Vereb, Balla et al. 2001). The catalytic function of MARK3 depends on ATP binding. Therefore, the activity of MARK3 served as an indicator of FSBA binding efficiency. In *in vitro* kinase assays different concentrations of FSBA (0.05 mM and 0.1 mM) were tested, considering their ability to inhibit MARK3 autophosphorylation. In these experiments 0.1 mM FSBA inhibited ~95% and 0.05 mM FSBA inhibited ~80% of the kinase activity of MARK3 compared to control experiments without FSBA (compare 0.1 mM FSBA Fig.: 13, lane 7A/B and 0.05 mM FSBA Fig.: 13, lane 8A/B to 0 mM

FSBA Fig.: 13, lane 2 A/B). The strength of the phosphorylation signals were analysed visually. Since the experiment does not reveal if the concentration of 0.1 mM FSBA is high enough to block ATP binding sites in the screening procedure different experiments should have been performed to define an appropriate FSBA concentration for the screening procedure. However, at the time being 0.1 mM FSBA seemd to be sufficient since the concentration is 4 times higher than the cold ATP concentration recommended by Fukunaga to block ATP binding sites of membrane bound proteins. FSBA interacts irreversible with ATP binding sites (Fukunaga et al. 2004).

Since the MARK3/KSR1 complex seems stable in 1 M salt conditions, the capacity of MARK3 KD-MBP to phosphorylate substrates was investigated under high salt conditions (Muller, Ory et al. 2001). Performing the screening using high salt condition might reduce the amount of non-specific MARK3 KD-MBP protein complexes during screening with solid phase phosphorylation. However, during the *in vitro* kinase assays, high salt reaction conditions (500 mM NaCl) led to decreased substrate phosphorylation compared to physiological salt conditions (Fig.: 13, lane 9A/B 500 mM NaCl compared to Fig.: 13, lane 3A/B 150 mM NaCl). The decrease in substrate phosphorylation might be due to the effect that if the salt concentration of a solution is increased, more of the bulk water becomes associated with the ions. Therefore, less water is available to partake in the solvation layer around the protein, which exposes hydrophobic patches on the protein surface and might lead to aggregation of the proteins and precipitation of the protein. Since in the screening

procedure a strong phosphorylation signal was desired to clearly identify substrates physiological salt conditions were applied.

To sum up, MARK3 KD-MBP phosphorylated KSR WT, while the same fragment in which the phosphorylation site was mutated showed no phosphorylation. Furthermore, the activity of MARK depends on manganese not magnesium as divalent cation to orientate the ATP. Addition of non-radioactive ATP to the kinase buffer next to [γ-^{32}P]-ATP (as recommended by Fukunaga et al. 2004) did decreased the overall incorporation of radioactive [γ-^{32}P] into the substrate probably due to a competitive effect between cold and hot ATP. FSBA was used to block ATP binding sites during the screening procedure. Besides, MARK3 could be shown to phosphorylate substrates under high salt conditions. However, the signal strength was so low that physiological conditions were preferred during the screening.

3.2.3 MARK3 KD-MBP is phosphorylated on T211 within the phosphorylation loop

Kinases of the MARK group are activated by phosphorylation of a threonine residue (MARK3 T211) within the T-loop (Timm, Li et al. 2003; Lizcano, Göransson et al. 2004). Since a highly active kinase was desired for the solid phase screening, the capacity of MARK3 KD-MBP to autophosphorylate on T211 was investigated. For this purpose, the bacterially expressed kinase domain of MARK3 was treated with lambda phosphatase (PP), which is known to dephosphorylate serine, threonine and tyrosine residues. Subsequently the phosphorylation state of phosphatase treated versus untreated MARK3 was analysed by Western blotting *via* an antibody directed against the phosphorylated threonine residue 211. The antibody was able to recognize the native kinase domain of MARK3 while the dephosphorylated kinase domain was not recognized (compare Fig.: 14A, lane 2 and 4 to Fig.: 14A lanes 3 and 5). MARK3 degradation products that contain threonine 211 are probably responsible for the bands below the MARK3 band. A second Western blot, with an antibody directed against the MBP tag authenticated the presence of the relevant proteins (Fig.: 14B, lanes 2-5). The MBP protein served as a negative control for the T211 phospho-specific MARK3 antibody and as a positive control for the MBP antibody (Fig.: 14B, lane 1). The experiment was performed as duplicate (Fig. 14: lane 2 + 3, and lane 4+5).

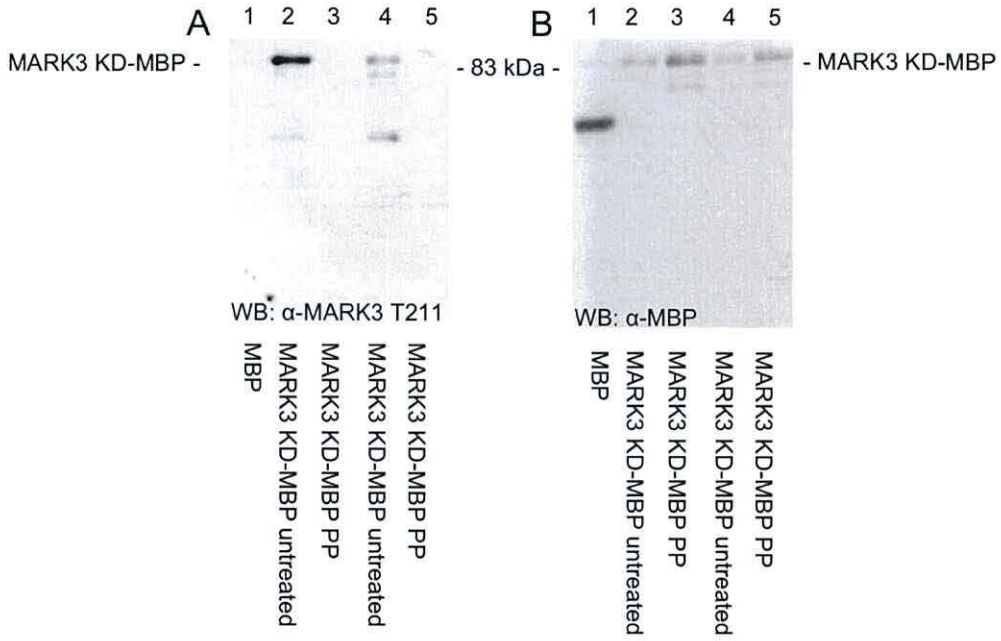


Figure 14: MARK3 KD-MBP is phosphorylated on T211 within the T-loop.
The phosphorylation state of MARK KD-MBP was analysed by Western blotting by means of a phospho-specific antibody directed against threonine 211 within the phosphorylation loop. For this purpose, lambda phosphatase (PP) treated MARK3 KD-MBP was compared to untreated MARK3 considering its threonine 211 phosphorylation (Fig.: 14A). A second Western blot (WB), with an antibody directed against the MBP tag, authenticated the presence of relevant the proteins (Fig.: 14B, lanes 1-5B). Lanes 4 and 5 show duplicates of lane 1 and 2. Identities of the samples are shown in the figure

3.2.4 MARK3 phosphorylates substrates immobilized on nitrocellulose filters

In the screening with solid phase phosphorylation potential substrates are transferred to nitrocellulose membranes. Prior to the screening it was necessary to confirm that the kinase domain of MARK3 (MARK3 KD-MBP) is not only capable of recognizing known substrates in solution but also when the substrates are immobilized on nitrocellulose filters. The fragments of KSR-1, KSR WT and KSR MT served as positive and negative control for MARK3-dependent phosphorylation in these

experiments. Furthermore, since most proteins are partially denatured if transferred onto nitrocellulose membranes, an additional control was applied to ensure that the kinase domain of MARK3 did not rely on secondary structure to recognize substrates. For this reason, a short KSR-1 fragment (AA385-399) comprising the MARK3 phosphorylation motif was expressed as MBP fusion protein (KSRODN ~42 kDa) and analysed considering its capacity to serve as MARK3 substrate. MBP (~50 kDa) served as negative control to verify that phosphorylation events were specific for the KSR-1 fragment of the fusion protein. All proteins were blotted or dotted respectively onto nitrocellulose membranes and underwent the complete screening with solid phase phosphorylation procedure. Subsequently, the resulting phosphorylation was analysed by autoradiography. It could be observed that independently of the protein transfer method – Western blot and dotting - KSR WT and KSR ODN were phosphorylated by MARK3, while KSR MT and MBP remained unphosphorylated (For Western blotting compare KSR WT Fig.: 15A, lane 1 and KSR ODN Fig.: 15A, lane 3 to KSR MT Fig.: 15A, lane 2 and MBP Fig.: 15A, lane 4 // For dotting compare KSR WT Fig.: 15C dot 1 and KSR ODN Fig.: 15C dot 3 to KST MT Fig.: 15C dot 2 and MBP Fig.: 15C dot 4) Performing a Western blot with an antibody directed against the MBP tag of the fusion proteins authenticated their presence (For Western blotting Fig.: 15B, lane 1-4 // For dotting Fig.: 15D, dot 1-4).

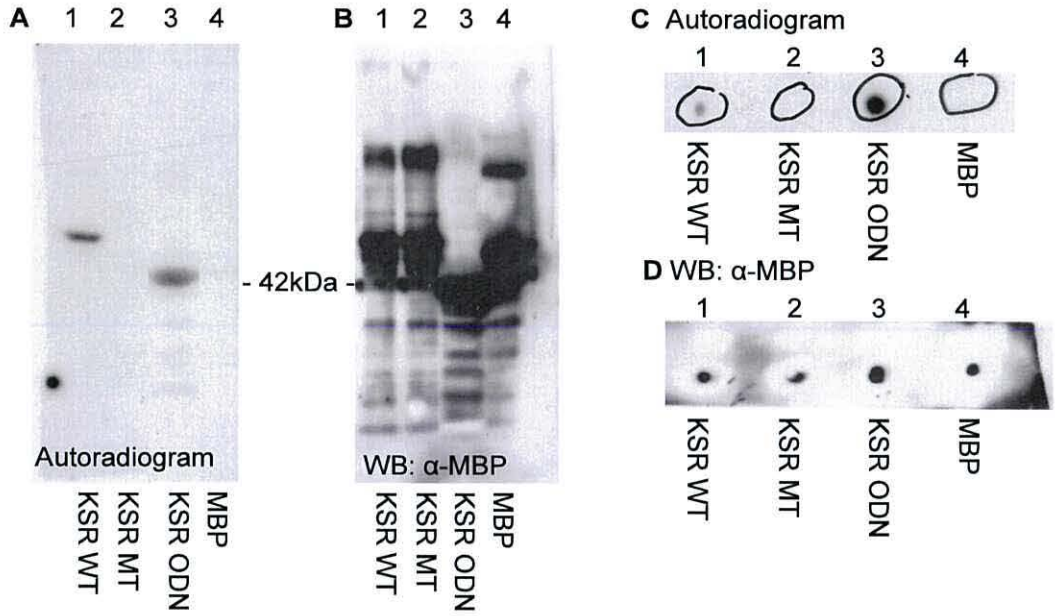


Figure 15: MARK3 phosphorylates substrates immobilized on nitrocellulose filters

MARK3 KD-MBP specifically phosphorylated a known substrate immobilized on a nitrocellulose filter. 100 μg of KSR WT, KSR ODN, KSR MT and MBP were blotted (Fig.: 15A/B) or dotted (Fig.: 15C/D) onto a nitrocellulose filter. Subsequently, the entire screening with solid phase phosphorylation procedure was performed with 2 μg/ml MARK3 KD-MBP and the filters were analysed by autoradiography for MARK3 derived substrate phosphorylation. A Western blot (WB) with an antibody directed against the MBP tag of the proteins confirmed their presence. The size difference of KSRODN and MBP is due to fact that the multiple cloning site of the vector is situated within the open reading frame of the *malE lacZα* fusiongene (~50 kDa) Cloning of a target gene inside the multiple cloning site leads to disruption of the open reading frame, which results in a fusion protein with a ~42 kDa MBP tag. Identities of the samples are shown in the figure.

To sum up, the bacterially expressed kinase domain of MARK3 specifically recognized substrates in solution and also when substrates are transferred onto nitrocellulose membranes. Moreover, KSR ODN phosphorylation by MARK3 indicates that MARK3 does not need any secondary structure to recognize its substrates.

3.2.5 Optimization of solid phase phosphorylation on the membrane

For the screening with solid phase phosphorylation, as for any screening method, a high signal to noise ratio is crucial to guarantee clear identification of *bona fide* phosphorylation signals. The signal to noise ratio can either be increased by enhancing the signal strength or by reducing the background phosphorylation. In this regard, the screening with solid phase phosphorylation protocol was modified and the ratio between KSR WT and KSR MT phosphorylation was used as an indicator for an improved signal to noise ratio. Two different amounts (250 ng, 500 ng) of KSR WT – positive control - and KSR MT – negative control- were dotted onto a nitrocellulose membrane and solid phase phosphorylation was performed. The modifications concerned a) the kinase concentration that was raised from 2 μg/ml to 5 μg/ml MARK3 KD-MBP to increase the signal strength and b) the kinase/substrate association was separated form the phosphorylation reaction by three washes, which should have reduced the amount of unspecically bound kinase and therefore the background signal. However, this modification would select for more stable associations that withstand the washes, which would antagonize the advantage of the method to detect transient kinase/substrate interactions.

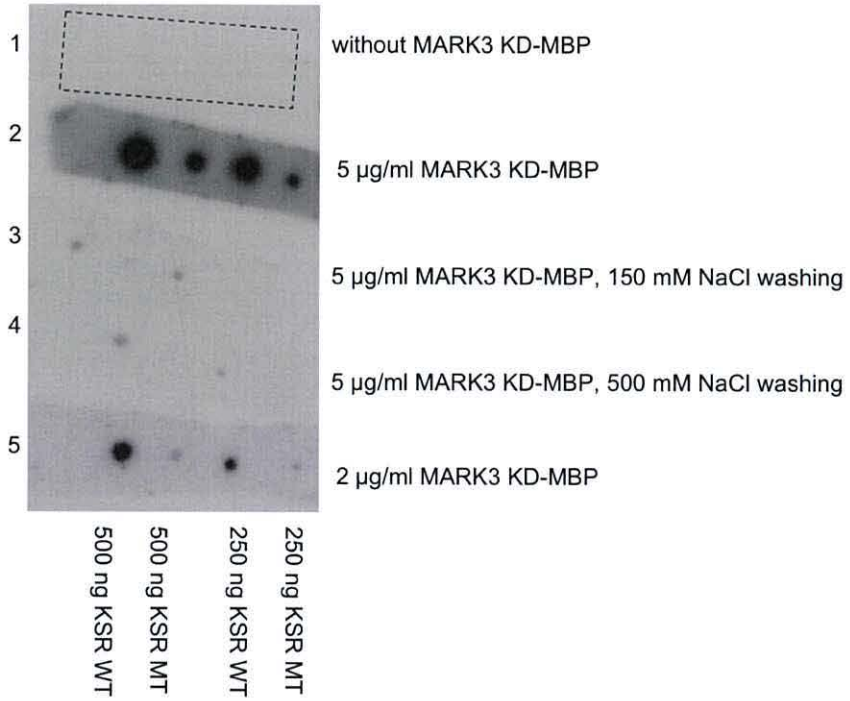


Figure 16: Optimization of solid phase phosphorylation on the membrane
The standard screening with solid phase phosphorylation protocol was modified to increase the signal to noise ratio. For this purpose, either the amount of kinase was raised from 2 µg/ml to 5 µg/ml or the kinase substrate association was separated from the phosphorylation reaction and interposed washes with different salt concentrations (150 mM and 500 mM NaCl) were performed to reduce the amount of non-specifically bound MARK3. Different concentrations - 250 ng and 500 ng - of KSR WT and KSR MT immobilized on filter membranes served as substrates for MARK3 KD-MBP. The signal to noise ratio was analysed by autoradiography. Identities of the samples are indicated in the figure.

Modification 1:

Raising the kinase concentration from 2 µg/ml (Fig.: 16, row 5) to 5 µg/ml (Fig.: 16, row 2) led to increased substrate phosphorylation for KSR WT. However, the observed stronger KSR WT phosphorylation was accompanied by enhanced non-specific background phosphorylation of the negative control KSR MT. Raising the kinase concentration did not seem to lead to an improved signal to noise ratio.

Modification 2:

To separate the kinase substrate association from the phosphorylation reaction, the nitrocellulose membranes were firstly exposed to 5 μg/ml kinase domain of MARK3 for 15 minutes. Then the membranes were then washed three times with MRB buffer containing either 150 mM NaCl (Fig.: 16, row 3) or 500 mM NaCl (Fig.: 16, row 4) to reduce the amount of non-specific bound kinase. Subsequently, the nitrocellulose filters were washed twice in MRB and then incubated in MRB containing 6.5 μCi/ml [γ-^{32}P]-ATP, so that phosphorylation could occur. Both approaches – 150 mM NaCl and 500 mM NaCl - led to a weaker overall phosphorylation signal of KSR WT and KSR MT but not to a significant change in the signal to noise ratio. Besides omitting the kinase did not result in [γ-^{32}P]-derived radioactive background signals (Fig.: 16, row 1)

To summerize, raising the concentration of kinase as well as separating the kinase substrate association from the phosphorylation reaction did not result in a convincing increase in the signal to noise ratio. Therefore, 2 μg/ml (Fig.: 16, row 6) of the kinase domain of MARK3 was set as a standard concentration for the screening protocol since a strong phosphorylation signal could be observed for the positive control KSR WT and a considerably low background phosphorylation for the negative control KSR MT. The higher levels of background phosphorylation for KSR MT in figure 16, row 5 compare to figure 15, lane 2 is due to an increased exposure time and less substrate. For the standard protocol with 2 μg/ml MARK3 KD-MBP, the signal to noise ratio (phosphorylation of KSR WT/KSR MT) was quantified by phosphorimaging.

Different concentrations of KSR WT and KSR MT (5 ng, 50 ng and 500 ng) were dotted onto a nitrocellulose membrane and the standard screening protocol was performed. For the standard protocol with 2 μg/ml MARK3 KD-MBP the phosphorylation of KSR WT was ~6.5 times stronger than the background phosphorylation of KSR MT. Concentrations as low as 5 ng could be detected (compare Fig.: 17 KSR WT 5 ng, 50 ng, 500 ng to KSR MT 5 ng, 50 ng, 500 ng). The higher background phosphorylation of 500 ng KSR MT in figure 17 compared to figure 16 is due to a longer exposure time that was necessary to detect 5 ng of KSR WT.

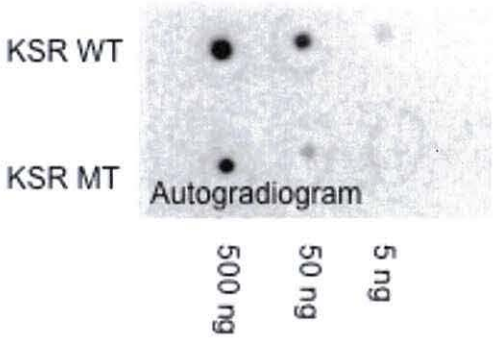


Figure 17: Quantification of the signal to noise ratio for the standard screening protocol
Different concentrations of KSR WT and KSR MT (500 ng, 50 ng, 5 ng) were dotted onto a nitrocellulose membrane and the standard screening with solid phase phosphorylation protocol with 2 μg/ml MARK3 KD-MBP was performed. The amount of radiolabelled phosphate incorporated into each substrate was assessed by phosphorimaging. Identities of the samples are shown in the figure.

3.2.6 Screening a cDNA expression library with solid phase phosphorylation

The screening with solid phase phosphorylation was performed, as described in detail, in the Materials and Methods. Briefly, bacteria were transduced with a phage brain cDNA expression library and grown until the plaques reached 0.5 mm diameter. Subsequently, the expressed proteins were immobilized on nitrocellulose membranes and then blocked with 5% BSA to reduce non-specific protein binding. In addition, the membranes were exposed to 0.1 mM FSBA to mask ATP binding sites (Menzel, Chari et al. 2007). After washing the filters extensively, these were incubated with 2 μg/ml kinase domain of MARK3 and 6.5 μCi/ml [γ-^{32}P]-ATP to perform the solid phase phosphorylation. After extensive washing, two autoradiograms with different exposure times were performed and analysed. Phosphorylation-like signals (false positives), derived by dust particles, - strong pinpoint sized signals (Fukunaga and Hunter 2004) - could be excluded from analysis by comparing the different exposure times of the filters (Fig.: 18C/D white arrow). These signals change their position since a new x-ray film is used for each exposure. True phosphorylation signals were described as fuzzy and dull points showing an increase in signal strength with increased exposure time (Fig.: 18C/D, black arrow Fukunaga and Hunter 2004). For authentication phages responsible for such phosphorylation signals underwent a second round of screening with solid phase phosphorylation. For this purpose, the autoradiogram was aligned with the plates and the phages of interest were plugged out and extracted from the agarose. Since the plaques of the primary screening were plated densely, the phage extract comprised a mixture of approximately 2-10 different phages. Applying the phage mixture to the secondary screening procedure should

have resulted in 10-50% MARK3-derived phosphorylation signals if the mixture had contained a phage encoding for a MARK3 substrate. In the primary as well as in the secondary screening, different concentrations of KSR WT and KSR MT were dotted as controls onto a nitrocellulose filter to confirm that the solid phase phosphorylation had occurred (Fig.: 18A/B).

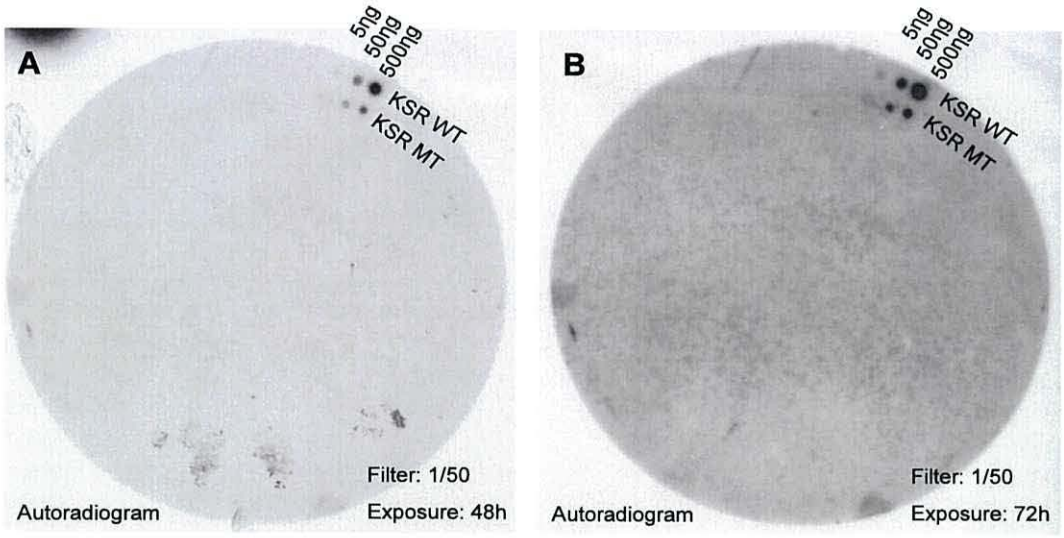


Figure 18: Primary solid phase phosphorylation screening
The expressed proteins of a brain phage library were transferred on nitrocellulose membranes and were exposed to MARK3 and [γ-^{32}P]-ATP. The phosphorylation was visualized by autoradiography. KSR WT and KSR MT were dotted onto the nitrocellulose filters as positive and negative control respectively to confirm that the phosphorylation reaction had occurred. Two exposures - 48h (Fig.: 18A/C) and 72h (Fig.: 18B/D) - were performed to exclude signals from the analysis derived by dust particles (Fig.: 18C/D white arrow). Radioactive signals, which appeared as fuzzy and dull points and showed an increase in signal strength with increased exposure time, underwent a second round of screening for authentication reasons (Fig.: 18C/D black arrow).

The primary screening with solid phase phosphorylation revealed thirty phosphorylation signals fulfilling the criteria mentioned above (Fig.: 18C/D indicated by a black arrow). All thirty respective phages underwent a second round of screening

but none could be confirmed to express a potential MARK3 substrate in the secondary screening. Either no phosphorylation signals were observed (Fig.: 19A) or the phosphorylation signals were of similar strength for all plaques on the plate. Since the phages were plated densely for the primary screening, phages encoding for different proteins were isolated at the same time. Therefore, only a percentage of the plaques in the secondary screening should have shown a positve signal. As examples, the pictures above show the autoradiograms of two filters of the primary screening (Fig.: 18A/B and Fig.: 18C/D) and two autoradiograms of filters of the secondary screening (Fig.: 19A/B, only one exposure time).

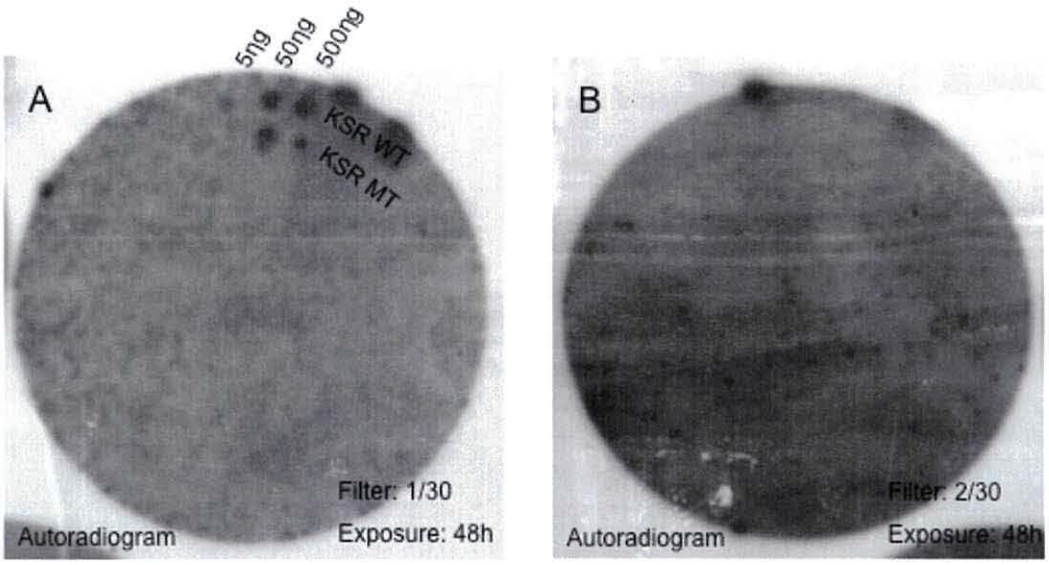


Figure 19: Secondary solid phase phosphorylation screening
Phages encoding for a protein identified as a potential MARK3 substrate in the primary screening, were isolated and rescreened. KSR WT and KSR MT were dotted onto the nitrocellulose filter to authenticate that the *in situ* phosphorylation reaction had occurred (Fig.: 19A).

3.2.7 Conclusions

Screening with solid phase phosphorylation was first established by Fukunage et al. and has been described as a valuable tool to identify new substrates for various kinases (Fukunaga and Hunter 2004). Large amounts of active kinase were required for the screening with solid phase phosphorylation. Therefore, the kinase domain of MARK3 was expressed as a MBP fusion protein in *E. coli* and purified *via* MBP column chromatography. Phosphorylation of a specific threonine residue (MARK3 T211) within the t-loop has been shown to activate kinases of the MARK group (Timm, Li et al. 2003; Lizcano, Göransson et al. 2004). Analysis of the phosphorylation state of the bacterially expressed catalytic domain of MARK3 revealed that the respective threonine residue is phosphorylated. In addition, *in vitro* kinase assays showed that MARK3 KD-MBP phosphorylated a fragment of KSR-1 (KSR WT), which contains the MARK3 phosphorylation site (S392) (Muller, Ory et al. 2001). Since potential substrates were immobilized on nitrocellulose membranes for the screening with solid phase phosphorylation, MARK3 KD-MBP was analysed to assess its capacity to phosphorylate membrane-immobilized proteins. For this purpose, KSR WT and KSR MT were transferred respectively onto nitrocellulose membranes and the entire screening with the solid phase phosphorylation protocol was performed. These experiments verified that MARK3 is capable of phosphorylating substrates on nitrocellulose membranes. However, despite the fact that the most basic parameters for the screening with solid phase phosphorylation were given – kinase amounts, kinase activity, kinase specificity - and an adequate amount of phages was screened, no novel MARK3 substrates could be identified.

The signal to noise ratio is a crucial parameter for substrate identification by screening with solid phase phosphorylation. This was determined for MARK3 KD-MBP as ratio of KSR WT phosphorylation to KSR MT. To improve this ratio, either the signal strength has to be increased or the background phosphorylation reduced. The phosphorylation signal strength depends on the kinase activity, kinase specificity, quantity of the substrate, reaction kinetics and the number of phosphorylation sites per substrate molecule. The amount of a potential substrate as well as the number of the MARK3 phosphorylation sites within the substrate were determined by the respective protein and the phage brain cDNA expression library respectively and can thus be regarded as constant. Furthermore, the kinase domain of MARK3 was confirmed as activated and showed substrate recognition at least for one known MARK3 substrate. Besides omitting non-radioactive ATP from the reaction, which seemed to enhance the signal strength tremendously, none of the experiments to improve the signal strength as well as the kinase specificity resulted in an improved signal to noise ratio. However, the level of substrate phosphorylation was only analysed visually in these experiments. Usage of a phosphor imager to analyse the level of substrate phosphorylation would have made the evaluation more significant.

In solid phase phosphorylation, as described here, background phosphorylation depends mainly on efficient blocking of the nitrocellulose filters, ATP binding sites and non-specific MARK3 phosphorylation. The blocking of membranes was performed adequately in 5% BSA as recommended by Fukunaga and Hunter (1997). The concentration of FSBA (0.1 mM) to block ATP binding sites was considered as sufficient, since a) FSBA binds irreversible to ATP binding sites and b) the

concentration used was 4 times higher than the recommended concentration of 25 μM ATP (Fukunaga and Hunter 1997). However, since the concentration has not been tested properly usage of 0.1 mM FSBA could have led to an indefinete amount of false positves phosphorylation signals that might have complicated the identification of true phosphorylation signals. Furthermore, the signal to noise ratio of ~6.5 might have been too low to discriminate *bona fide* phosphorylation events from that background. Therefore, it seems that the expressed kinase was not specific enough to distinguish under the chosen conditions between substrates and none substrates.

In addition, general aspects need to be considered why no substrates could be identified. Firstly, the method of cDNA library construction should be mentioned in this context since tissue specific cDNA libraries express only a subset of the human transcriptome of which a significant amount is only partially expressed. Therefore, if only a few MARK3 substrates exist, these might a) not be expressed in the specific tissue, b) not be in frame with the expression promoter or c) only partially expressed and lack the MARK3 phosphorylation site. Moreover, a potential substrate, which is toxic for bacteria, would, if at all, only be expressed in marginal amounts and therefore probably not be detectable by this method.

3.3 Analysis of MARK3 protein complexes

Association of kinase and substrate often initiates the phosphotransfer from ATP to the acceptor amino acid residue(s) of the substrate which leads to conformational changes within the three dimensional structure of the substrate. These changes can decrease the affinity of kinase and substrate and finally lead to the dissociation of the complex (Stryker et al. 2008). However, some kinases such as MARK3 have been demonstrated to associate constitutively with their substrates. The substrates of MARK3 - KSR1, Cdc25c, HDAC7 and PKP2 - for which the protein-protein interaction with MARK3 was analysed have been shown to associate constitutively with the kinase and could be co-immunoprecipitated in MARK3 complexes (Zhang, Kobayashi et al. 1997; Peng, Graves et al. 1998; Muller, Ory et al. 2001; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006). Therefore, analysis of MARK3 protein complexes was assumed to be a promising approach to reveal novel MARK3 substrates. The main advantage of this method is that co-factors, which might be inevitable for kinase substrate phosphorylation, are present. Identified substrates show a high likelihood to be *bona fide* interaction partners since phosphorylation and association is authenticated in one step.

3.3.1 Purification of MARK3 MBP fusion proteins and MBP in *E. coli*

The active kinase domain of MARK3 (MARK3 KD-MBP, ~87 kDA) was prepared as previously described. Moreover, the inactivated kinase domain of MARK3 (MARK3

KD-MBP IA, ~87 kDA) and MBP (MBP, ~50 kDa) were expressed in *E. coli* as MBP fusion proteins and purified in the same manner as the active kinase domain of MARK3.

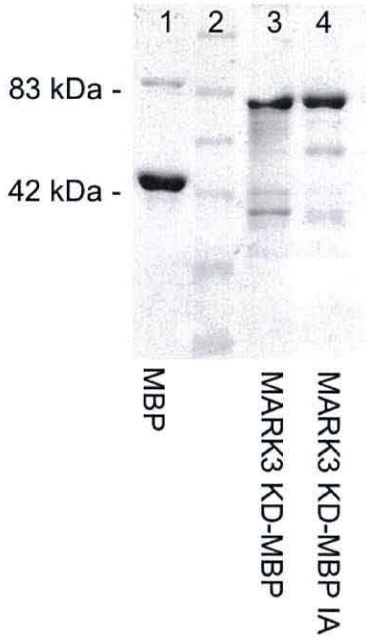


Figure 20: Purification MBP, MARK3 KD-MBP, MARK3 KD-MBP IA
MBP (Fig.: 20, lane 1), Protein Marker (Fig.: 20, lane 2), MARK3 KD-MBP (Fig.: 20 lane 3) and MARK3 KD-MBP IA (Fig.: 20, lane 4) were expressed in *E. coli* BL21 (de3) pRIPL and purified *via* MBP column chromatography. The proteins were resolved by SDS-PAGE. The gel was stained using Coomassie blue and subsequently dried. Identities of the samples are indicated in the figure.

The kinase domain of MARK3 was inactivated by mutagenesis of lysine 85, which coordinates the ATP binding, to alanine (Manning et al 2002). Analysis of the purified proteins by SDS-PAGE and subsequent Coomassie staining showed for MBP (Fig. 20, lane 1), MARK3 KD-MBP (Fig.: 20, lane 3) and MARK3 KD-MBP IA (Fig.: 20, lane 4) protein bands that constitute for ~80-90% of the total protein mass loaded in

each lane. This indicates that the proteins are fairly pure and only minor degradation occurred during the purifcation process.

3.3.2 MARK3 protein complex analysis

For MARK3 protein complex analysis the kinase domain of MARK3 (MARK3 KD-MBP) was expressed in bacteria and purified *via* column chromatography. But instead of eluting the kinase at the end of the purification process, it was kept bound to the beads and incubated with HEK293 cell lysates so that complex formation could occur. After the incubation period the MARK3 protein complexes were washed thoroughly and a radioactive kinase assay was performed to distinguish between MARK3 substrates and non-substrate MARK3 interaction partners. Then, the MARK3 protein complexes were denatured and resolved by SDS-PAGE. The gels contained 7% or 12% bisacrylamide to obtain a higher resolution for different molecular weights. Phosphorylation bands were visualised by autoradiography and determined whether they were potential MARK3 substrates. In addition, the gels were silver stained to confirm that phosphorylation bands align with protein bands. The inactive kinase domain of MARK3 and MBP served as controls to authenticate that a phosphorylation signal was specific for the active kinase domain and not derived by a) co-purified extrinsic kinases of the lysates or b) potential substrates that bind the MBP tag and not the kinase domain. In further control experiments the active kinase domain, the inactive kinase domain and MBP were incubated with kinase buffer instead of

HEK293 cell lysates prior to the kinase assay to detect radioactive protein bands derived from MARK3 autophosphorylation and subsequent protein degradation.

For the identification of MARK3 substrates by analysis of MARK3 protein complexes it was crucial a) to verify that the kinase domain of MARK3 was active and specific for MARK3 substrates b) that the catalytic function of the mutated kinase domain was completely disrupted and c) that MARK3 protein complex formation occurred.

The bacterially expressed kinase domain of MARK3 was authenticated as active and could be demonstrated to recognize KSR-1, a known MARK3 substrate, within the previous section (Fig.: 13, lane 1-3). Furthermore, *in vitro* kinase assays confirmed that the mutated kinase domain of MARK3 was catalytically inactive since no autophosphorylation could be observed compared with the active kinase domain of MARK3 (compare Fig.: 21A lane 4 to Fig.: 21A: lane 5). Moreover, phosphorylation of the inactivated catalytic domain of MARK3 could be observed when incubated with HEK293 lysates. Since the inactive kinase domain is not able to autophosphorylate the phosphorylation must be due to extrinsic kinases, which would authenticate that at least transient complexes were formed during the incubation period (7% SDS-PAGE compare Fig.: 21A lane 2 Fig.: 21A lane 5 // 12% SDS-PAGE compare Fig 22A lane 2 to Fig 22A lane 5).

Despite the fact that the kinase domain was active, specific for at least one known MARK3 substrate - KSR-1 - and protein-protein interaction was authenticated, the analysis of the MARK3 complexes did not reveal any substrates. All phosphorylation bands were either derived from MARK3 autophosphorylation and subsequent protein degradation or by extrinsic kinases since the bands appeared either for the control

reactions without HEK293 or for the control reactions containing the kinase inactive domain of MARK3 respectively (7% SDS-PAGE: compare Fig.: 21A lane 1 to Fig.: 21A lane 4 // 12% SDS-PAGE: compare Fig.: 22A lane 1 to Fig 22A lane 4). The silver stained SDS-PAGEs confirmed that the active kinase domain of MARK3, the inactive kinase domain of MARK3 and MBP were present in equal amounts (7% SDS-PAGE: Fig.: 21B // 12% SDS-PAGE: Fig. 22B)

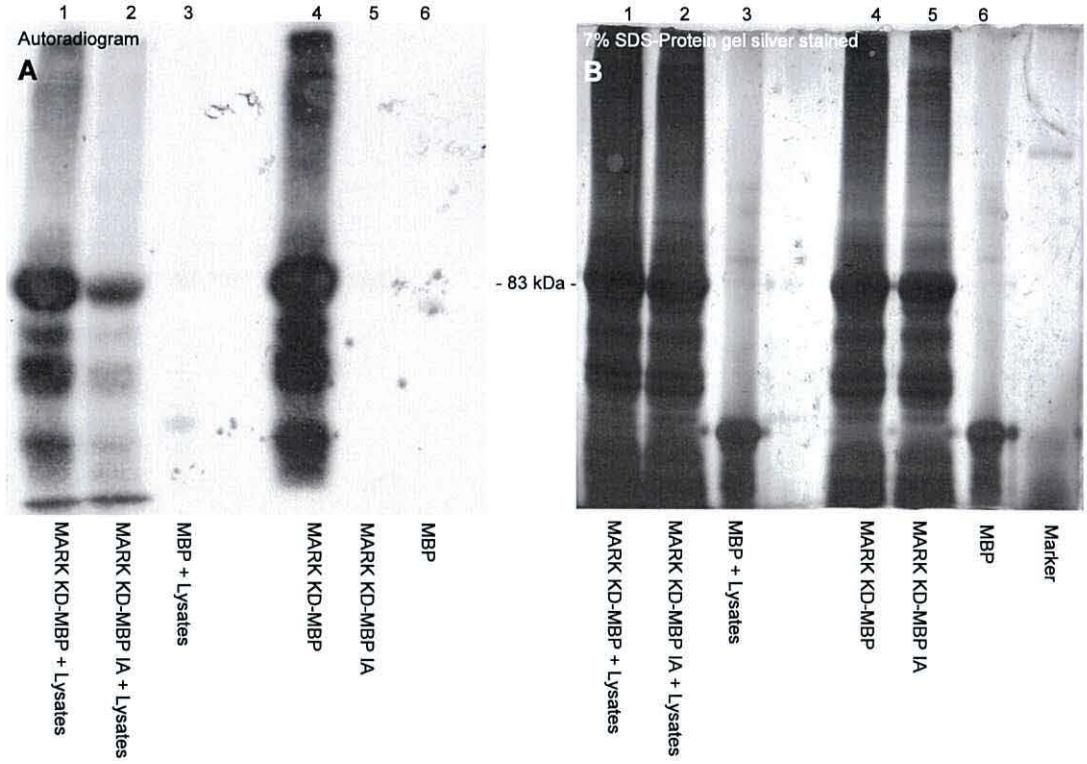


Figure 21: MARK3 KD-MBP protein complex analysis 7% SDS-PAGE
The analysed MARK3 protein complexes did not reveal any MARK3 substrates for the 7% gel. The indicated proteins were incubated with HEK293 cell lysate to form protein complexes. Subsequently a kinase assay was performed to distinguish between substrates and non-substrate interaction partners. The protein complexes were denatured and resolved by SDS-PAGE. Then, the gel was silver stained (Fig.: 21B) and the phosphorylation was visualised by autoradiography (Fig.: 21A). Identities of the samples are shown in the figure.

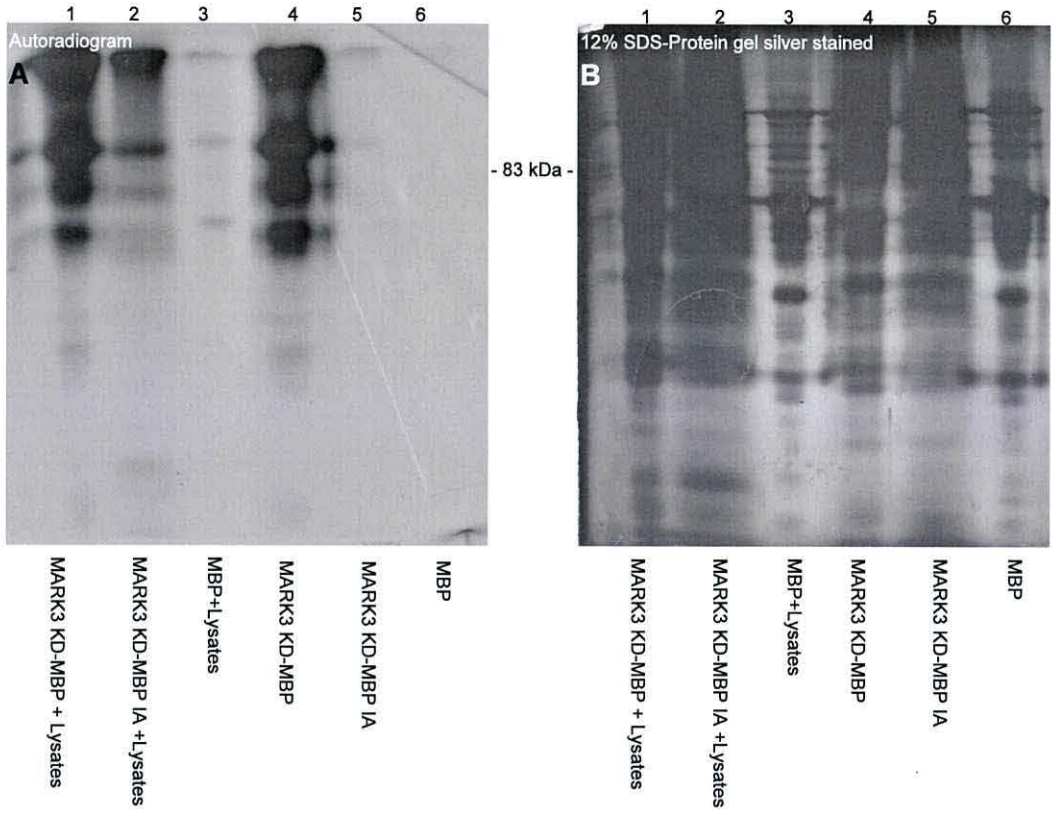


Figure 22: MARK3 KD-MBP complex purification 12% SDS-PAGE
The analysed MARK3 protein complexes did not reveal any MARK3 substrates for the 12% gel. The indicated proteins were incubated with HEK293 cell lysate to form protein complexes. Subsequently a kinase assay was performed to distinguish between substrates and non-substrate interaction partners. The protein complexes were denatured and resolved by SDS-PAGE. Then, the gel was silver stained (Fig.: 22B) and the phosphorylation was visualised by autoradiography (Fig.: 22A). Identities of the samples are shown in the figure.

3.3.3 Conclusions

MARK3 has been shown to associate constitutively with the majority of its known substrates (Peng, Graves et al. 1998; Muller, Ory et al. 2001; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006). Therefore analysis of protein complexes, a standard

method to investigate protein-protein interaction, combined with kinase assays, appeared feasible and promising in identifying new MARK3 substrates (Johnson and Hunter 2005). For this purpose the active, as well as the mutationally inactivated kinase domain of MARK3 were purified from bacteria and incubated with HEK293 cell lysates to promote complex formation. After the incubation period a kinase assay was performed to distinguish between MARK3 substrates and non-MARK3 substrates within the complex. The inactive kinase domain of MARK3 and MBP served as controls to authenticate that a potential substrate phosphorylation was MARK3 kinase domain specific. By incubating the protein with kinase buffer instead of HEK293 cell lysates phosphorylation signals derived by autophosphorylation and degradation of the active kinase domain were revealed and excluded from further analysis. Despite the observation that the kinase domain of MARK3 was active, specific towards a known MARK3 substrate and protein-protein interaction occurred during the incubation period no MARK3 substrates could be identified.

It is possible that the majority of unknown substrates associate only transiently and not stably with MARK3 as assumed by the present data. Thus, phosphorylation of these would have led to kinase substrate dissociation. Only marginal undetectable amounts would have been co-immunoprecipitated with the kinase domain. The same applies for the detection of substrates which a) show a low abundance in the cell lysates since the transcription/translation levels are low or the proteins are instable, b) show a higher affinity to proteins that block mark3 binding and c) were localized mainly in the nucleus bound to DNA. Furthermore, recently post-translational

modifications of MARK3 have been demonstrated crucial for MARK3 14-3-3 association (Göransson, Deak et al. 2006). The "bait" proteins applied in this assay were bacterially expressed, thus lack most post-transcriptional modifications. Therefore, they might have been restricted in their capability to form functional protein complexes, which would have reduced the number of potential interaction partners in an indefinable way. Moreover, since expression of bacterial full length MBP tagged MARK3 failed, only the NH_2-terminal part (AA1-393) of MARK3 was used to precipitate potential MARK3 targets. Substrates, which additionally rely on COOH-terminal MARK3 sequence to form a functional kinase substrate complex would have not been detected by this approach. However, the question has to be addressed why none of the known MARK3 substrates was co-immunoprecipitated with MARK3?

KSR-1 (18%) and HDAC7 (3%) show a low expression profile in HEK293 cells compared to other cell lines. In addition, all class IIa HDACs as well as PKP2 are subject to nuclear shuttling. The protein amounts within the cytosol of these known MARK3 substrates might have been below the detection limit of the assay (Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006; Web reference 2). Furthermore, autophosphorylation and subsequent degradation of the MARK3 kinase domain led to four distinct phosphorylation signals on the autoradiogram between ~60 and ~85 kDa. Despite using gels containing different bisacrylamide concentrations to obtain a higher resolution for specific molecular weights, potential substrates of a molecular weight within the mentioned range might have been superimposed by the autophosphorylation signals. The superimposition effect could account for the

observation that PTPH1 ~58kDa, Cdc25c ~60 kDa and PKP2 ~82 kDa were not detected in this assay. Furthermore, the time course and kinetics of phosphorylation in the system used might be different from the systems previously used in literature to describe the substrates. In addition, the bacterially expressed kinase domain was only demonstrated to be specifc for KSR-1. Therefore, other MARK3 substrates may not be recognized.

However, certain changes considering the experimental setup could have increased the likelihood to identify novel MARK3 substrates by analysis of MARK3 complexes. Instead of using the bacterially expressed MARK3 fusion protein to precipitate MARK3 interaction partners, full length MARK3 could have been overexpressed in mammalian cells and then precipitated *via* affinity tags. For this purpose MARK3 and kinase inactive MARK3 would have been expressed as tandem affinity tagged fusion proteins. Using tandem affinity purification ensures that the amount of unspecifically bound proteins is minimal. After the purifcation of the complexes, similar to the experiments described in this thesis, a radioactive kinase assay would have been performed. Then, complexes would have been analysed *via* SDS-PAGE and autoradiography for MARK3 substrates. However, the described changes were never realized, since the microarray approach to identify novel MARK3 substrates produced promising results

3.4 Identification of MARK3 substrates by microarray technology

The ProtoArray® Human Protein Microarray Kit for kinase substrate identification allows the rapid and efficient identification of potential human kinase substrates using a protein kinase of interest. Approximately eight thousand human proteins are printed in duplicates on the ProtoArray® Human Protein Microarray (referred to as human protein microarray). The ProtoArray® technology is based on the protein microarray technology developed by Zhu et al. to detect molecular interactions with proteins (Zhu, Bilgin et al. 2001). The new generation of functional protein microarrays has overcome some of the limitations of the previously used peptide microarrays to identify kinase substrates. Peptide microarrays are spotted with short amino acid sequences that code for potential phosphorylation sites. Probing the array with a kinase of interest leads to the identification of potential phosphorylation motifs of the applied kinase. These motifs are then used to screen protein databases for potential substrates (Schutkowski, Reineke et al. 2005). However, it is difficult to predict which cellular proteins are *bona fide* kinase substrates solely based on short primary sequence information, since it has been demonstrated that the ability of a kinase to modify a protein at a specific site is influenced by its structural context such as post-translational modifications, inhibitory interaction partners, secondary structure and solvent accessibility (Kreegipuu, Blom et al. 1998; Graves, Bartleson et al. 1999; Harwood 2002; Gual, Le Marchand-Brustel et al. 2005; Park, Ryu et al. 2005; Sato, Takahashi et al. 2005). The use of microarrays containing native or functional proteins for the identification of kinase substrate relationships overcome these

limitations and are considered as one of the finest methods to determine the substrate spectrum of a kinase. Firstly, microarrays are a "high throughput" technology so that thousand of proteins can rapidly be screened. Following the assay, the identity of the substrates is immediately known. Secondly, the probability that a phosphorylated protein revealed in a functional protein microarray experiment is indeed a cellular substrate should be much higher than candidates identified from experiments based on peptide arrays (Meng, Michaud et al. 2008). Recent publications demonstrated the successful application of this new generation of functional protein microarrays to identify various kinase substrates (MacBeath and Schreiber 2000; Ptacek, Devgan et al. 2005; Boyle, Michaud et al. 2007)

3.4.1 Identification of new MARK3 substrates by the ProtoArray® Human Protein Microarray kit

The ProtoArray® Human Protein Microarray kit consists of four microarrays. Two control protein microarrays that serve to verify the probing and detection protocol and two human protein microarrays for substrate identification.

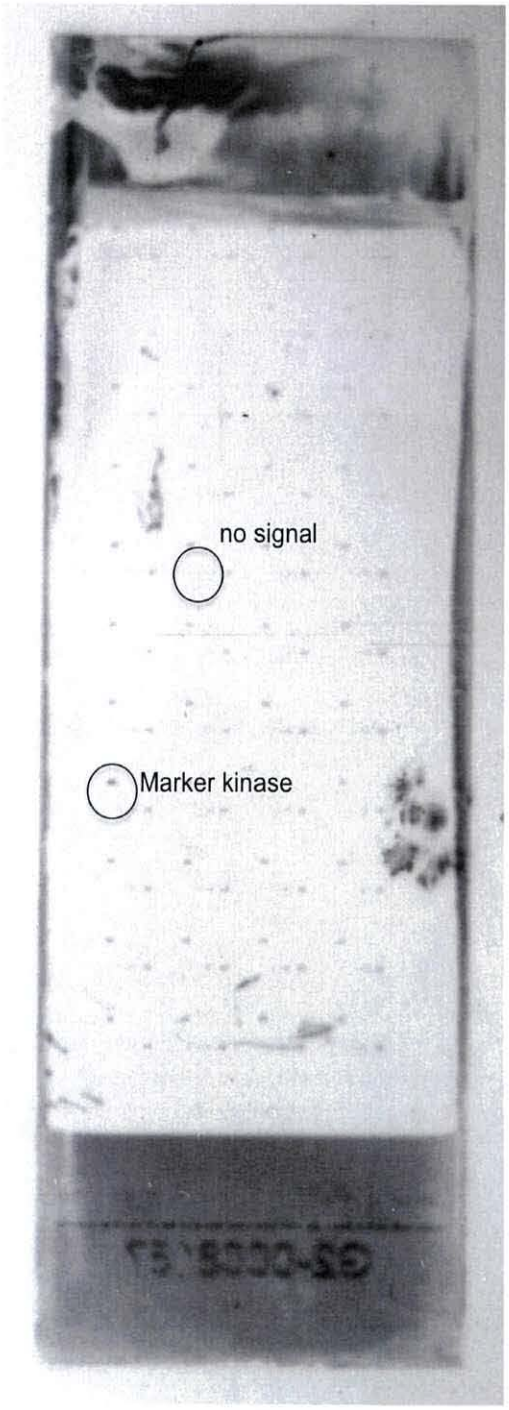
To verify the probing and detection protocol one of the control protein microarrays (Fig.: 23B) is probed with an unknown kinase (provided by Invitrogen) in the presence of [γ-^{33}P]-ATP, while the other control microarray (Fig.: 23A) is probed with [γ-^{33}P]-ATP. Exposing the microarray to the test kinase (Fig.: 23B) demonstrated phosphorylation of test kinase substrates and autophosphorylation of marker kinases. Incubating the other control protein microarray with [γ-^{33}P]-ATP served as negative

control (Fig.: 23A) and also confirmed autophosphorylation of marker kinases. One example for specific substrates phosphorylation and one example for a marker kinase detected on the microarrays are highlited within figure 23A/B (Compare the circled areas of the microarray incubated with the test kinase Fig.: 23B to the circled areas of the microarray incubated without test kinase Fig.: 23A). During the data evaluation the marker kinases are used to allocate potential substrate phosphorylation signals to proteins on the microarray.

One of the two human protein microarrays for substrate identification was probed with MARK3 in the presence of [γ-^{33}P]-ATP. The other array was probed with maternal embryonic leucine zipper kinase (MELK) in the presence of [γ-^{33}P]-ATP instead of [γ-^{33}P]-ATP only as recommened in the manufacturer's protocol. Probing the array with MELK is not an appropriate negative control, since proteins that serve as substrates for both kinases will not be detected. This modification of the experimental setup was chosen out of economic reasons.

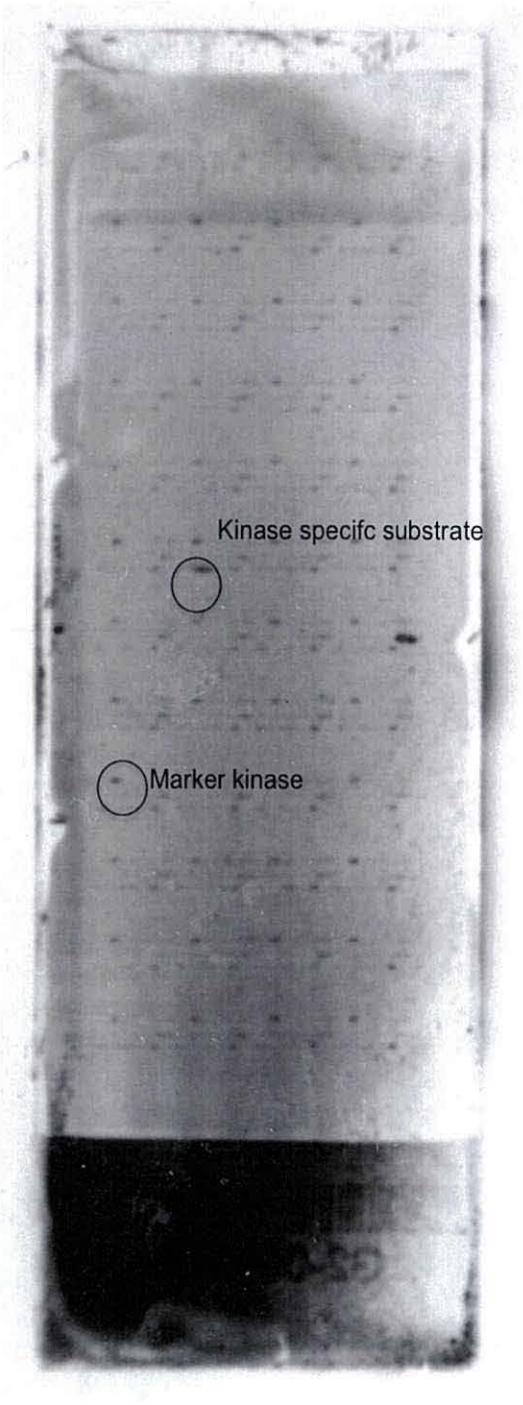
Probing the microarray with 150 ng of MARK3 (Fig.: 23D) for 60 minutes at 30°C in the presence of [γ-^{33}P]-ATP led to phosphorylation of MARK3 substrates, while incubation of the other microarray with MELK in the presence of [γ-^{33}P]-ATP (Fig.: 23C) led to phosphorylation of MELK substrates. Phosphorylation events were visualised by autoradiography. The localisation of 2 of 71 specific MARK3 phosphorylation signals are highlited in Figure 23C/D (Compare the circled areas of the microarray incubated with MARK3 Fig.: 23D to the circled areas of the microarray incubated with MELK Fig.: 23A).

A



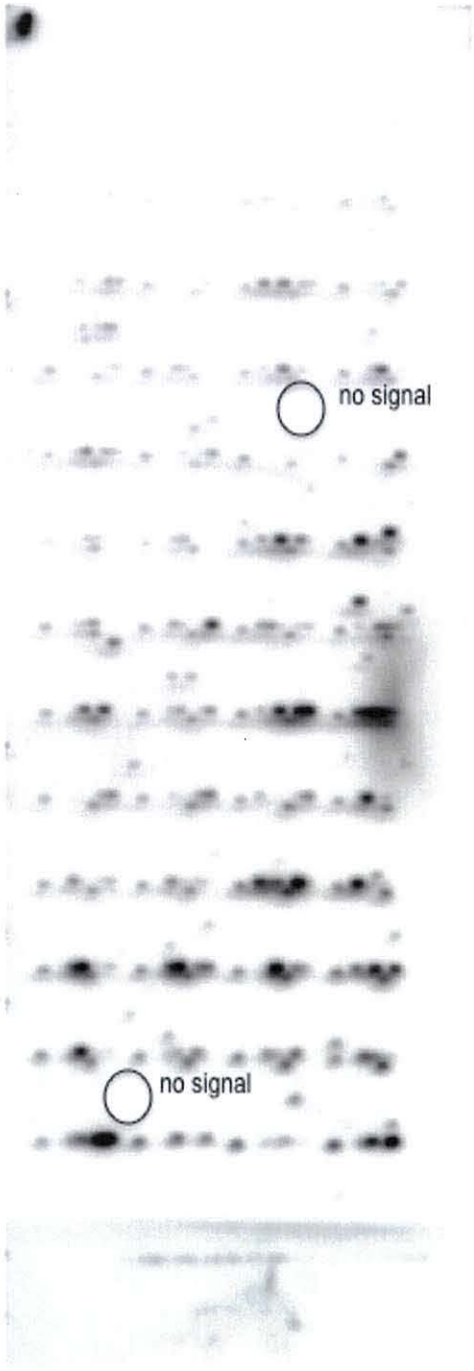
Control microarray probed with [γ-^{33}P]-ATP

B



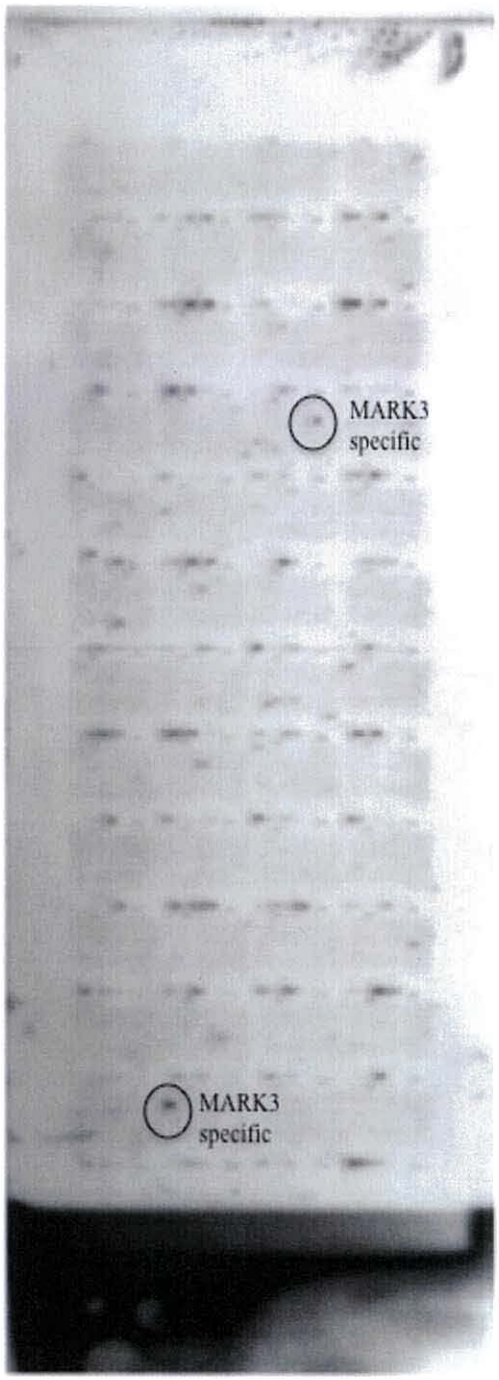
Control microarray probed with [γ-^{33}P]-ATP and control kinase (Invitrogen)

C



Microarray probed with [γ-^{33}P]-ATP and MELK

D



Microarray probed with [γ-^{33}P]-ATP and MARK3

Figure 23: Protein microarrays, MARK3 substrate identifcation
The ProtoArray® Human Protein Microarray kit consists of four microarrays two control protein microarrays (A, B) and two human protein microarrays (C, D). The control protein microarrays were probed with (B) and without a control kinase (A) respectively in the presence of ^{33}P-ATP to verify the probing and detection protocol. After the control protein microarrays were performed successfully the human protein microarrays were probed with 150 ng of MARK3 (D) and 150 ng of MELK (C) for 60 minutes at 30°C respectively in the presence of [γ-^{33}P]-ATP to identify MARK3 substrates. The circled areas in figure 23A/B highlite marker kinases on both arrays (Fig.: 23A/B, lower circles) and a phosphorylation signal specifc for the test kinase (Fig.: 23B, upper circle). The circled areas in figure 23C/D highlite two substrates that have been phosphorylated by MARK3 (Fig.: 23D) but were not recognized by MELK (Fig.: 23C). Identities of the microarrays and samples are shown in the figure.

3.4.2 Evaluation of the protein microarray derived data and substrate verification by *in vitro* kinase assays.

The obtained microarray data was analysed by the ProtoArray® Prospector software version 4.0. Briefly, the MARK3 probed human protein microarray was analysed considering MARK3-specific phosphorylation signals. With the help of the known location of autophosphorylated marker kinases, phosphorylation signals of potential substrates were allocated to proteins on the microarray. Depending on the signal to noise ratio the Prospector software calculated the likelihood of a phosphorylation signal to be derived by MARK3. This likelihood is then transformed into a Z-Score, a lower Z-Score than 3 indicates that the signal is unspecific. Comparison of the MARK3 and the MELK probed microarray led to identification of 71 MARK3 specifc phosphorylation events with a higher Z-Score than 3. None of these has been described in the literature before as a MARK3 substrate. 55 of these proteins belong to the kinase superfamily, 13 are non-kinases and 3 are hypothetical proteins that have only been predicted yet. The following table shows the 25 MARK3 phosphorylation

targets with the highest Z-Score and their localisation on the array (Array ID → B=Block, R=Row and C= Column).

Table 12: Microarray identified MARK3 substrates

	Potential MARK3 substrate	Array ID	Z-Score
1	Casein kinase 1, gamma 1	B05R19C17	12.46835
2	Kinase insert domain receptor	B11R19C17	11.49376
3	Protein kinase C, iota	B18R19C13	11.22657
4	Casein Kinase 1 delta/CK1 delta	B05R19C19	11.17707
5	CUE domain containing 1	B47R05C19	10.21439
6	Protein Kinase C, Delta	B05R19C11	9.45136
7	Protein kinase C, gamma	B01R19C11	9.18258
8	IKB Kinase Beta	B37R19C11	7.1006
9	Mitogen-activated protein kinase kinase kinase 3	B35R19C11	6.90144
10	Protein domain Z binding kinase	B27R19C15	6.80198
11	Epidermal growth factor receptor L858R	B13R19C17	6.63594
12	Proviral integration site 1	B01R19C19	6.51309
13	Protein kinase C, alpha	B01R19C17	6.39543
14	Abelson murine leukemia viral oncogene 1 Y253F	B28R19C15	5.99602
15	Protein kinase C, beta	B03R19C09	5.71502
16	Ancient conserved domain-containing protein 1	B05R15C05	5.71417
17	RUN domain containing 3A	B26R12C17	5.53654
18	**Kinesin family member C3**	**B31R06C11**	**5.31876**
19	Macrophage stimulating 1 receptor	B10R19C13	5.31043
20	**Brain-enriched guanylate kinase-associated homolog**	**B37R08C05**	**5.29369**
21	Transglutaminase	B37R19C09	5.18676
22	Chromosome 15 open reading frame 63	B47R06C19	5.14802
23	Serine/threonine protein kinase MST4	B07R19C11	4.83693
24	hypothetical protein LOC158376	B48R13C19	4.67111
25	**Kinesin light chain 4**	**B01R05C03**	**4.57689**

Proteins considered for further analysis had to fulfill three main criteria:

- High Z-Score that means a high signal to noise ratio.

- Kinases were excluded from the analysis since the signal could be due to autophosphorylation.

- The protein should contain at least one high stringency 14-3-3 binding site, since MARK3 has been shown to regulate all known substrates by phosphorylation and subsequent 14-3-3 binding. Scansite a bioinformatics program was used to predict 14-3-3 binding sites within the potential MARK3 substrates (Web reference 3)

Most of the proteins, which showed strong phosphorylation signals were kinases (55). These were excluded since the observed signal could be derived by autophosphorylation of the kinase that has not been detected on the control array because of handling variations during the assay performance. However, these hits could well be *bona fide* phosphorylation targets since kinase cascades have been shown to be key modules of cell signalling pathways (Dhanasekaran 1998; Bluthgen and Legewie 2008). Three of the thirteen non-kinase identified proteins that matched the previously described criteria were chosen for further analysis. To authenticate the microarray data Begain, KLC4 and KIFC3 were overexpressed as MYC-tagged fusion proteins in HEK293 cells and purified *via* their affinity tag. Cell lysis and affinity

purification was performed in RIPA buffer to destroy protein complexes and ensure that no extrinsic kinases were co-purified with the proteins. Then, Begain, KIFC3 and KLC4 served as substrates for MARK3 in *in vitro* kinase assays. This experiments confirmed kinesin family member C3 (KIFC3) and kinesin light chain 4 (KLC4) as *in vitro* MARK3 substrates. However, Begain could not be authenticated as MARK3 target (data not shown). Autophosphorylation and non-MARK3 dependent phosphorylation by extrinsic kinases could be excluded since KLC4 and KIFC3 respectively only showed phosphorylation if incubated with MARK3 (For KIFC3: compare Fig.: 24A/B lane 3 to Fig.: 24A/B, lane 4 // For KLC4: compare Fig.: 24A/B, lane 5. to Fig.: 24A/B, lane 6). In control reactions immunoprecipitations of untransfected cells were incubated with and without MARK3 to demonstrate that substrate phosphorylation was specific and not derived from resin/antibody bound proteins or degradation of autophosphorylated MARK3 (Fig.: 24A/B, lane 1 and Fig.: 24A/B, lane 2). Phosphorylation was analysed by autoradiography. Western blots with an antibody directed against the epitope tag of the kinase and substrate respectively verified that the proteins were present (Fig.: 24B, lane 1-6).

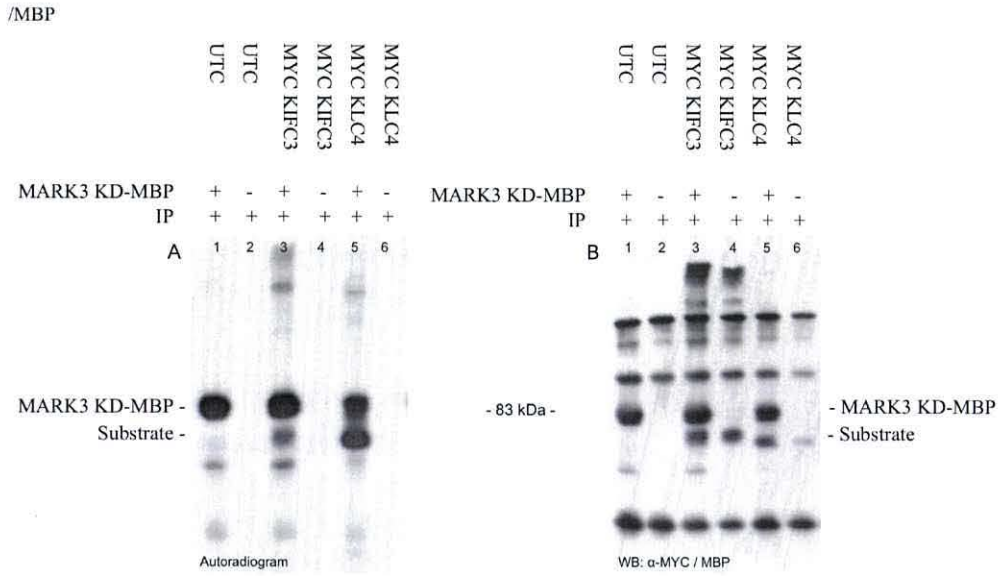


Figure 24: Authentication of microarray identified MARK3 substrates
Authentication of the human protein microarray identified MARK3 substrates by *in vitro* kinase assays. MYC-tagged KIFC3 and KLC4 were overexpressed in HEK293 cells and purified *via* immunoprecipitation (IP). In *in vitro* kinase assays the purified proteins served as substrates for MARK3. The samples were resolved by SDS-PAGE and phosphorylation was analysed by autoradiography. Untransfected (UTC) cells served as control to ensure detected phosphorylation bands/ protein bands are specific for KIFC3 and KLC4 respectively. Western blots (WB) with an antibody directed against the epitope-tags of the relevant proteins (MYC and MBP) confirmed their presence. Identities of the samples are indicated in the figure.

3.4.3 Conclusions

In contrast to screening with solid phase phosphorylation and MARK3 complex analysis, probing a protein microarray with MARK3 led to specific phosphorylation of 71 proteins. None of these 71 proteins has been previously described as MARK3 substrate. Phosphorylated proteins, considered for further analysis, had to fulfill three criteria to increase the likelihood that these are relevant *in vivo*: 1) They had to show a high signal to noise ratio. 2) Only non-kinases were further analysed since

phosphorylation signals derived by kinases could be derived by autophosphorylation. 3) Potential substrates had to contain at least one high stringency 14-3-3 binding site, since MARK3 phosphorylation of known MARK3 substrates results in binding of 14-3-3 (Zhang, Kobayashi et al. 1997; Peng, Graves et al. 1998; Muller, Ory et al. 2001; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006).

To ensure that the observed phosphorylation was not a microarray-based artefact potential substrates that matched the above-mentioned criteria, were retested in liquid phase *in vitro* kinase assays. In these experiments Kinesin family member C3 (KIFC3) and Kinesin light chain 4 (KLC4) could be authenticated as *in vitro* MARK3 substrates. Both proteins are involved in cargo trafficking, which indicates a presently undescribed role for MARK3 in cellular transport and transport-dependent functions respectively. However, *in vitro* phosphorylation is only a moderate evidence for *in vivo* relevance of protein interactions. In order to sustain the microarray data the novel MARK3 substrate relationships were characterized in more detail.

3.5 Discussion

MARK3 is a serine/threonine specific kinase, which has been implicated in cell signalling, cell cycle control, transcription regulation and cell adhesion *via* its substrates KSR-1, PTPH1, Cdc25c, HDAC7 and PKP2. The aim of the project was the identification of novel MARK3 substrates, which characterize the cellular signalling network of MARK3. Since identification of novel kinase substrate pairs is a

challenging task three different approaches – screening with solid phase phosphorylation, MARK3 complex analysis and microarray technology – had to be applied to identify novel MARK3 substrates. Each approach covered different characteristics, considered important in finding new MARK3 substrates.

In situ phosphorylation screening and protein microarrays are *in vitro* screening systems, which have the advantage that potential substrates are immobilized and transient kinase substrate interactions can be determined. Since the microarray technology for substrate finding is restricted to eight thousand proteins, a cDNA library was also screened to complement this approach.

MARK3 has been shown to associate stably with most of its known substrates (Peng, Graves et al. 1998; Muller, Ory et al. 2001; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006). Therefore, MARK3 complex analysis combined with kinase assays seemed to be a reasonable approach to detect new MARK3 substrates. Complex analysis compared to *in situ* phosphorylation screening and microarray technology has the advantage that potential substrate are native and co-factors such as adaptor proteins, which might be required for the kinase substrate interaction, are present.

Screening with solid phase phosphorylation and MARK3 complex analysis did not reveal any novel MARK3 substrates. The most probable reasons these methods failing to detect MARK3 substrates have been discussed in detail in the respective conclusion sections. In contrast to this, probing a protein microarray with MARK3 led to the identification of two novel *in vitro* MARK3 substrates, KIFC3 and KLC4. Both proteins belong to the kinesin superfamily and are involved in cellular transport. This finding suggests a presently undescribed role for MARK3 in regulation of cellular

transport or transport-dependent functions respectively. What is noteworthy in this regard might be that other kinases of the MARK family have been shown to regulate microtubule stability (Ebneth, Drewes et al. 1999; Schneider, Biernat et al. 1999). Microtubules serve as transport routes on which kinesin complexes transport their cargoes (Wade 2007). The interaction of the proteins was investigated in more detail to support the hypothesis that KLC4 and KIFC3 are *bona fide* MARK3 substrates in the following section.

Chapter IV: Characterisation of the novel MARK3 substrates KIFC3 and KLC4

4.1 Introduction

Two novel *in vitro* MARK3 substrates, KIFC3 and KLC4, were identified by protein microarray technology. KIFC3 and KLC4 belong to the Kinesin super family of proteins (KIFs), which serve as molecular motors that move along microtubules carrying cargoes such as membranous organelles, protein complexes and mRNAs (Hirokawa 1998). Most kinesins are heteromeric protein complexes that consist of kinesin heavy chains (KHCs) to which KIFC3 belongs and kinesin light chains (KLCs) to which KLC4 belongs. While KHCs are responsible for locomotion and cargoe binding, KLC are mostly involved in cargoe binding. On the basis of the position of the motor domain, the most kinesin motor proteins are classified into three major types: NH_2-terminal motor domain type (N-kinesin), middle motor domain type (M-kinesin), and COOH-terminal motor domain type (C-kinesin) to which KIFC3 belongs (Miki, Setou et al. 2003). N-type kinesins and M-type kinesins move exclusively towards the plus-end of the microtubules such as the cell periphery or synapse terminal, whereas C-type kinesins move towards the minus-end, for example the apical membrane of a polarized epithelial cell or towards the microtubule organizing centre (MTOC).

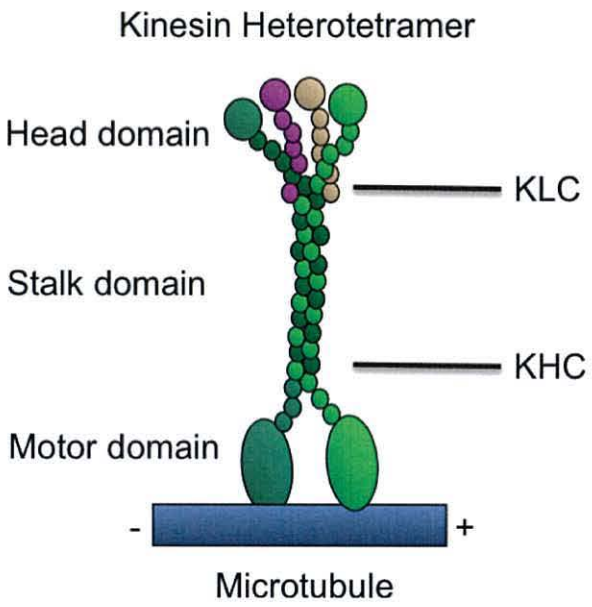


Figure 25: Organization of a kinesin heterotetramer
Kinesins move along microtubules and transport cargoes such as membranous organelles, protein complexes and mRNAs. The kinesin light chains (KLCs, purple and red) and the kinesin heavy chains (KHCs, light and dark green) form a heterotetrameric complex. This complex has a rod-like structure composed of two globular heads, a stalk, and a fan-like end. The globular heads are composed of kinesin heavy chains, which serve as motor domains in order to move along microtubules in an ATP-dependent manner. The Kinesin light chains constitute the fan-like end, which is involved in cargo recognition and transport.

The original or "conventional" kinesin was shown to be a tetrameric protein composed of two heavy chains (KHCs) and two light chains (KLCs) (Hirokawa 1998; Miki, Setou et al. 2001; Miki, Setou et al. 2003). The heterotetrameric complex has a rod-like structure composed of two globular heads (10 nm in diameter), a stalk, and a fanlike end (Hirokawa, Pfister et al. 1989). The globular heads are composed of KHCs, which serve as motor domains to move along microtubules in an ATP dependent manner. The KLCs constitute the fanlike end, which is involved in cargo recognition and transport (Fig.: 25) (Hirokawa, Pfister et al. 1989). KLCs were shown

to associate with c-jun NH_2-terminal kinase (JNK)-interacting proteins (JIPs), JIP-1, JIP-2, and JIP-3, which are scaffolding proteins for the JNK signalling pathway and bind kinases. Furthermore, KLCs contain tetratricopeptide repeat (TPR) motifs shown to be involved in cargo recognition and binding (Verhey, Meyer et al. 2001). However, the transport of macromolecular complexes containing mRNAs seems to be mediated mainly by KHC (Bowman, Kamal et al. 2000). The potential implication of MARK3 in cellular transport and/or cellular transport dependent functions will be discussed in detail later.

4.2 Characterisation of the novel MARK3 substrates KIFC3 and KLC4

4.2.1 MARK3 associates with KLC4 and KIFC3

Four out of five known MARK3 substrates – KSR-1, Cdc25c, HDAC7 and PKP2 - have been shown to constitutively associate with MARK3 and could thus be co-immunoprecipitated (Peng, Graves et al. 1998; Muller, Ory et al. 2001; Kanai, Dohmae et al. 2004; Dequiedt, Martin et al. 2006). For PTPH1, the fifth known MARK3 substrate, phosphorylation was authenticated but association of substrate and kinase was not investigated (Zhang, Kobayashi et al. 1997). Therefore, MARK3 is assumed to form stable complexes with at least some of its substrates.

To demonstrate the MARK3/KIFC3 and MARK3/KLC4 interactions *in vivo* HIS tagged MARK3 and either MYC tagged KLC4 or MYC tagged KIFC3 were

simultaneously overexpressed in HEK293 cells. Subsequently, MARK3 or the respective substrate was immunoprecipitated from these transformed HEK293 cells *via* its affinity tag. Depending on the immunoprecipitated protein the relevant binding partner was determined by Western blot.

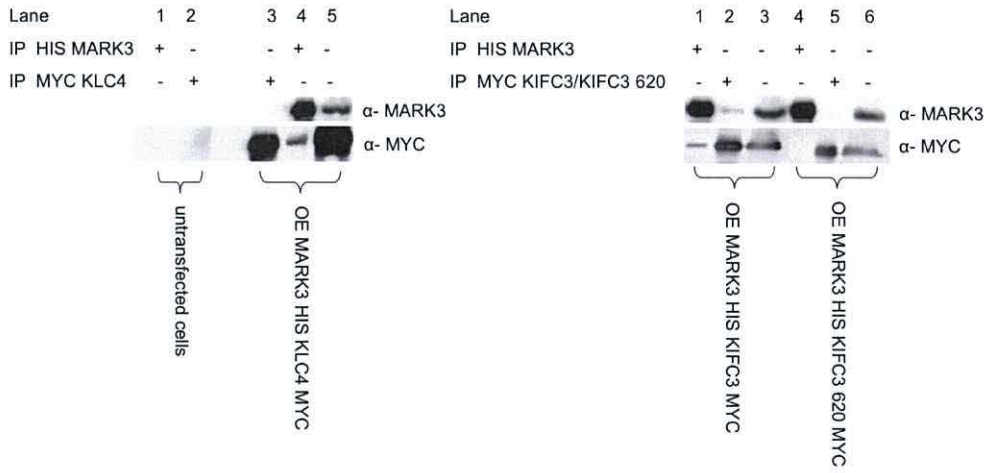


Figure 26: MARK3/KLC4 and MARK3/KIFC3 association
Histidin tagged MARK3 (MARK3 HIS) was overexpressed (OE) together with either MYC tagged KLC4 (KLC4 MYC), MYC tagged KIFC3 (KIFC3 MYC) or MYC tagged KIFC3 620 (KIFC3 620 MYC) in HEK 293 cells. Subsequently, MARK3 or the MYC tagged substrate was immunoprecipitated (IP) and analysed considering its relevant *in vivo* binding partners. Western blots with antibodies directed against the epitope tag of the overexpressed proteins (KLC4, KIFC3) or the protein itself (MARK3) confirmed their presence. Untransfected cell lysates served as negative control to confirm that the detected associations were significant and not antibody or resin related. Identities of the samples are indicated in the figure.

These experiments showed that KLC4 could be co-immunoprecipitated with MARK3, (Fig.: 26, lane 5) whereas MARK3 could not be co-immunoprecipitated with KLC4 (Fig.: 26, lane 4). This might be due to the antibody/bead complex that could impair the association between KLC4 and MARK3. The association seems not to be stochiometric since only small amounts of KLC4 co-precipitate with MARK3. HEK293 lysates confirmed that the relevant proteins were overexpressed in sufficient

amounts (Fig.: 26, lane 6). Similar to KLC4, MARK3 was demonstrated to co-immunoprecipitate with KIFC3 (Fig.: 26, lane 7) and *vice versa* (Fig.: 26, lane 8). Using a KIFC3 deletion mutant (KIFC3 620 MYC) lacking the COOH-terminal 68 amino acids instead of wild type KIFC3 led to abrogation of the MARK3 KIFC3 association, which localises the MARK3 binding site within the last 68 amino acids of KIFC3 (compare Fig.: 26, lane 7 and 8 to Fig.: 26, lane 10 and 11). HEK293 lysates confirmed that the relevant proteins were overexpressed in equal amounts (MARK3 KIFC3 Fig.: 26, lane 6 // MARK3 KIFC3 620 (Fig.: 26, lane 12). Control experiments with untransfected cells authenticated that the results were specific and not due to antibody or resin derived artefacts (Fig.: 26, lane 1 and 2).

4.2.2 Phosphopeptide mapping of KLC4 and KIFC3

Localisation of the MARK3 phosphorylation sites in KLC4 and KIFC3

Based on the mutational analysis of three MARK3 phosphorylation sites (KSR-1, Cdc25c and PKP2) it could be shown that MARK3 recognizes and phosphorylates the $\phi^a xRxx_pS\phi xxx\phi^a$ motif (ϕ^a and ϕ are hydrophobic aliphatic residue and a hydrophobic residue respectively) (Muller, Ritt et al. 2003). However, Dequiedt et al. recently discovered a novel MARK3 phosphorylation site in HDAC7 (S155), which did not completely match the predicted MARK3 phosphorylation motif (Dequiedt, Martin et al. 2006). Therefore, the phosphorylation site analysis in this thesis focused on a rather basic phosphorylation motif R/KxxS/T of basophilic kinases.

To narrow down the localisation of the phosphorylation site(s), FLAG tagged fragments of KIFC3 and KLC4 were expressed in HEK293 cells to served as substrates for MARK3. For KLC4 the NH_2-terminal fragment comprised the amino acids 1-451 and the COOH-terminal fragment comprised the amino acids 451-620. For KIFC3 the NH_2-terminal fragment comprised the amino acids 1-515 and the COOH-terminal fragment the amino acids 515-688. In *in vitro* kinase assays only the COOH-terminal fragment of the substrates was phosphorylated (For KLC4 compare Fig.: 27A, lane 1 NH_2-terminal fragment to Fig.: 27A, lane 2 COOH-terminal fragment and for KIFC3 compare Fig.: 26B, lane 1 NH_2-terminal fragment to Fig.: 27B, lane 2 COOH-terminal fragment). The presence of the fragments was confirmed by Western blot with antibodies directed against the epitope tags of the proteins (For KLC4 Fig.: 27A lane 3 NH_2-terminal fragment Fig.: 27A lane 4 COOH-terminal fragment and for KIFC3 Fig.: 27B lane 3 NH_2-terminal fragment and Fig.: 27B lane 4 COOH-terminal fragment). Since MARK3 only phosphorylated the COOH-terminal fragment of KLC4, the number of potential phosphorylation sites was reduced from initially 11 to 8 (S460, T498, S519, S554S, S566, S582, S590 and S609). For KIFC3 phosphorylation of the COOH-terminal fragment narrowed down the number of potential phosphorylation sites from 11 to 5 (S563, S614, S628, S666 and S678). It is noteworthy regarding the phosphorylation of the protein fragments that MARK3 has been demonstrated to phosphorylate a KSR-1 fragment of 15 amino acids containing the phosphorylation site S392 fused to MBP. Therefore, MARK3 dependent substrate

phosphorylation seems - at least for KSR-1 - independent of amino acid sequences outside the phosphorylation motif (Fig.: 15, lane 3).

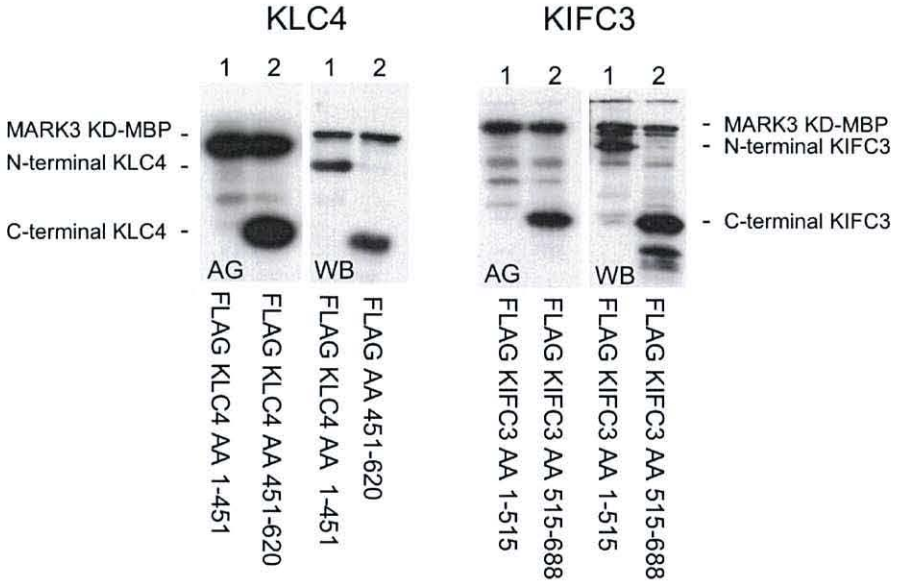


Figure 27: Phosphorylation of KLC4 and KIFC3 fragments
Only the COOH-terminal part of KIFC3 and KLC4 is phosphorylated by MARK3 in *in vitro* kinase assays. FLAG tagged NH_2- and COOH-terminal fragments of KLC4 - KLC4 AA1-451 KLC4 AA451-620 - and KIFC3 - KIFC3 AA1-515; KIFC3 AA515-688 - were overexpressed in HEK293 cells and purified by immunoprecipitation with antibodies directed against the epitope tag. The fragments served as substrates for MARK3 KD-MBP in radioactive *in vitro* kinase assays. Phosphorylation of the substrates was analysed by autoradiography (AG). Western blots (WB) with antibodies directed against the FLAG tag of the substrate fragments and the MBP tag of the kinase authenticated the presence of the relevant proteins. Identities of the samples are shown in the figure.

Phosphopeptide mapping of KLC4

Since all known MARK3 substrates are phosphorylated on one residue each of the remaining 8 potential MARK3 phosphorylation sites in KLC4 was mutated to alanine.

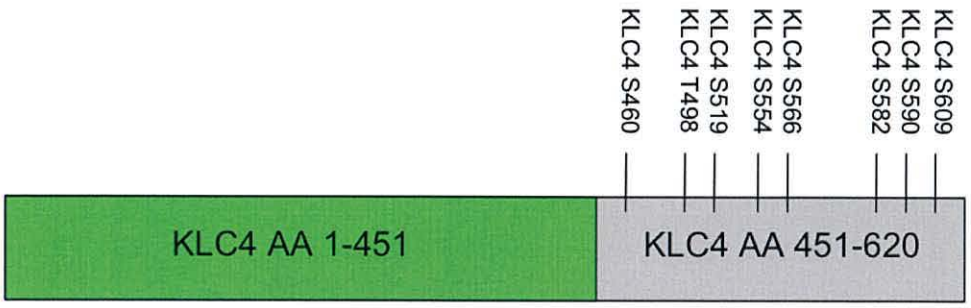


Figure 28: Localisation of potential MARK3 phosphorylation sites in KLC4
Figure 28 shows the location of 8 potential MARK3 phosphorylation sites within the COOH-terminal part of KLC4 (AA451-620, grey). The NH_2-terminal part of KLC4 (AA1-451, green) probably does not contain any MARK3 phosphorylation site as above (Figure 27, KLC4 lane 1). For each potential MARK3 phosphorylation within the COOH-terminal part of KLC4 (AA451-620, grey) a mutant was prepared that encoded for an alanin instead of serine or threonine respectively. The mutagenesis primers are stated within the methodology section.

The mutants were then assessed considering their capability to serve as MARK3 substrate *via in vitro* kinase assays. Unexpectedly, each single mutant was phosphorylated by MARK3. This finding let to the conclusion that either more than one residue is phosphorylated or that MARK3 phosphorylates not only the basic K/RxxS/T phosphorylation motif in KLC4 (Fig.: 29 lane 2-8). Performing the kinase assays for MARK3 KD-MBP without KLC4 ensured that the KLC4 phosphorylation signals were not derived by degradation of autophosphorylated MARK3 (compare Fig.: 29, lane 1 to Fig.: 29, lane 2-8).

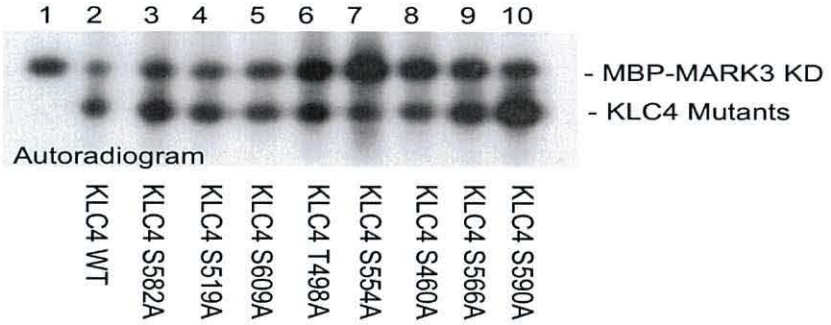


Figure 29: Phosphorylation of KLC4 wild type and 8 single mutants by MARK3
MYC tagged KLC4 wild type (KLC4 WT) and 8 KLC4 single mutants of potential MARK3 phosphorylation sites (KLC4 S582A, KLC4 S519A, KLC4 S609A, KLC4 T498A, KLC4 T554A, KLC4 S460A, KLC4 S566A, KLC4 S590A) were overexpressed in HEK293 cells and purified *via* immunoprecipitation. Subsequently, the proteins were analysed regarding their capability to become phosphorylated by the kinase domain of MARK3. KLC4 wild type and the 8 KLC4 single mutants were all phosphorylated to a similar extent (lane 2-10). MARK3 alone served as control to ensure KLC4 phosphorylation bands were not derived by autophosphorylation and subsequent protein degradation (lane 1). Identities of the samples are shown under the figure.

Since all single mutants showed MARK3 phosphorylation two-dimensional phosphopeptide mapping was used to precisely determine the site(s) of phosphorylation. For this purpose the [γ-^{32}P]-labeled KLC4 proteins - shown in figure 29 - were excised and trypsin digested. The resulting peptides were dotted onto thin layer chromatography plates and separated electrophoretically and chromatographically into horizontal and vertical directions respectively. MARK3 phosphorylated peptides could be detected by autoradiography, whereas unphosphorylated peptides remained invisible.

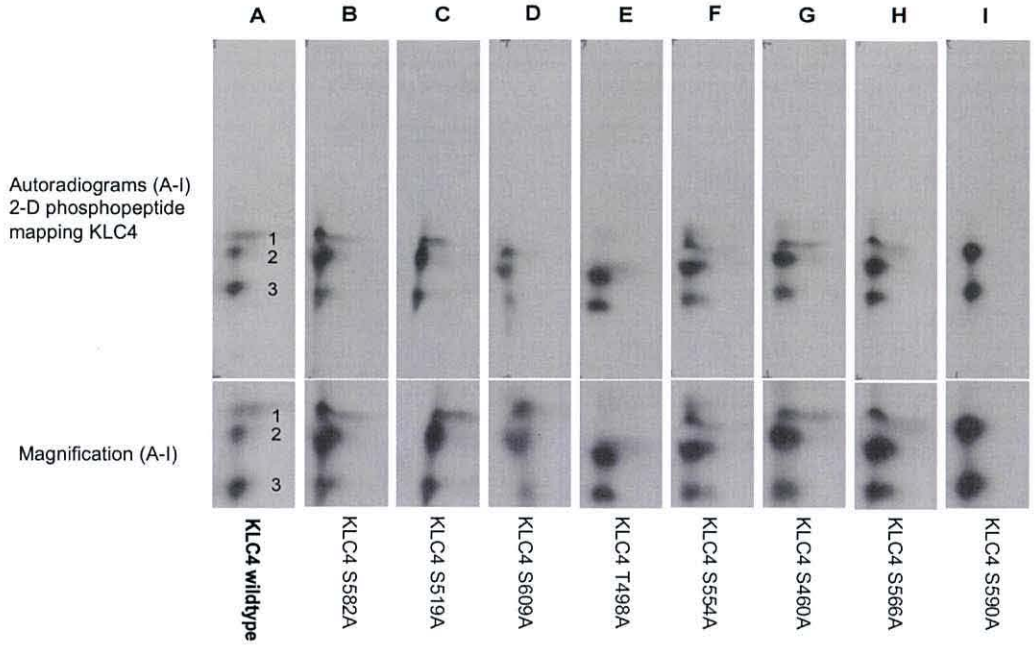


Figure 30: Identification of three MARK3 *in vitro* phosphorylation sites in KLC4
KLC4 wild type (Fig.: 29A) and KLC4 8 single mutants – KLC4 S582A, KLC4 S519A S609A, KLC4 T498A, KLC4 S554A, KLC4 S460A, KLC4 S556A, KLC4 S590A, (Fig.: 30B-I) – were phosphorylated *in vitro* by MARK3. The [γ-^{32}P]-labeled proteins were resolved by SDS-PAGE, excised and trypsine digested. The peptides were dotted onto thin layer chromatography plates and separated electrophoretically and chromatographically. [γ-^{32}P]-labeled peptides were detected by autoradiography. For KLC4 wild type three phosphorylated peptides could be detected (Fig.: 30A indicated by the numbers 1,2 and 3). Identities of the samples are shown in the figure. Each lower panel shows a magnifcation of the respective upper panel.

For wild type KLC4 three different phosphorylated peptides were identified (Fig.: 30A, number 1-3), which indicates that there is likely more than one MARK3 phosphorylation site in KLC4. Analysing the KLC4 single mutants revealed that three mutants – KLC4 S609A, KLC4 T498A and KLC4 S590A – only showed two phosphopeptides (compare Fig.: 30D, Fig.: 30E and Fig.: 30I with Fig.: 30B, Fig.: 30C, Fig.: 30F, Fig.: 30G and Fig.: 30H). Loss of the phosphorylation signal due to a mutation of a potential phosphorylation site indicates that the specific site was radio

labeled. Therefore, S609, T498 and S590 might be the KLC4 MARK3 *in vitro* phosphorylation sites of MARK3. S609 of these potential phosphorylation sites seems to be less convincing since the phosphorylation signal did not completely disappear for the mutant. Further analysis is necessary to authenticate these sites as MARK3 phosphorylation sites.

PKP2	S082	N	L	H	R	T	S	$_pS$	V	P	E	Y	V
KSR1	S392	R	L	R	R	T	E	$_pS$	V	P	S	D	I
Cdc25c	S216	G	L	Y	R	S	P	$_pS$	M	P	E	N	L
MARK3	P-Motif	X	Φ^a	X	R	X	X	$_pS$	Φ	X	X	X	Φ^a
PTPH1	S359	P	A	M	R	R	L	$_pS$	V	E	H	L	E
HDAC7	S155	P	L	R	K	T	V	$_pS$	E	P	N	L	K
KLC4	S609	Q	V	S	R	G	L	$_pS$	A	S	T	M	D
KLC4	S590	N	M	K	R	A	A	$_pS$	L	N	Y	L	N
KLC4	T498	R	S	R	R	Q	G	$_pT$	D	P	I	S	Q

Figure 31: Canonical MARK3 phosphorylation motif
The canonical MARK3 phosphorylation motif predicts that hydrophobic aliphatic (ϕ^a) residues at -5, +5, a hydrophobic residue (ϕ) at +1 and an arginine relative to the phosphorylated serine ($_pS$) are crucial for optimal MARK3 phosphorylation. Abbreviations: Plakophiline 2 (PKP2); Kinase suppressor of ras 1 (KSR-1); Cell devision cycle homolog c (Cdc25c); MAP/Microtubule Affinity Regulating Kinase 3 (MARK3); Protein tyrosine phosphatase H1 (PTPH1); Histone deacetylases 7; Kinesin light chain 4 (KLC4)

An earlier analysis of MARK3 phosphorylation sites based on three known substrates (KSR-1, PKP2, Cdc25c) predicted that hydrophobic residues at -5, +1 and +5 an arginine at the -3 position relative to the phosphorylated serine are crucial for optimal MARK3 phosphorylation (Fig.: 31) (Muller, Ritt et al. 2003). The potential *in vitro* phosphorylation sites identified for KLC4 only partially match the canonical MARK3

phosphorylation motif. The phosphorylation sites KLC4 S609 and KLC4 S590 lack the hydrophobic residue at +5. The phosphorylation site T498 lacks all predicted residues except the arginine at position +3 relative to the phosphorylation site.

KLC4 associates with 14-3-3 proteins

MARK3 regulates all known substrates by phosphorylation of a specific serine residue, which leads to 14-3-3 binding and subsequent translocation of the substrate (Peng, Graves et al. 1998; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006). Therefore, KLC4 was analysed to determine its capacity to associate with 14-3-3. For this purpose MYC tagged KLC4 and FLAG tagged 14-3-3β were simultaneously overexpressed (OE) in HEK293T cells. KLC4 was immunoprecipitated and 14-3-3β binding was assessed by western blotting with an antibody directed against the epitope tag of 14-3-3β (Fig.: 32). Cells transfected with KLC4 or 14-3-3β respectively as well as untransfected cells served as controls to show that the binding 14-3-3β was specific for the KLC4 immunoprecipitation.

This experiment demonstrated that KLC4 associates with 14-3-3β (Compare OE FLAG-14-3-3β and MYC-KLC4 Fig.: 32 lane 4 with untransfected cells Fig.: 32 lane 1, OE FLAG-14-3-3β Fig.: 32 lane 2 and OE MYC-KLC4 Fig.: 32 lane 3). To authenticate that 14-3-3β co-immunoprecipitation is specific for KLC4 overexpression a western blot was performed with an antibody directed against the epitope tag of KLC4 (Compare OE MYC-KLC4 and FLAG-14-3-3β Fig.: 32 lane 8

and OE MYC-KLC4 Fig.: 32 lane 7 with untransfected cells Fig.: 32 lane 5). Future experiments will investigate whether this interaction is MARK3 dependent.

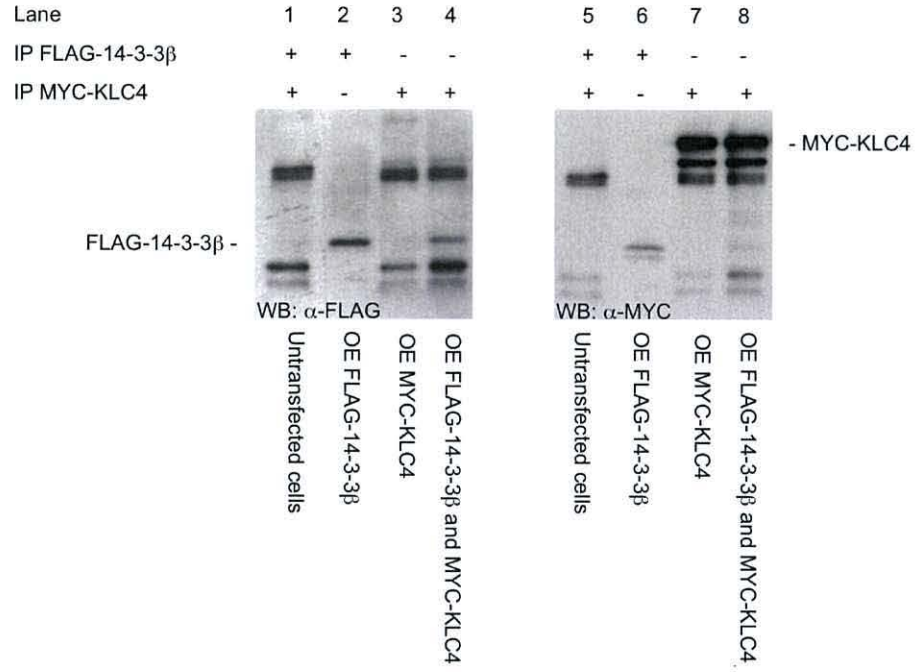


Figure 32: KLC4 associates with 14-3-3β
KLC4 was analysed considering its capacity to associate with 14-3-3β. For this purpose MYC tagged KLC4 and FLAG tagged 14-3-3β were simultaneously overexpressed (OE) in HEK293T cells. KLC4 was immunoprecipitated and analysed considering 14-3-3β binding by western blotting with an antibody directed against the epitope tag of 14-3-3β. A western blot (WB) with an antibody directed against the MYC tag of KLC4 confirmed the presence of KLC4. Cells transfected with KLC4 or 14-3-3β respectively and untransfected cells served as controls to show that the observed protein interactions are specific. Identities of samples are indicated in the figure.

4.3 Discussion

So far MARK3 has been implicated in a diversity of cellular functions such as cell cycle control, intracellular signalling, transcription regulation and cell adhesion (Peng,

Graves et al. 1998; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006). This work aimed to characterize the position of MARK3 within the cellular signalling network in more detail. One route to achieving this aim was the identification of novel MARK3 substrates, which might reveal as yet undescribed cellular functions of MARK3. Applying the ProtoArray® Human Protein Microarray System to this task led to the identification of two novel *in vitro* MARK3 substrates, namely kinesin family member C3 (KIFC3) and kinesin light chain 4 (KLC4), both of which are involved in cellular cargo trafficking. Subsequently the microarray-based findings were authenticated by *in vitro* kinase assays and protein-protein association studies, which demonstrated that MARK3 not only phosphorylates KIFC3 and KLC4 *in vitro,* but also forms complexes with both substrates in cells. These two observations together - *in vitro* phosphorylation and *in vivo* association - show that KIFC3 and KLC4 are most likely *bona fide* MARK3 substrates, which indicates a novel role for MARK3 in cellular cargo transport or transport-dependent cellular programs. However, during the interaction analysis the proteins of interest have been overexpressed. This could have led to unspecific protein interactions due to the increased amounts of the expressed proteins. Therefore, further interaction studies in which endogenous MARK3, KIFC3 and KLC4 are immunoprecipitated and analysed regarding their interaction partners are necessary.

Mapping of the phosphorylation site(s) was performed to further characterize the MARK3 substrate relationships. Since all known MARK3 substrates have been shown to be phosphorylated on one serine residue, it was surprising that two-

dimensional phosphopeptide mapping revealed two (T498, S590), possibly three (S609), different MARK3 phosphorylation sites for KLC4 (Peng, Graves et al. 1998; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006). Furthermore, these phosphorylation sites only partially matched the predicted phosphorylation motif $\phi^a xRxx_pS\phi xxx\phi^a$ (ϕ^a and ϕ are respectively a hydrophobic aliphatic residue and a hydrophobic residue) (Muller, Ritt et al. 2003). Noteworthy in this regard is that Dequiem et al. challenged the canonical MARK3 phosphorylation motif by demonstrating that the phosphorylation motif of HDAC7, the most recently identified MARK3 target, as well lacks most residues that are predicted for optimal MARK3 substrate phosphorylation. They could additionally show that in HDAC7 potential phosphorylation motifs, which are more homologous to the canonical MARK3 phosphorylation motif, were not phosphorylated by MARK3 (Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006). The MARK3 phosphorylation site analysis of Dequiem et al. and the prelimery phosphorylation site mapping data for KLC4 presented in this thesis together would suggest a modified phosphorylation motif $\phi^a xR/Kxx_pS/T\phi xxx\phi^a$ for MARK3. The hydrophobic residue at the +5 position seems to be less crucial than the hydrophobic residues at +5 and +1 relatively to the phosphorylation site. Furthermore, a positively charged lysine residue can replace the positively charged arginine residue at the -3 position relative to the phosphorylation site. Moreover, the results in this thesis show, for the first time, that MARK3 might phosphorylate threonine residues as well as serine residues. This finding is substantiated by the observation that bacterially expressed MARK3 is phosphorylated on the threonine 211 within the phosphorylation loop. However, the phosphorylation site mapping

performed in this thesis has not been substantiated by kinase assays experiments with proteins in which the potential sites have been mutated. Moreover, trypsin predominantly cleaves peptide chains at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline (Olsen et al. 2004). Two (T498, S590) of the three identified potential phosphorylation sites show a double argine and argine lysine respectively at the cleavage site, which might have resulted in two different phosphopeptides that would migrate differentially during 2 dimensional phosphomapping analysis and result in two independent spots on the autoradiogram. Therefore, the drawn conclusions about the canonical MARK3 phosporylation motif have to be regarded with extrem caution and clearly need further analysis.

MARK3 phosphorylation of all known substrates leads to 14-3-3 binding and translocation of the substrate. In mutational analysis two high stringency phosphorylation dependent 14-3-3 binding motifs - RSXpSXP (mode 1) and RXXXpSXP (mode 2) - were revealed, which are recognized by all seven 14-3-3 isoforms (Muslin, Tanner et al. 1996; Yaffe, Rittinger et al. 1997; Rittinger, Budman et al. 1999). However, phosphorylation dependent 14-3-3 has been demonstrated independently of these motifs for various proteins (Göransson et al. 2004). Scanning - with help of the online program scansite - KLC4 for these motifs identified S590 as moderate, and T498 as low stringency binding sites for 14-3-3, whereas S609 - the least convincing MARK3 phosphorylation site detected by 2D-phosphopeptide mapping - did not show any stringency for 14-3-3 binding (Web reference 3). Moreover, in association studies KLC4 was shown to bind 14-3-3β. Therefore, KLC4 phosphorylation by MARK3 might lead to KLC4/14-3-3 association. However, since

a) the KLC4 phosphorylation sites have not been authenticated *in vivo,* b) computer based predictions of protein-protein interactions have to be regarded with extreme caution c) it is not clear if the detected 14-3-3β/KLC4 association is MARK3 dependent and d) the predicted 14-3-3 binding sites for KLC4 have not been experimentally confirmed yet. Further experiments are necessary to unravel the exact mechanism of how MARK3 regulates KLC4.

If KLC4 and KIFC3 are considered as *bona fide* targets of MARK3 the question of the physiological relevance of the interaction must be addressed, what means elucidating the cellular functions of the MARK3 phosphorylation of KLC4 and KIFC3. Since no cellular functions have been described for KLC4 at present any conclusions except that MARK3 might be involved in cellular transport *via* KLC4 would remain entirely speculative. Conversely, KIFC3 has been demonstrated to be involved in epithelial cell polarity and cell-cell adhesion (Noda, Okada et al. 2001; Xu, Takeda et al. 2002; Meng, Mushika et al. 2008). Both are cellular programs that have been shown regulated by kinases of the MARK group and by upstream regulators of MARK3 aPKC and LKB1 (Henrique and Schweisguth 2003; Hurov and Piwnica-Worms 2007; Hezel and Bardeesy 2008). Therefore, the next part of the discussion tries to illustrate how MARK3 could be involved in these cellular functions *via* KIFC3 and what implications this might have for the assumed role of MARK3 in cancer development.

MARK3 KIFC3 interaction might be involved in the formation of apical basal cell polarity

The plasma membrane of polarized epithelial cells shows an apical and a basolateral domain separated by tight junctions. A distinct subset of functional proteins and membrane composition can be found in each domain. Differential transport is a hallmark in establishing and maintaining the apical and the basolateral cell polarity (Matter and Mellman 1994). Four regulatory mechanisms have been implicated in this polarisation process: packaging, transport, docking and retention and/or removal (Muth et al. 2003). Proteins transported to the apical or basolateral domain are packed into their domain specific targeted transport vesicles. These vesicles are then transported *via* microtubules, the actin networks, to their specific destination. At the plasma membrane specific docking mechanisms ensure that the transport vesicles fuse with the proper plasma membrane region (Hunziker, Harter et al. 1991; Calakos, Bennett et al. 1994; Calakos and Scheller 1994; Harder and Simons 1997).

KIFC3 could be involved in the apical transport process of annexin IIIB and haemagglutinin in epithelial polarized cells *via* its function as a motorprotein (Noda, Okada et al. 2001). Wild type KIFC3 and its cargoes could be located at the apical membrane, whereas dominant negative KIFC3 (AA1-515), which lacked parts of the motor domain, was evenly distributed throughout the cell (Noda, Okada et al. 2001). In this current work MARK3 was demonstrated to bind and phosphorylate KIFC3 within the COOH-terminal part of KIFC3, deleted in the dominant negative KIFC3. These findings, along with the knowledge that MARK3 is known to regulate the localisation of its substrates, give rise to the hypothesis that MARK3 might be

involved in formation of apical basolateral cell polarity *via* regulating the apical localization of KIFC3 and the associated transport of annexin IIIB and haemagglutinin in epithelial cells respectively.

Furthermore, in adrenocortical cells KIFC3 and cytoplasmic dynein play a distinct role in Golgi integration and positioning *via* their function as motor proteins (Xu, Takeda et al. 2002). Similar to the organization and localisation of the mitotic spindle, the positioning of the Golgi apparatus is assumed to be the result of a balance between plus and minus end directed motors, known as a "tug-of-war" mechanism (Xu, Takeda et al. 2002; Kozlowski, Srayko et al. 2007). Due to its role in protein trafficking, the Golgi apparatus serves as a marker of cell polarization and reorientates between the nucleus and the polarized surface (Saraste and Goud 2007). Since MARK3 binds and phosphorylates the motor domain of KIFC3 it might be involved in Golgi positioning, which would indicate a role for MARK3 in protein trafficking and/or cellular polarisation.

One of the most recent study implicates KIFC3 in maintaining and modulating the apical belt of adherence junctions, the *zonula adherence*. KIFC3 is proposed to access adherence junctions *via* PLEKHA7-Nezha anchored microtubules and works together with these proteins to regulate the cadherin assembly (Meng, Mushika et al. 2008). Depletion of any of these proteins – KIFC3, PLEKHA7 or Nezha - led to disorganisation of the cadherins and concomitant flattening of the cells, suggesting that the complex is crucial for cells to maintain their tension (Meng, Mushika et al. 2008). Cell-cell adhesion in epithelial cells is closely linked to cell polarity. Proteins involved in regulation of cell polarity are frequently involved in the regulation of cell-

cell adhesion (Caplan, Seo-Mayer et al. 2008). MARK3 has been shown to be implicated in cell adhesion *via* the desmosomal protein PKP2 (Muller, Ritt et al. 2003). Therefore, it might be conceivable that MARK3 is also involved in the integrity of adherence junctions *via* KIFC3 phosphorylation.

All three described KIFC3 functions are related to cell polarity and cell-cell adhesion. The upstream regulators of MARK3, aPKC and LKB1, have been established as master regulators of these cellular programs (Suzuki and Ohno 2006; Williams and Brenman 2008). In this regard it might be notable that in recent observations LKB1 has been implicated in defective Golgi positioning and aberrant lung cell polarity (Zhang, Schafer-Hales et al. 2008). Therefore, the MARK3 KIFC3 interaction might partially reveal how cell polarity and cell-cell adhesion is regulated downstream of LKB1 and aPKC. This has not yet been fully understood.

MARK3 KIFC3 interaction and cancer development

The present data might indicate a role for MARK3 in cancer development. This proposal is based on two observations. Firstly, all identified substrates of MARK3 either participate in or control cell programs deregulated in tumour cells (Draviam, Stegmeier et al. 2007; Fields, Frederick et al. 2007; Raman, Earnest et al. 2007; Shah, Pang et al. 2008; Partanen, Nieminen et al. 2009). Secondly, tumour suppressor proteins seem to activate MARK3, while oncogenes inactivate or regulate the localisation of MARK3 (Bachmann, Hennemann et al. 2004; Hurov, Watkins et al. 2004; Lizcano, Göransson et al. 2004). Furthermore, MARK3 has been shown to be

downregulated in later stages of melanoma development (Haqq et al. 2005). In osteoclast differentiation MARK3 has been shown to play a role by regulating the localisation of microphthalmia-associated transcription factor (MITF). Besides its function in osteoclast differentiation, MITF is a master regulator of melanocyte development and a melanoma oncogene (Levy et al. 2006; Bronisz et al. 2006). These observations led to the hypothesis that MARK3 might be involved in melanoma development *via* regulation of MITF localisation.

The following paragraph discusses if the observations made in this thesis support the hypothesis that MARK3 could be a tumour suppressor protein. KIFC3 has only been marginally investigated. No direct link between KIFC3 and tumour formation could yet be established. However, the cellular programs in which KIFC3 participates, cell polarity and cell-cell adhesion, have been identified as key players in tumour development and progression (Wodarz and Nathke 2007; Gloushankova 2008). Loss of normal plasma membrane polarisation in epithelial cancers was an early observation and became substantiated when tumour suppressor genes were identified that are physiological determinants of epithelial polarity (Molitoris and Nelson 1990; Vasioukhin, Bauer et al. 2001; Bilder 2004). Alteration of cell polarity is assumed to be involved in early and late phases of tumour development. Cell polarity alterations are hard to detect in early phases, since the polarity machinery is still intact (Wodarz and Nathke 2007). Abberant cell polarity is obvious in late phases, such as tumour invasion, which shows some characteristics of epithelial to mesenchymal transition (EMT) (Wodarz and Nathke 2007). Reductions of cell-cell adhesions, apical-basolateral polarity and epithelial markers, as well as an acquisition of motility,

spindle-cell shape and mesenchymal markers are features of EMT a process first recognized in a central differentiation process in early embryogenic morphogenesis (Takanori et al. 2009). The term "EMT" has been more liberally referred to in cancer development as a loss in adhesion junctions, change of cell polarity and along with this a gain of migratory behaviour (Thiery 2003). Cell adhesion and polarity in epithelial cells depends on the formation of adherence junctions in which E-cadherin is a key player, providing the physical scaffold for both cell-cell attachment and the recruitment of signalling complexes (Perez-Moreno, Jamora et al. 2003). After losing their attachment invasive cells need to change their baso-apical polarity into a anterior-posterior polarity axis, which allows the cell to migrate (Ridley, Schwartz et al. 2003). The MARK3 upstream regulators, aPKC and LKB-1, have both been established as regulators of cellular polarity/cell adhesion and could be implicated *via* these functions in the development and progression of neoplasms (Fields, Frederick et al. 2007; Zhang, Schafer-Hales et al. 2008). The exact molecular mechanisms of how these signalling molecules are involved in cell transforming processes are not yet understood. MARK3 as a substrate of LKB1 and aPKC might be involved downstream in these processes *via* a function of KIFC3 to participate in epithelial cell polarization or cell adhesion (Noda, Okada et al. 2001; Hurov, Watkins et al. 2004; Lizcano, Göransson et al. 2004).

Furthermore, KIFC3 could lately be involved in chemoresistance of breast cancer cells by destabilising tubulin (De et al. 2009). Docetaxel resistant clones were isolated and showed elevated levels of KIFC3 expression. In further experiments overexpression of KIFC3 or the additional kinesins KIFC1, KIF1A, or KIF5A in

MDA-MB231 and MDA-MB 468 breast cancer cells led to increased docetaxel resistance (De et al. 2009)

Furthermore, kinesins are essential for cell cycle progression. The creation of the metaphase spindle and the subsequent segregation of sister chromatids in anaphase require the actions of dynein and several members of the kinesin superfamily (Scholey *et al.*, 2003) Microtubule minus end motors, such as KIFC3, are essential for correct chromosome segregation. MARK3 has been shown to be involved in regulation of the G2/M transition by phosphorylation of Cdc25c (Peng, Graves et al. 1998; Heald 2000). In addition, MARKK, a MARK3 upstream regulator, could recently be implicated in spindle checkpoint signalling (Draviam et *al.*, 2007). MARKK seems to be required for chromosome congression and checkpoint-induced anaphase delay (Draviam et *al.*, 2007). Therefore, it could be speculated that MARK3 is not only involved in G1/M transition *via* Cdc25c phosphorylation but also in the regulation of chromosome segreggation *via* KIFC3 downstream of MARKK.

Furthermore, motorproteins of the KIF family, such as KIF1Bβ, have been shown to be tumour suppressor proteins and kinesin spindle proteins, as potential cancer targets, are currently studied in clinical trials (Schlisio, Kenchappa et al. 2008; Sarli and Giannis 2008). Thus emphasizing the importance of kinesins in cancer development.

Further experiments are necessary to either reject or confirm any of the previously stated hypotheses. However, finding KIFC3 as a MARK3 target raises the possibility that MARK3 functions as a tumour suppressor kinase and/or might shed light on how

the cellular master regulators such as MARKK, aPKC and LKB1 are involved in carcinogenesis.

4.4 Future experiments and perspectives

The mechanism of how MARK3 regulates KIFC3 and KLC4 should be revealed in detail in future experiments. This knowledge can be used to identify the physiological context of the interactions. Depending on the context, it might then be interesting to investigate if known upstream regulators of MARK3 are involved.

The mechanism of KLC4 and KIFC3 regulation

The identified MARK3 phosphorylation sites of KLC4 have to be authenticated by *in vitro* kinase assays in which double and triple mutants of KLC4 serve as substrate. Once the sites have been authenticated *in vitro*, MARK3 knock down experiments - using short interference RNA, short hairpin RNA or zinc finger nucleases - as well as overexpression experiments with dominant-negative MARK3 in human cells should lead to loss of phosphorylation of the potential phosphorylation site(s). This loss of phosphorylation can then be detected by mass spectroscopic analysis of the substrate and should reveal which sites are phosphorylated *in vivo*.

For KIFC3 the *in vitro* phosphorylation sites have to be determined by mass spectroscopy or by conventional phosphopeptide mapping technique. The authentication of these sites *in vivo* can be performed as described for KLC4.

Since MARK3 has been shown to regulate all known substrates by phosphorylation of a specific serine residue, which leads to 14-3-3 binding and translocation of the substrate, KIFC3 and KLC4 should be analysed considering their capacity to associate with 14-3-3 in a MARK3 dependent manner. Furthermore, the cellular localisation of KLC4 and KIFC3 in dependency of MARK3 phosphorylation should be analysed. Together, these results will reveal if MARK3 regulates KLC4 and KIFC3 similarly or differentially from the already described substrates.

Physiological context of the MARK3 and KIFC3 and KLC4 interaction

Identification of the physiological context of a protein-protein interaction is often difficult. However, MARK3 knock down or over expression combined with the expression of KLC4 and KIFC3 wild type or mutant proteins might lead to cellular phenotypes, which indicate the physiological context of the MARK3 phosphorylation. The considerations concerning overlapping functions of KIFC3 and the MARK group and MARK3 upstream regulators respectively might serve as guideline which cellular phenotypes could be expected. Nevertheless, to predict any phenotypes at this point would be purely speculative.

Chapter V: Identification of MARK3 upstream regulators

5.1 Introduction

Twenty-one different phosphorylation sites were mapped for MARK3 *in vivo* (Göransson, Deak et al. 2006). However, only four of these could be matched to the four known upstream kinases of MARK3. Both the tumour suppressor master kinase of the AMPK family, LKB1 (responsible for Peutz-Jeghers syndrome) and MARKK (a Ste20-like kinase) have been described to activate MARK3 by targeting T211 within the phosphorylation loop (Timm, Li et al. 2003; Lizcano, Göansson et al. 2004). Oncogenic serine/threonine kinase PIM-1 functions as a negative regulator of MARK3 *via* phosphorylating S90 as well as either S95 or S96 within the kinase domain (Bachmann, Hennemann et al. 2004). Moreover, atypical kinase C is assumed to regulate MARK3 localisation by phosphorylation of T564 within the KA1 domain (Hurov, Watkins et al. 2004). However, the protein kinases that phosphorylate the majority of the identified MARK3 phosphorylation sites and the regulatory consequences of these phosphorylations remain elusive. The in-gel kinase assay (IGKA) is an excellent molecular tool to investigate kinase substrate relationships (Wooten 2002) and was applied to identify protein kinases that phosphorylate MARK3.

5.2 Identification of MARK3 upstream regulators

5.2.1 Purification of the inactive kinase domain of MARK3 and MBP

For the in-gel kinase assays the mutationally inactivated MBP tagged kinase domain of MARK3 (MARK3 KD-MBP IA) and MBP were expressed in *E. coli* and purified *via* MBP column chromatography as described in the former chapter (MBP, Fig.: 20, lane 1// MARK3 KD-MBP IA Fig.: 20, lane 4). The mutated kinase was authenticated as catalytically inactive by *in vitro* kinase assays (compare active MARK3 KD-MBP Fig.: 21A, lane 4 to inactive MARK3 KD-MBP IA Fig.: 21A, lane 5). Since substantial amounts of substrate were needed for the in-gel kinase assay (0.25 mg/ml), proteins from ten litres of bacterial culture were purified, resulting in a yield of ~1.5 mg protein per litre culture for both proteins.

5.2.2 Identification of upstream regulators by in-gel kinase assay

The in-gel kinase assay was used to identify protein kinases, which phosphorylate the MARK3 kinase domain. The inactive kinase domain of MARK3 (MARK3 KD-MBP IA) was copolymerized within the gel matrix to serve as substrates for samples containing putative upstream kinases. Lysates of different unstimulated cell lines - HeLa cells and COS-1 cells - were used as samples. The lysates were separated by electrophoresis. Kinase activity was determined by soaking the gel in radiolabelled ATP. An autoradiogram of the gel revealed radiolabelled bands corresponding to

kinases that phosphorylated the kinase domain of MARK3. Performing an in-gel kinase assay with MBP as a substrate served as a control to authenticate that potential upstream regulators phosphorylated the MARK3 kinase domain specifically but not the MBP tag. Therefore, any phosphorylation band appearing in both gels resulted either from MBP phosphorylation or autophosphorylation of a kinase and was excluded from further analysis. Phosphorylation bands that were detected only on the gel with co-polymerized MARK3 kinase domain, were most likely caused by upstream regulators of MARK3. The molecular weight of these bands indicated the apparent molecular weight of the putative upstream kinases.

In both lysates – Cos-1 (Fig.: 33, lane 1) and HeLa cells (Fig.: 33, lane 2) - a kinase of 60 kDa was identified, which specifically phosphorylated the kinase or UBA domain of MARK3 but not the MBP tag (Compare MARK3 KD-MBP IA Fig.: 33A with MBP Fig.: 33B).

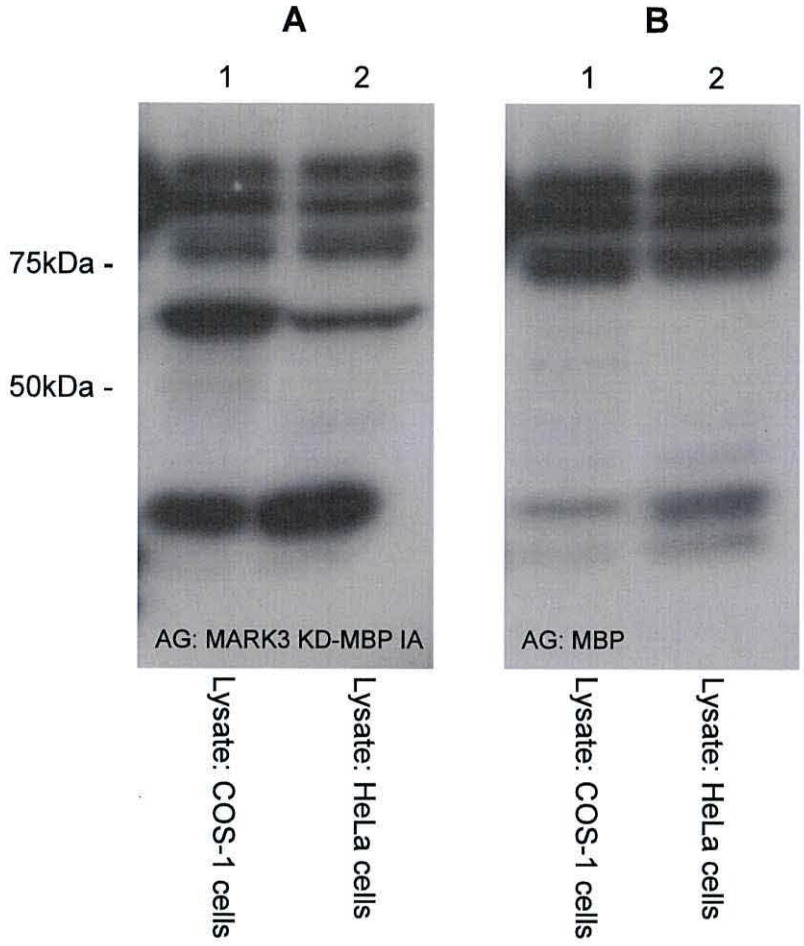


Figure 33: Identification of MARK3 upstream regulators
The in-gel kinase assay is a molecular tool to investigate kinase substrate relationships and was applied to identify protein kinases, which phosphorylate the kinase domain of MARK3. The inactivated kinase domain of MARK3 (MARK3 KD-MBP IA) was copolymerized in the gel matrix of a protein gel to serve as substrate for samples containing putative upstream kinases. The lysates of COS-1 cells and HeLa cells were applied as samples. Analysis of the autoradiogram (AG) of the in-gel kinase assay revealed that both lysates contained a kinase of 60 kDa, which significantly phosphorylated the kinase domain of MARK3. The respective phosphorylation signal was not detected for the control gel containing MBP as substrate. Identities of the samples are shown under the figures.

5.3 Discussion

Four upstream regulators of MARK3 have been identified to date, which have been shown to phosphorylate four of the 21 known MARK3 phosphorylation sites. Both MARKK (TAO-1), a kinase involved in mitosis regulation, MAPK signalling and apoptosis, and, the tumour suppressor master kinase of the AMPK family, LKB1 have been shown to activate MARK3 by phosphorylation of the phosphorylation loop. The oncogenic serine/threonine kinase PIM-1, involved in the development of various tumours, functions as a negative regulator of MARK3 by phosphorylating two serine residues within the kinase domain. Moreover, atypical kinase C is assumed to regulate MARK3 localisation by phosphorylation of the COOH-terminus within the KA1 domain (Hurov, Watkins et al. 2004). However, the protein kinases that phosphorylate the majority of the identified MARK3 phosphorylation sites and the regulatory consequences of these phosphorylations have so far remained elusive. Upstream regulators of MARK3 comprise oncogenic kinases, which inactivate MARK3 as well as tumour suppressor kinases, which activate MARK3. This regulation pattern implies an important role for MARK3 during tumour development and progression. In addition, three out of four known upstream regulators of MARK3 are assumed to be valuable therapeutic targets considering anti cancer drug development (Jin, Ying et al. 2008; Li et al. 2009; Thorac et al. 2009) Therefore, the identification of novel upstream regulators could not only help to confirm the participation of MARK3 in neoplasm formation but also bears the chance to identify a new potential target for novel cancer treatments.

The in-gel kinase assays revealed a putative MARK3 regulator of approximately ~60kDa for Cos-1 and HeLa cell lysates. Three of the four known upstream kinases of MARK3 - MARKK (~116 kDa) PIM-1 (~44 kDa) and LKB1 (~50 kDa) cannot be responsible for the phosphorylation signal since their molecular weight differs significantly from the molecular weight of the identified kinase. In addition, it might be worth mentioning for LKB1 that the kinase is only active when in complex with its co-factors MO25 (~40 kDa) and STRAD (~45 kDa) (Bass et al. 2003). aPKC (~ 67 kDa) the fourth known upstream regulator of MARK3 has approximately the same molecular weight as the identified kinase but was shown to phosphorylate the KA-1 domain of MARK3 and not the kinase domain (Hurov, Watkins et al. 2004). Hence, the identified kinase might be a novel MARK3 upstream regulator. The phosphorylation event seems not to be a cell line or a method-specific artefact, since the upstream kinase was detected for two different cell lines. In further experiments the identity of the kinase as well as the physiological meaning of the interaction has to be revealed in detail.

5.4 Future experiments and perspectives

The potential MARK3 upstream kinase should be identified in future experiments. For this purpose HELA as well as COS-1 cell lysates will be fractionated by ion exchange and hydrophobic interaction chromatography. The binding and the eluting fraction of each column will be analysed *via* an IGKA in which MARK3 KD-MBP IA serves as substrate for the presence of the potential MARK3 upstream kinase. Then,

the chromatography for the fractions containing the upstream regulator will be performed sequentially. Performing two chromatographies sequentially should result in a final fraction that is enriched for the upstream regulator of MARK3. This final fraction will then be used in a radioactive *in vitro* kinase assay in which kinase inactive MARK3 serves as substrate. The sample will be electrophretically size separated by SDS-PAGE and then analysed *via* autoradiography. Phosphorylation of MARK3 MBP-KD IA authenticates that MARK3 upstream kinases are present in the fraction. In addition, the gel will be silver stained to detect protein bands. The protein bands around 60 kDa will then be excised and analysed *via* mass spectroscopy. Identified kinases will be immunoprecipitated from cell extracts and tested in an *in vitro* kinase assay in which the inactive bacterially-expressed MARK3 fusion protein will serve as substrate. If the protein is confirmed to be a MARK3 kinase the phosphorylation site(s) will be determined by mass spectroscopy as well as conventional phosphopeptide mapping techniques and characterized considering its potential to regulate MARK3. To reveal if the identified kinase has an activating or inhibiting effect on MARK3, two consecutive kinase assays will be performed. In the first kinase assay, the upstream kinase will be allowed to react with MARK3. Then, MARK3 will be used for a second kinase assay in which the previously described KSR-1 fragment (KSR WT) serves as a substrate. MARK3 that has not been treated with the upstream kinase will be used as a standard in comparison to the kinase activity of the modified MARK3 proteins. Proteins mutated at the identified phosphorylation sites will also be included as a control. The proteins will then be separated by SDS-PAGE and the amount of incorporated radioactive [γ-^{32}P]

subsequently measured and quantitated by phosphor-imaging. Since known regulators of MARK3 comprise oncogenes or tumour suppressors, the identified regulators might be also involved in cancer development or progression, making them attractive anti-cancer therapy targets. To investigate the carcinogenic potential of the upstream kinase, soft agar assays will be used. These assays measure the potential for anchorage independent growth and are considered as stringent assays for detecting malignant transformation. If the identified protein kinase activates MARK3 in the above kinase assays, this protein may be considered a tumour suppressor. RNAi will be used to decrease its expression in the cells and analyse if this increases anchorage-independent growth. On the other hand, if the upstream kinase inactivates MARK3, this protein is a potential oncogene. Therefore, this protein will be overexpressed in the soft agar assay to demonstrate its transforming potential. These experiments will thus clearly demonstrate the direct implication of these proteins in cancer development, either as a promoter or suppressor of malignant transformation

Chapter VI: Final Discussion

MARK3 is a serine/threonine specific kinase, which belongs to the MARK group of kinases. In general MARK kinases are considered to regulate microtubule stability and thereby affecting cell polarity (Tassan and Le Goff 2004). However, so far MARK3 could not be implicated in cell polarity or microtubule stability but has been shown to participate in the regulation of crucial cellular functions such as cell signalling, cell adhesion, transcription and cell cycle regulation (Zhang, Kobayashi et al. 1997; Peng, Graves et al. 1998; Muller, Ory et al. 2001; Muller, Ritt et al. 2003; Dequiedt, Martin et al. 2006). Since these are cellular programs that are often deregulated in cancer development, MARK3 is assumed to participate in cell transformation. This assumption is sustained by the fact that upstream regulators of MARK3 comprise tumour suppressor as well as oncogenic kinases (Fields, Frederick et al. 2007; Shah, Pang et al. 2008). This project aimed to identify novel MARK3 substrates and upstream regulators to characterize more precisely the position of MARK3 within the cellular signalling network.

Identification of kinase substrate pairs is a challenging task, therefore, a combination of molecular techniques is often necessary to identify new kinase substrate interactions. Substrates of the kinase of interest for which association or phosphorylation motifs have been already described might help to choose a promising approach to identify novel kinase targets. However, this should be considered with caution since each kinase substrate relationship is unique. Therefore, three different

approaches - screening with solid phase phosphorylation, analysis of MARK3 complexes and a protein microarray - were applied to maximise the likelihood of identifying a novel MARK3 substrate. Each approach covered different characteristics, which were considered important in finding new MARK3 substrates.

Screening with solid phase phosphorylation as well as the human protein microarray are *in vitro* screening systems. These have the advantage that a large number of potential substrates can be screened at the same time and the substrates are immobilized. Therefore substrate pairs, which only transiently associate can be determined. While the human protein microarray analysis was restricted to approximately eight thousand potential substrates printed on one microarray chip, the screening with solid phase phosphorylation used a cDNA library to express the transcriptome of a specific tissue, which might contain proteins not present on the chip. In contrast to *in situ* phosphorylation screening and microarray technology MARK3 complex analysis has the advantages that potential interaction partners are native and co-factors such as adaptor proteins, which might be indispensible for the interaction, are present.

Screening with solid phase phosphorylation and MARK3 complex analysis did not reveal any MARK3 substrates. The probable reasons for the failure of these methods to detect novel MARK3 substrates are discussed in detail within the respective chapters. However, probing the protein microarray with MARK3 revealed two novel substrates namely KIFC3 and KLC4. This finding indicates a presently undescribed role for MARK3 in cellular transport and/or cellular transport-dependent functions.

Microarray data considering protein interactions has to be regarded with caution since *in vitro* protein interactions have only moderate *in vivo* significance. Therefore, association studies were performed authenticating *in vivo* interaction of the relevant proteins. *In vitro* phosphorylation combined with *in vivo* association make it more than likely that KIFC3 and KLC4 are *bona fide* MARK3 substrates. In addition, the interaction domain between MARK3 and KIFC3 could be localized within the COOH-terminal tail near the motor domain of KIFC3.

If KLC4 and KIFC3 are *bona fide* MARK3 substrates the physiological significance of this interaction has to be investigated. Since no data considering cellular functions of KLC4 are available at present, it is extremely difficult to draw any conclusions considering the physiological significance of the MARK3 KLC4 interaction. For KIFC3, three publications have recently been published, implicating KIFC3 in cell polarity and cell-cell adhesion respectively (Noda, Okada et al. 2001; Xu, Takeda et al. 2002; Meng, Mushika et al. 2008). Interestingly, in the past family members of the MARK group as well as upstream regulators of MARK3 have been shown to participate in these cellular programs. This gives rise to the assumption that MARK3 might also be involved in cell polarity and/or cell adhesion *via* KIFC3. Therefore, the MARK3 KIFC3 interaction might partially unravel how MARK3 upstream kinases such as aPKC and LKB-1, which are both master regulators of cell-cell adhesion and cell polarity, exhibit their cellular functions and how deregulation of these can lead to carcinogenesis respectively. Further experiments are necessary to support these hypotheses. However, an involvement of MARK3 in cellular polarization would corrobate the recently expressed opinion that all kinases of the MARK group are

involved in cell polarity and metabolism as members of the AMPK family but also perform isotype specific functions (Hurov et al. 2007). Besides identifying two undescribed MARK3 substrates, one potential novel MARK3 upstream kinase could be identified. This could be of substantial interest since known MARK3 upstream kinases are involved in tumour development and considered valuable therapeutic targets.

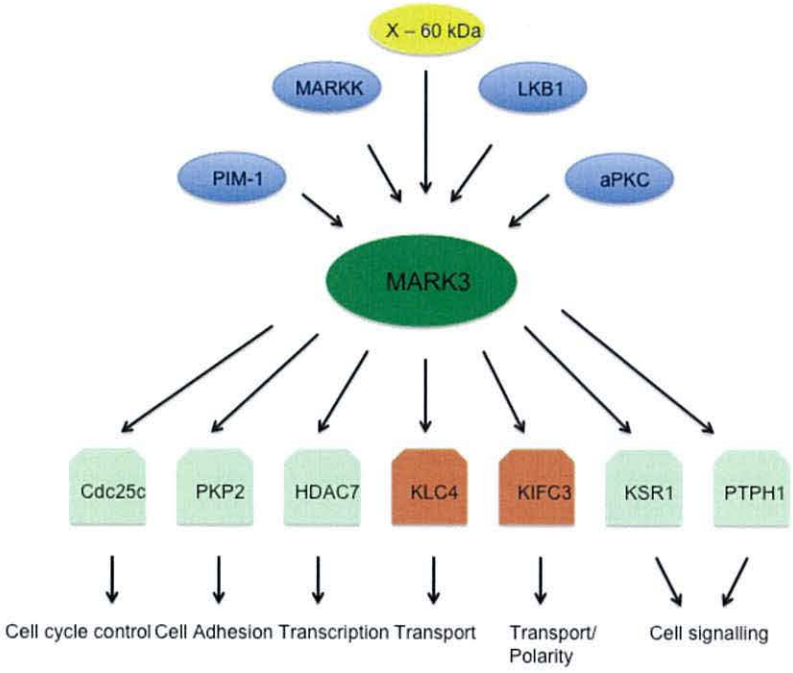


Fig. 34: MARK3 signalling network
MARK3 a serine/threonine specific kinase has been shown to participate in crucial cellular functions such as cell signalling, cell adhesion, cell cycle control and transcription reglation. The work presented in this thesis extends the known MARK3 signalling network by identifying two new MARK3 substrates - KLC4 and KIFC3 - and one novel MARK3 upstream regulator (x-60 kDa). The substrates involve MARK3 in protein trafficking and protein trafficking dependent functions respectively, such as polarity, respectively. Since most MARK3 upstream regulators are considered valuable therapeutic targets the identification of a new upstream regulator might be of substantial interest.

Appendices

Appendix A: Protein sequences

Microtuble affinity regulating kinase 3 (MARK3 AA 776 accession number: P27448)

MSTRTPLPTVNERDTENHTSHGDGRQEVTSRTSRSGARCRNSIAS
CADEQPHIGNYRLLKTIGKGNFAKVKLARHILTGREVAIKIIDKTQ
LNPTSLQKLFREVRIMKILNHPNIVKLFEVIETEKTLYLIMEYASGG
EVFDYLVAHGRMKEKEARSKFRQIVSAVQYCHQKRIVHRDLKAE
NLLLDADMNIKIADFGFSNEFTVGGKLDTFCGSPPYAAPELFQGK
KYDGPEVDVWSLGVILYTLVSGSLPFDGQNLKELRERVLRGKYRI
PFYMSTDCENLLKRFLVLNPIKRGTLEQIMKDRWINAGHEEDELK
PFVEPELDISDQKRIDIMVGMGYSQEEIQESLSKMKYDEITATYLL
LGRKSSELDASDSSSSSNLSLAKVRPSSDLNNSTGQSPHHKVQRSV
FSSQKQRRYSDHAGPAIPSVVAYPKRSQTSTADSDLKEDGISSRKS
SGSAVGGKGIAPASPMLGNASNPNKADIPERKKSSTVPSSNTASG
GMTRRNTYVCSERTTADRHSVIQNGKENSTIPDQRTPVASTHSISS
AATPDRIRFPRGTASRSTFHGQPRERRTATYNGPPASPSLSHEATP
LSQTRSRGSTNLFSKLTSKLTRRNMSFRFIKRLPTEYERNGRYEGS
SRNVSAEQKDENKEAKPRSLRFTWSMKTTSSMDPGDMMREIRKV
LDANNCDYEQRERFLLFCVHGDGHAENLVQWEMEVCKLPRLSL
NGVRFKRISGTSIAFKNIASKIANELKL*

Kinesin light chain 4 (KLC4 AA1-620 accession number: Q9NSK0)

MSGLVLGQRDEPAGHRLSQEEILGSTRLVSQGLEALRSEHQAVLQ
SLSQTIECLQQGGHEEGLVHEKARQLRRSMENIELGLSEAQVMLA
LASHLSTVESEKQKLRAQVRRLCQENQWLRDELAGTQQRLQRSE
QAVAQLEEEKKHLEFLGQLRQYDEDGHTSEEKEGDATKDSLDDL
FPNEEEEDPSNGLSRGQGATAAQQGGYEIPARLRTLHNLVIQYAA
QGRYEVAVPLCKQALEDLERTSGRGHPDVATMLNILALVYRDQN
KYKEAAHLLNDALSIRESTLGPDHPAVAATLNNLAVLYGKRGKY
KEAEPLCQRALEIREKVLGTNHPDVAKQLNNLALLCQNQGKYEA
VERYYQRALAIYEGQLGPDNPNVARTKNNLASCYLKQGKYAEAE
TLYKEILTRAHVQEFGSVDDDHKPIWMHAEEREEMSKSRHHEGG
TPYAEYGGWYKACKVSSPTVNTTLRNLGALYRRQGKLEAAETLE
ECALRSRRQGTDPISQTKVAELLGESDGRRTSQEGPGDSVKFEGG
EDASVAVEWSGDGSGTLQRSGSLGKIRDVLRRSSELLVRKLQGTE
PRPSSSNMKRAASLNYLNQPSAAPLQVSRGLSASTMDLSSSS*

Kinesin family member C3 (KIFC3 AA1-688 accession number: A8K6S2)

MVENERLRQEMRRCEAELQELRTKPAGPCPGCEHSQESAQLRDK
LSQLQLEMAESKGMLSELNLEVQQKTDRLAEVELRLKDCLAEKA
QEEERLSRRLRDSHETIASLRAQSPPVKYVIKTVEVESSKTKQALS
ESQARNQHLQEQVAMQRQVLKEMEQQLQSSHQLTARLRAQIAM
YESELERAHGQMLEEMQSLEEDKNRAIEEAFARAQVEMKAVHEN
LAGVRTNLLTLQPALRTLTNDYNGLKRQVRGFPLLLQEALRSVK
AEIGQAIEEVNSNNQELLRKYRRELQLRKKCHNELVRLKGNIRVI
ARVRPVTKEDGEGPEATNAVTFDADDDSIIHLLHKGKPVSFELDK
VFSPQASQQDVFQEVQALVTSCIDGFNVCIFAYGQTGAGKTYTME
GTAENPGINQRALQLLFSEVQEKASDWEYTITVSAAEIYNEVLRD
LLGKEPQEKLEIRLCPDGSGQLYVPGLTEFQVQSVDDINKVFEFGH
TNRTTEFTNLNEHSSRSHALLIVTVRGVDCSTGLRTTGKLNLVDL
AGSERVGKSGAEGSRLREAQHINKSLSALGDVIAALRSRQGHVPF
RNSKLTYLLQDSLSGDSKTLMVVQVSPVEKNTSETLYSLKFAERV
RSVELGPGLRRAELGSWSSQEHLEWEPACQTPQPSARAHSAPSSG
TSSRPGSIRRKLQPSA

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