Functional characterisation of the mammalian NDR1 and NDR2 protein kinases and their regulation by the mammalian STE20-like kinase MST3

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A) Tamaskovic, R., Bichsel, S.J., Rogniaux, H., Stegert, M.R. and Hemmings, B.A. (2003) Mechanism of Ca^{2+}-mediated regulation of NDR protein kinase through autophosphorylation and phosphorylation by an upstream kinase. *J Biol Chem*, **278**, 6710-18.

B) Bichsel, S.J., Tamaskovic, R., Stegert, M.R. and Hemmings, B.A. (2004) Mechanism of activation of NDR (nuclear Dbf2-related) protein kinase by the hMOB1 protein. *J. Biol. Chem.*, **279**, 35228-35.

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ABBREVIATIONS

Frequently used abbreviations are listed below; other abbreviations are defined within the text.

ATP	adenosine triphosphate
cAMP	cyclic andenosin monophosphate
CDK	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitors
cGMP	cyclic guanosine monophosphate
CREB	cAMP responsive element-binding protein
EGF	epidermal growth factor
ER	endoplasmatic reticulum
ERK	extracellular signal regulated kinase
GCK	germinal centre kinase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
KRS	kinase responsive to stress
LATS	large tumour surpressor
MAPK	mitogen-activated protein kinase
MASK	Mst3 and Sok related kinase
MOB	Mps one binder
MST	mammalian STE20-like
NAD	nicotinamiddinucleotide
NDR	nuclear Dbf2-related
PAK	p21 activated kinase

PKB	protein kinase B
SGK	serum glucocorticoid kinase
PRMT	protein arginine methyltransferase
SOK	STE20 like oxidative stress response kinase
SUMO	small ubiquitin-related modifier
STAT	signal transducer and activator of transcription
YSK	yeast SPS1/STE20-related kinase

SUMMARY

Protein modification is a common regulatory mechanism in order to transduce a signal from one molecule to another. One of the best-studied protein modifications is phosphorylation. The enzymes that are capable of transferring phosphate groups onto other proteins are called protein kinases. Depending on the acceptor group, kinases can be distinguished into tyrosine, serine/threonine and dual-specificity kinases. This work describes the characterisation of human and mouse NDR1 and NDR2 kinases, members of the AGC group of serine/threonine kinases. The NDR protein kinase family is highly conserved between yeast and human, and several members have been shown to be involved in the regulation of cell morphology and the control of cell cycle progression. For example, the yeast NDR kinases Sid2p (Schizosaccharomyces pombe) and Dbf2p (Saccharomyces cerevisiae) are central components of the septation-initiation network and the mitosis exit network, respectively. The closest yeast relatives Cbk1p and Orb6p, members of the regulation of Ace2p transcription and morphogenesis network and Orb6 signalling pathways, are implicated in the coordination of cell cycle progression and cell morphology. This study, as well as studies using worms and flies, provide evidence that not only NDR is conserved, but also the NDR signalling pathway and regulation. Similar to yeast, NDR kinase activation is regulated by phosphorylation at the activation segment phosphorylation site and the hydrophobic motif phosphorylation site. This phosphorylation is regulated by a conserved signaling module consisting of MOB proteins and a STE20–like kinase. Here we show that the STE20-like kinase MST3 activates NDR by phosphorylation specifically at the hydrophobic motif *in vitro* and *in vivo*. Furthermore, MOB1A binding is important for the release of autoinhibition and full kinase activation. The data also indicate that NDR is part of a feedback mechanism, which induces cleavage and nuclear translocation of MST3. The data presented here also show that NDR1 and NDR2 are differentially expressed, but regulated in a similar manner. Mouse *Ndr1* mRNA is mainly

expressed in spleen, thymus and lung, whereas *Ndr2* mRNA is more ubiquitously expressed, with the highest levels in the gastrointestinal tract. Both, NDR1 and NDR2, are activated by S100B protein and okadaic acid stimulated phosphorylation; NDR1 and NDR2 are also indistinguishable in the biochemical assays used: membrane targetting, phosphorylation by MST3, and activation by MOB. Further, this work describes the generation and initial characterisation of a mouse model for NDR1 deficiency. Protein analysis using NDR1 knockout mouse embryonic fibroblasts suggest a compensation of the loss of NDR1 by upregulation of NDR2 expression.

I. GENERAL INTRODUCTION

1. MECHANISMS OF SIGNAL TRANSDUCTION

(This first chapter gives a short overview about the current textbook knowledge signal transduction (see Gomperts *et. al.*, 2002; Krauss *et al.*, 1997) and serves as a general introduction to the thesis.)

Multicellular organisms rely on coordinated interactions between organs and cells. In order to ensure a well-ordered course of events during development and in the mature organism, animals developed a variety of forms of intercellular signalling. Bioactive molecules (e.g. peptides, steroids, retinoids, nucleotides or amino acids) can be released from one type of cell, and provoke timely, coordinated responses in target cells. Depending on the range of signalling events, this can be classified as endocrine (long-range; signalling over long distances throughout the whole body), paracrine (short-range; signalling to neighbouring cells) and autocrine (self; sending cell can also be receiving cell) signalling.

Communication between neighbouring cells can also occur via “gap junctions”. Gap junctions are channels which directly link two neighbouring cells and allow the direct exchange of metabolites or signalling molecules. Another form of cell-cell interaction is direct interaction of cells via cell surface proteins. Lastly, cells can also communicate via electrical processes and/or neurotransmitters.

Typically, signals in a sender cell are initiated by a mostly external trigger signal, which is then transported or transduced to a target cell (which can be the sender cell itself). There, the signal is received by a receptor protein, and then subsequently converted into a sequence of biochemical or electrical reactions. Signalling pathways are often regulated by mechanisms, which allow the termination or attenuation of the signal.

Intercellular signalling occurs typically by the release of bioactive compounds, via diffusion or exocytosis, into the extracellular space. The molecules (ligands) either diffuse into target cells or bind to specific receptors at the cell surface. Alternatively, cell surface proteins can directly interact with each other. The binding of ligands induces conformational changes in the receptor protein, which result in a dimerisation/oligomerisation and enzymatic activation of the receptor, or the recruitment, association or release of other molecules/proteins or domains. For instance, ligand-induced oligomerisation of receptor tyrosine kinases such as PDGF (platelet derived growth factor) or ERB receptors results in an autophosphorylation and activation of the intracellular domain (Pawson *et al.*, 2002); serine/threonine kinase receptors, such as the BMP (bone morphogenetic protein) or TGF (transforming growth factor) receptors, are activated by ligand binding driven heterodimerisation of type I and II receptors (Piek *et al.*, 1999); and activation of NOTCH proteins by their ligands (JAGGED, DELTA or SERRATE) trigger the release of the intracellular domain by two steps of proteolytic cleavage (Lai, 2004).

Components of the intracellular signalling are low molecular weight substances, so-called "second messenger" molecules, or proteins. Second messenger molecules are either stored or released from intracellular organelles (e.g. Ca^{2+}), or can be created or removed by enzymatic reactions (e.g. cAMP, cGMP, inositoltrisphophate, phosphatidyl-inositol-phosphates or diacylglycerol). Their binding to effector molecules leads to the rapid and local activation or inhibition of signalling enzymes.

There are various methods for the transduction of signals by proteins. For example, proteins can function as "adaptor proteins". Adaptor proteins serve as "bridging molecules" between signalling components. These proteins play an important role in regulating the co-localisation of signalling components by creating a close proximity between proteins, and therefore enable an effective and specific transduction of signals. Adaptor proteins often contain specific

modular domains which recognise specific protein sequences or modifications. Src-homology 2 (SH2) or phosphotyrosin-binding (PTB) domains recognise specific phospho-tyrosine-containing protein sequences. SHC (Src-homology 2/α-collagen) contains both SH2 and PTB domains, which link the EGF (epidermal growth factor) receptor protein with the GRB2 (growth factor receptor bound protein 2) adaptor protein (Downward, 1994; Tari and Lopez-Berestein, 2001). SH3 domains recognise proline-rich sequences. For example, the SH3 domain of CRK (CT10 regulator of kinase) interacts with the ABL (Abelson) tyrosine kinase (Donaldson *et al.*, 2002); PDZ domains (Post-synaptic density/Discs-large/ZO1 domain) are modular protein interaction domains that are specialised for binding to short peptide motifs at the extreme carboxy termini of other proteins, although they can also have other modes of interaction. Their target proteins are frequently transmembrane receptors or ion channels. PDZ-containing proteins often result in the assembly of large protein complexes at specific subcellular localisations (Hung and Sheng, 2002). For example, erbin bridges the ERBB2 receptor and G proteins such as RAS or RHO (Kolch, 2004).

Proteins can also function as activator or inhibitor proteins, which signal by inducing conformational changes of themselves or of their target proteins. Differential expression of activator or inhibitor proteins can regulate the activity of target molecules. For example, CYCLINS and CKI's regulate the cell cycle progression by specifically stabilizing or inducing an active or inactive conformation of CDK's, which drive the cell forward to the next phase of the cell cycle (Li and Blow, 2001).

Ligand binding can also induce conformational changes, which activate or inhibit signalling molecules. Seven-transmembrane domain receptors (also called serpentine receptors) such as the β-adrenergic receptor or the glucagon receptor are G-protein coupled receptors that cross the plasma membrane seven times. Upon ligand binding in the extracellular domain, the

intracellular domain changes into an active conformation and recruits and activates heterotrimeric G-proteins (Pierce *et al.*, 2002).

Binding of the second messenger ligands also controls the conformation of regulatory GTPases, which include monomeric and heterotrimeric GTPases. GTP binding induces an active conformation, whereas bound GDP stabilises the inactive conformation. Guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and GDP dissociation factors (GDIs) can regulate the activity of the GTPases. In the active state, GTPases modulate effector protein: for example RAF kinase is recruited by active RAS·GTP, and adenylate cyclase activity is regulated by the α-subunits of heterotrimeric G-proteins (Bhattacharya *et al.*, 2004).

One of the most common ways of transducing signals to downstream components of signalling pathways is protein modification. Enzymes regulate the activity, localisation and/or affinity of downstream signalling molecules by posttranslational alteration of one or more amino acid residues via introducing a covalent bond with another chemical subunit. There is a wide variety of protein modifications; some of the most prominent of these are outlined in the next section.

2. SIGNAL TRANSDUCTION BY PROTEIN MODIFICATIONS

The regulation of proteins in the long term depends mostly on their expression levels. However, the complexity of multicellular organisms requires fast changes in enzymatic activities or binding affinities in order to react to environmental or intrinsic changes. One of the best-studied strategies of acute regulation is the use of posttranslational protein modifications. The covalent modification of proteins by removal or addition of chemical subunits alters the properties of the protein, or targets proteins to different subcellular

complexes. This chapter outlines the features of some of the most common modes of modification.

2.1 PROTEOLYTIC CLEAVAGE

The removal of peptide sequences by enzymatic cleavage can have multiple effects on proteins. Some proteins are synthesized as inactive precursors, so call proproteins that are activated by proteolysis. Caspases are one example of this. Procaspases reside as latent precursors in cells. Initiator caspases (e.g. caspase-2, -8, -9 or -10) are activated upon dimerisation, and then cleave and activate effector or executioner caspases (e.g. caspase-3 or -7) (Boatright and Salvesen, 2003), which subsequently cleave their substrates. For example, caspase-mediated cleavage of ERK2 results inactivation of the kinase (Marchetti *et al.*, 2004). In contrast, caspase cleavage of RAF kinase or ROCK1 results in a constitutively active kinase (Cornelis *et al.*, 2005; Sebbagh *et al.*, 2001). Proteolytic cleavage can alter not only enzyme activity: in several cases it also plays a role in the localisation of proteins.

NOTCH proteins undergo several steps of proteolytic cleavage during the activation process. Processing of synthesized NOTCH in the *trans*-Golgi network by furin proteases is constitutive and necessary for signalling in mammals. Upon ligand binding, NOTCH is sensitised to cleavage of the extracellular domain by extracellular proteases from the ADAM/Kuzbanian family. This induces a further processing step by γ-secretases, which enables the NOTCH intracellular domain (NICD) to translocate to the nucleus. There, it associates with CBF-1 (CREB binding factor1) and MAM (mastermind), and leads to the activation of target genes (Schweisguth, 2004).

2.2. GLYCOSYLATION

The attachment of carbohydrates to proteins is called glycosylation. Glycosylation can occur on oxygen, nitrogen or carbon atoms of proteins. This modification mainly takes place while

the proteins are being transported from the ER through the Golgi apparatus to the plasma membrane. Glycosylation of cell surface proteins plays an important role in cell-cell communication, maintenance of the cell structure and self-recognition by the immune system. Carbohydrate modifications are also involved in protein transport in the secretory pathway, or the membrane linkage of proteins via glycosylphosphatidylinositol (GPI) anchors. For example, the importance of carbohydrate structures for self-recognition by the immune system is shown in the ABO blood group antigens: different glycosylation patterns of the antigen are recognised by antibodies (Morgan and Watkins, 2000). The modification of proteins in the secretory pathway is required for proper sorting of the proteins; e.g. a mannose-6-phosphate modification targets proteins to the lysosome (Scheiffele and Fullekrug, 2000). The affinity of protein-protein interactions can also be regulated by glycosylation. The level of O-fucosylation of serine and threonine residues of the NOTCH EGF repeats determines the affinity for its ligand DELTA (Schweisguth, 2004).

Cytoplasmic and nuclear proteins are also described as targets of glycosylation. O-GlcNAc modifications of serine residues are involved in numerous processes such as nuclear transport (e.g. nucleoporin), transcription (e.g. RNA polymerase II), macromolecular assembly processes (tau proteins, prions) or protein stability (p^{67}) (Van den Steen *et al.*, 1998).

2.3. METHYLATION

The methylation of proteins occurs on lysine and arginine residues or carboxy groups. Methylation often alters the affinity of proteins for each other. One of the most prominent cases of methylation is histone methylation. The lysine methylation of histones regulates the transcription of specific chromosomal loci. Histone methylation is often associated with the transcriptional repression of chromosomal regions: histone H3-K9 promotes the recruitment of HP1 (heterochromatin protein 1) and leads to the propagation of heterochromatin. H3-K27

methylation is recognized by the Polycomb repressor complex, which mediates repression at the gene level in euchromatic regions. In contrast, H3-K4 methylation impairs methylation of H3-K9, thereby keeping the chromatin in a transcriptionally active state (Sims 3rd *et al.*, 2003).

Arginine methylation has also been shown to regulate protein-protein interactions. Recent studies have suggested that arginine methylation is involved in a variety of processes, including RNA processing, transcription and polyadenylation, regulation of cytoskeleton proteins, signal transduction and DNA repair (Boisvert *et al.*, 2003). For instance, arginine methylation of STAT1 by the protein methyltransferase PRMT1 is required for the cellular interferon response (Mowen *et al.*, 2001).

The RAS protein can exemplify carboxymethylation. The CAAX motif of RAS is endoproteolytically cleaved after the attachment of the prenyl-group. Subsequent carboxymethylation of the cysteine creates a hydrophobic moiety, which allows binding to the membrane (Maurer-Stroh *et al.*, 2003). Another example for carboxyl group methylation is the catalytic subunit of PP2A. The methylation of the C-terminal leucine is important for the association of the PR55/B regulatory subunit (Evans and Hemmings, 2000)

2.4 ACETYLATION

Proteins can be acetylated on amino-terminal residues or the ε-amino group of lysine residues. Amino-terminal acetylation occurs on the majority of eukaryotic proteins during the translation process. Some proteins require acetylation for activity or stabilisation, but for most proteins amino-terminal acetylation has no apparent biological significance (Polevoda and Sherman, 2002). Regulatory peptides and hormones are also acetylated at the amino terminus. This posttranslational modification is important for regulating the biological activity of peptides and hormones (e.g. α-melanocyte-stimulating factor) (Fu *et al.*, 2002). The most

studied proteins that are acetylated are histones. Acetylation on lysine residues decreases the positive charge of histone tail structures, and therefore weakens the DNA-histone binding, which results in a greater accessibility for transcriptional complexes. Histone acetylation is regulated by histone acetylases (HATs) and histone deacetylases (HDACs) (Eberharter and Becker, 2002). Non-histone chromosomal proteins such as HMG (high mobility group) proteins are also subject to acetylation, which is thought to be important for their binding to distorted DNA (Ugrinova *et al.*, 2001). Several transcription factors are also well-characterised targets of protein acetylation. Acetylation can alter their DNA binding ability (e.g. in the cases of E2F1, p53, EKLF (erythroid Krueppel-like factor) or HNF-4 (hepatocyte nuclear factor)), their protein interactions (e.g. in the cases of c-JUN, TCF (T-cell factor) or HNF-4) or their localisation (HNF-4) (Polevoda and Sherman, 2002). Acetylation is also implicated in the regulation of nuclear import by modifying import factors such as RCH1/IMPORTIN-α (Bannister *et al.*, 2000). In recent years, much effort has also been put towards understanding the role and regulation of α-TUBULIN acetylation. Acetylation is mostly associated with stable microtubule structures, and is thought to influence cell motility (Westermann and Weber, 2003).

2.5. UBIQUITINATION

Ubiquitination is a conserved, reversible, posttranslational modification that results in the covalent attachment of ubiquitin to the ε-amino group of a lysine residue on the target molecule. This multi-step process requires the coordinated activity of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) (Pickart, 2001). Polyubiquitin chains consisting of Lys48-Gly76 polymers target proteins for ATP-dependent proteolysis by the 26S proteasome, while Lys63-Gly76 chains modulate protein function or label proteins for destruction via non-proteasome-dependent mechanisms (Sun and Chen,

2004). In contrast, monoubiquitination has been linked to receptor endocytosis, lysosomal and peroxisomal degradation of proteins, virus budding, transcription, DNA repair or caspase recruitment or modulate protein function (Lee and Peter, 2003).

2.6. SUMOYLATION

Sumoylation is the reversible, posttranslational modification of proteins on lysine residues with the ubiquitin-like SUMO protein. The mechanisms of sumoylation and ubiquitination are similar. The SUMO conjugation pathway also involves the E1, E2 and E3 enzymes. SUMO acceptor sites contain a conserved ΨKXE (Ψ is a large hydrophobic amino acid) site. In contrast to ubiquitination, sumoylation has not been associated with protein degradation, but is also involved in the regulation of protein activity and localisation (Gill, 2004). Sumoylation is used to regulate protein translocation (e.g. RAN·GAP1 or adenoviral E1B), transcription (e.g. SP3, HSF1 and 2 or TEL), DNA replication and repair (e.g. PCNA or topoisomerase I) and chromosome segregation (Seeler and Dejan, 2003).

2.7. PHOSPHORYLATION

Protein phosphorylation involves the transfer of a phosphate group from an energetic phosphate donor (mostly ATP) to specific phosphoacceptor sites on a protein. The reaction is generally catalysed by protein kinases, but another mode of protein phosphorylation involving inositol-pyrophosphates has been described recently (York *et al.*, 2004). Phosphorylation is a reversible protein modification. The dephosphorylation reaction is catalysed by protein phosphatases or can occur by hydrolysis. The phospho-status of proteins is tightly regulated by the relative activities of protein kinases and protein phosphatases. Protein phosphorylation occurs mostly on serine, threonine or tyrosine residues. However, protein phosphorylation has also been reported on histidine and aspartate residues. For example, the two-component systems in bacteria (e.g. *Bacillus subtilis*) consist of a His-Asp relay network that transfers

phosphate from histidine to aspartate. However, due to the chemical properties these modifications are very unstable (Oka *et al.*, 2002; Wolanin *et al.*, 2002). In mammals histidine kinases are also known for many years, but little is still known about their biological functions (Besant and Attwood, 2005).

Protein phosphorylation is one of the best-studied protein modifications, and is involved in almost all cellular processes ranging from transcription, replication, translation, splicing and protein degradation to the regulation of cell cycle progression, cell morphology and many more. The modification of proteins with a phosphate group can alter protein localisation. For example, the localisation of the Forkhead transcription factor FKHRL1 (FOXO3a) is regulated by phosphorylation through PKB and SGK (Brunet *et al.*, 2001; You *et al.*, 2004). Via phosphorylation of serine and threonine residues, FKHRL1 transcription is modified or abolished and FKHRL1 translocates to the cytoplasm.

Phosphorylation can also determine the localisation of proteins to specific protein complexes. Phosphorylation of the scaffolding molecule DOK-R by c-SRC leads to the co-recruitment of the SRC family kinase inhibitory kinase CSK to the EGF receptor, and results in an attenuation of EGF signalling (Van Slyke *et al.*, 2005). Phosphorylation of CREB by protein kinase A allows the recruitment of the coactivator protein CBP (CREB-binding protein) and enables transcription at CRE sites (Cardinaux *et al.*, 2000).

Phosphorylation signals are often used by adaptor proteins to link signalling proteins to each other in order to regulate signalling cascades or biological responses (Cherezova *et al.*, 2002). For example, SH2 or PTB domains are used to bind specific phospho-tyrosine residues of target molecules.

The dimerisation of proteins can also be regulated by phosphorylation. For example, tyrosine phosphorylation STAT5 induces the formation of protein dimers, which allows subsequent translocation of the dimer to the nucleus, where it modulates the expression of target genes

(Imida and Leonard, 2000). Another effect of phosphorylation is the change of the electrostatic potential of proteins: phosphorylation adds a negative charge to proteins, which can result in the decrease or increase in protein affinities. For example, phosphorylation of the C-terminal domain (CTD) of RNA polymerase II is important for the promoter to release the polymerase. Unphosphorylated RNA polymerase is preferentially bound to the promoter. After initiation of transcription, kinases such as CDK7 phosphorylate the CTD and release the polymerase from the initiation complex (Prelich, 2002).

Phosphorylation can influence protein stability. Phosphorylation of β-CATENIN on Ser21, Thr102 and Thr112 by casein kinase II destabilises the protein by targeting it to the ubiquitin-proteasome pathway (Bek *et al.*, 2005). But phosphorylation can also have a stabilizing effect on proteins. After activation, RHO-associated coiled-coil forming kinase (ROCK I) phosphorylates the GTPase-deficient RHO E on Ser11 and stabilises the protein (Riento *et al.*, 2005).

Protein phosphorylation is also a common way of altering enzyme activities. The phosphorylation of enzymes can alter their structure towards an active or inactive conformation. For example, the phosphorylation of type 1 and type 2 phosphatases on their C-terminal regulatory domain is reported to inhibit the phosphatase activity (Brautigan, 1995). In contrast, phosphorylation of the regulatory subunit of ATP-Mg-dependent protein phosphatase inhibitor-2 on Thr72 by MAPK or GSK3 results in an activation of the phosphatase (Wang *et al.*, 1995). Inactivation of glycogen-synthase is associated with a multiple phosphorylation of the enzyme (Nielsen and Wojtaszewski, 2004). Protein kinases are not only performing phosphorylation, they are a class of enzymes which is regulated by phosphorylation. Phosphorylation of kinases is essential for their activity. However, in some cases phosphorylation of regulatory domains also has an inhibitory role. For example, CDK4 is activated by activation segment phosphorylation, but inhibited by phosphorylation on

Tyr17 (Ekkholm and Reed, 2000). Protein kinases often form signalling cascades where one kinase transduces a signal by phosphorylation and activation of another protein kinase. For example, MAPKs are activated by a conserved kinase subfamily, the MAPK kinases (MAPKK), which are in turn phosphorylated and activated by MAPKK kinases (Schaeffer and Weber, 1999). This thesis describes the phosphorylation and activation of NDR by an upstream kinase in detail in the results section.

2.8. OTHER MODIFICATIONS

Apart from the modifications listed above, many more modifications have been described. For example, adenylation is an important step in the ubiquitin transfer cascade for the ubiquitin-like protein NEDD8 (Walden *et al.*, 2003). Prenylation, myristoylatation palmitoylation, farnesylation and geranyl-geranyl modifications are lipid modifications which help proteins associate with membranes (Magee and Seabra, 2005). Sulfatation of tyrosine residues in selectin increases its binding affinity to sialyl-Lewis X antigen (Van den Steen *et al.*, 1998). Deamidation of proteins is thought to serve as a molecular clock for protein turnover, ageing and development using the intrinsic instability of asparagines and glutamine residues (Robinson and Robinson, 2001a,b). Biotinylation of proteins increases their affinity to the cell surface of monocytes and granulocytes (Storm *et al.*, 1996). Formylation occurs mostly as modification of the initiator methionine in bacteria (Ramesh *et al.*, 2003). Vitamin K-dependent proteins require carboxylation of glutamyl residues for their biological activity as regulators of bone morphogenesis, haemostasis and growth (Berkner and Pudota, 1998). ADP-ribosylation of proteins is known to be involved in the modulation of the immune response (e.g. modification of the human neutrophil protein (HNP1)) (Corda and Di Girolamo, 2002); and poly-ADP-ribosylation plays an important role in DNA repair (Oei *et al.*, 2005). Oxidative stress marks itself by oxidation and hydroxylation of proteins. However, there are a

few more modifications, e.g. pyroglutamylation, selenocysteine and selenomethionine, but a detailed coverage of all of them is not the aim of this study. This study mainly focuses on the effect of phosphorylation and protein binding on NDR kinase activity and function.

2.9. THE CONCEPT OF 'SIGNALLING PATHWAYS'

Protein modifications and protein-protein interactions are the basis for most signaling pathways. Signalling pathways transduce an extrinsic and/or intrinsic signal via consecutive signalling events (protein modifications, complex formation, second messenger binding etc.) to downstream effectors in order to change a transcriptional or physiological output. This section exemplifies the concept of signaling pathways using the PI3K/PKB and the JAK/STAT signalling pathway.

2.9.1. THE PI3K/PKB KINASE SIGNALLING PATHWAY

Upon stimulation with growth factors such as PDGF, EGF or IGF-1 the corresponding growth factor receptors (PDGFR, ErbB, or IGF1-R) homo- and/or hetero dimerise and get activated by autophosphorylation (Tallquist *et al.*, 2004; Roskoski, 2004, Adams *et al.*, 2004). The phosphorylated receptor tyrosine kinase recruits phosphoinositide 3-phosphat-kinase (PI3K) to the receptor, either directly or via the phosphorylation and recruitment of adaptor molecules such as insulin receptor substrate (IRS) proteins or SHC/GRB2/GAB (Wymann and Marone, 2005). The recruitment of PI3K results in the increase of phosphoinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) levels at the membrane. The opposing player for PtdIns(3,4,5)P_3 production is the 3'-phosphoinositide phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) (Sulis and Parson, 2003). Increasing levels of PtdIns(3,4,5)P_3 result in the recruitment of several phosphoinositide binding domain (eg. pleckstrin homology domain) containing proteins to cellular membranes (Cozier *et al.*, 2004). Within those PKB and its activation segment upstream kinase PDK1 (phosphoinositide

dependent kinase) are recruited to the membrane, which facilitates phosphorylation of PKB by PDK1 (Meier and Hemmings, 1998). Furthermore, membrane recruited PKB is phosphorylated at the C-terminal hydrophobic motif phosphorylation site by mTOR/rictor (Sarbassov *et al.*, 2005) or DNA-PK (Feng *et al.*, 2004). The C-terminal hydrophobic motif phosphorylation is impeded by the binding of inhibitory proteins such as CTMP1 (C-terminal modulator protein) or TRB3 (tribble homologue 3) (Maira *et al.*, 2001; Du *et al.*, 2003), which therefore prevent activation of PKB. Furthermore, PKB also is negatively regulated by protein phosphatase 2A, which is able to dephosphorylate and inactivate the kinase (Andjelkovic *et al.*, 1996). Activated PKB itself transduces the signal to several downstream components of the signalling pathway by phosphorylation. Phosphorylation of target molecules such as FOXO transcription factor, IκB kinase, hTERT, p21CIP p27KIP, PDE3B, PFK2, RAC, eNOS , NUR77, IRS-1, TSC2, GSK3, CHFR, MDM2, MYT1, CREB, BRCA-1, B-RAF or C-RAF results in multiple cellular responses encompassing protein synthesis, cell metabolism, cell proliferation and cell survival (Brazil and Hemmings, 2001; Brazil *et al.* 2004; Hay, 2005). However, this is only a simplified presentation of the PI3K/PKB signaling pathway, the real picture is still under development. Many more molecules have been identified (eg. Actin, Periplakin, JIP1, POSH or ERK1/2) and likely will be identified, which bind to PKB or affect PKB activity (Brazil *et al.*, 2004). Similarly, bioinformatic approaches as well as peptide library screens just point out the potential multitude of PKB substrates (Obata *et al.*, 2000; Obenauer *et al.*, 2003). (For illustration of the PI3K/PKB signalling pathway see Figure 1 of Brazil *et al.*, 2004).

2.9.2 THE JAK/STAT SIGNALLING PATHWAY

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway is another well studied pathway that transduces signals of cytokines and growth factors in order to alter the transcriptional response of the cell. Cytokines such as interferons and interleukins

bind to their receptors and induce multimerisation of receptor subunits (O'Shea *et al.*, 2002). This allows the activation and transphosphorylation of two neighbouring receptor associated JAKs, which subsequently phosphorylate STATs. This phosphorylation can be assisted STAT interacting proteins (StIP), which serve as adaptors (Aaronson and Horvath, 2002). Phosphorylated STATs dimerise through the interaction of SH2 domains and are translocated to the nucleus, where they alter the transcription of target genes such as MYC, NOS (nitrogen oxide synthase), p21CIP, NMI (N-Myc interacting protein), BCL2-Xi (B cell lymphoma 2 Xi) or SOCS (suppressor of cytokine signalling) (Rawlings *et al.*, 2004). SOCS itself is a negative regulator of the JAK/STAT signaling pathway, which modulate the signal intensity in a negative feedback loop. SOCS binds to phosphotyrosin residues at the receptor and inhibits STAT recruitment. Furthermore SOCS recruits E3-ligases which target JAKs and receptors for ubiquitination and subsequent degradation by the proteasome (Alexander, 2002). Other negative regulators of the JAK/STAT pathway are tyrosine phosphatases such as SHP-1 (SRC homology region 2 containing phosphatase), which reverse the activation of JAKs by dephosphorylation of the kinase. (Rawlings *et al.*, 2004). STAT proteins are also negatively regulated by the binding of protein inhibitors of activated STATs (PIAS), which prevent the binding of STATs to DNA. Furthermore PIAS is reported to have E3 conjugase activity which results in the sumoylation of STATs (Rogers *et al.*, 2003). This again, only gives a simplified view on the JAK/STAT signalling pathway, an few more molecules have been described to affect this signalling pathway (e.g. STAM (signal transducing activator molecule), PKCδ or IMPORTIN α-5) (Rawlings *et al.*, 2004). (For illustration of the JAK-STAT signaling pathway see Figure 1 of Rakesh and Agrawal, 2005).

In general, almost every step of signalling cascades is regulated by multiple modes to ensure the correct signal intensity and output. This includes cross talks of different signalling cascades and feedback regulatory loops, which contribute to the complexity of cellular signal

transduction observed in cells and organisms. Here we describe first steps towards the characterisation of the MOB/NDR signaling pathway, namely the identification of its upstream kinase MST3.

3. PROTEIN KINASES

Protein phosphorylation is one of the most widespread and well-studied signalling mechanisms in eukaryotes, and is involved in the control of many, if not all, cellular processes. The extent of protein phosphorylation is reversibly controlled by the activity of protein kinases and protein phosphatases, the 'Yin and Yang' of protein phosphorylation (Hunter, 1995).

Comparisons of the protein kinase complements ('kinomes') of yeast, worm, fly and human revealed that many kinase families are conserved from yeast to human. These kinases mediate mainly unicellular functions. However, metazoan-specific kinase families and groups (tyrosine kinase and tyrosine kinase-like) exist. This expansion during metazoan evolution is most likely due to the increased complexity of signalling in multicellular organisms in order to control cell-cell communication, development and differentiation (Manning *et al*., 2002a).

The eukaryotic protein kinases are classified into several groups: AGC (containing cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and protein kinase C (PKC)), CAMK (Ca^{2+}/calmodulin-dependent protein kinase), CK1 (casein kinase 1), CMGC (containing cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK3) and Cdc2-like kinase (CLK) families), STE (homologues of yeast sterile 7, 11 and 20-like kinases), TK (tyrosine kinase), TKL (tyrosine

kinase-like), RGC (receptor guanylate cyclase), 'Atypical', the 'Other' group, the worm-specific Fer and the yeast Hal group of protein kinases (Manning *et al.*, 2002a).

3.1 THE HUMAN KINOME

The human kinome consists of AGC, STE, CK1, CMGC, TK, TKL, Atypical and 'the Other' groups of protein kinases. Despite the fact that that the human kinome contains 518 kinases compared to 454 worm, 239 fly and 130 yeast kinases, only 13 kinase families are unique for humans. This indicates that most of the large-scale divergence of the kinase families had already occurred in their most recent common ancestor (Manning *et al*., 2002a). The conservation of protein kinase families between different organisms allows us to transfer conclusions gained by studying lower organisms to the mammalian systems. This work makes extensive use of this conservation and describes a conserved interaction between STE20-like kinases and NDR kinases. The conservation of protein kinases not only allows vertical transfers of information between organisms, but also horizontal transfers between members of kinase families and groups. A common feature of almost all protein kinases (except several Atypical protein kinases) is the structure of their catalytic domain.

3.2. STRUCTURE OF PROTEIN KINASES

The superfamily of eukaryotic protein kinases (ePKs) shares a conserved catalytic domain. This conservation is marked by a high degree of similarity in their primary structure, which is thought to result in a similar tertiary structure (Hanks and Hunter, 1995). The primary structure of the catalytic domain can be further divided into twelve subdomains which contain regions of higher homology (Hanks and Quinn, 1991). Subdomains I-IV form the smaller N-terminal lobe, which is responsible for the binding of Mg^{2+}·ATP. The N-terminal lobe consists of a five-stranded antiparallel β-strand: subdomain I – a β-strand-turn-β-strand with a conserved glycine-rich loop – is important for anchoring ATP to the protein; subdomain II – a

β-sheet with an α-helical extension – contains the invariant catalytic lysine which interacts with and stabilises the α- and β-phosphates of ATP; subdomain III – a large α-helix – contains a glutamate residue which stabilizes the interaction of the catalytic lysine with ATP; subdomain IV is also a β-strand. Subdomain V contains a hydrophobic β-strand and chain that connects the two lobes of the catalytic domains, which anchor ATP to the kinase and are important for substrate recognition. The larger C-terminal lobe consists mainly of α-helical structures encompassing subdomains V-VII and is involved in substrate binding and catalysis: it consists of the α-helical part of subdomain V; subdomain VIA which is a large α-helix; two hydrophobic β-strands connected by the so-called catalytic loop which form subdomain VIB and contain important residues (D-X-K-X-X-N motif) for the catalysis of the phosphate transfer to the substrate; and subdomain VII also has a β-strand-loop-β-strand structure containing the DFG motif which plays a role in orientating the γ-phosphate of ATP by chelating the Mg^{2+} which bridges the β- and γ-phosphate of ATP. Subdomain VIII contains a chain encompassing the so-called T- or activation loop, followed by the APE motif which has an important function in stabilising the large lobe of the kinase; a large α-helix forms subdomain IX and encompasses residues responsible for substrate recognition; the α-helix and the helix loop helix structure of subdomains X and XI, respectively, conclude the large lobe of the kinase (Hanks and Quinn, 1991). Most protein kinases share this primary structure similarity. However, several protein kinases of the 'Atypical' group are known that lack this sequence similarity, but several of them are shown to have structural similarities (Manning *et al.*, 2002b).

3.3 REGULATION OF SERINE/THREONINE KINASES

A common regulatory element of protein kinase activity is phosphorylation of the kinase activation segment. Phosphorylation on additional phosphorylation sites can stabilize the

kinases in an active or inactive conformation. Kinase activity is often also regulated by additional regulatory elements such as binding to coactivators or inhibitory proteins. For example, CYCLINS bind to one side of the catalytic cleft of CDKs and induce a large conformational change in the T-loop of the kinase (Jeffrey *et al.*, 1995). In contrast, binding of the inhibitor protein p27KIP1 to the cyclin A-CDK2 complex rearranges the amino lobe of the kinase and interacts with the catalytic cleft by mimicking ATP binding (Russo *et al.*, 1996). Also, the localisation of serine/threonine kinases plays an important role in the activation process of protein kinase. For example, adaptor molecules such as NOD1 mediate the interaction with RIP (receptor interacting protein)-like interacting caspase-like apoptosis-regulatory protein kinase (RICK) and IκB kinase (IKK) and form specific signalling modules (Inohara *et al.*, 2000). Several lines of evidence suggest that cellular membranes are an active compartment for kinase activation and phosphorylation. For example, the binding of GTP-bound GTPases such as RAS, RHO or RAC results in a membrane recruitment and activation of RAF, ROCK or PAK kinases, respectively (Wennerberg and Der, 2004). Several kinases are recruited to cellular membranes by their lipid-binding domain. For example, PKC is activated by membrane recruitment induced by binding to diacylglycerol (DAG) or phorbol esters (Brose and Rosenmund, 2002). PKB (also termed AKT) is translocated to the membrane by binding of the pleckstrin homology (PH) domain to phosphatidylinositol 3,4,5-trisphosphate lipid molecules, which leads to the subsequent phosphorylation and activation of the kinase (Brazil and Hemmings, 2001; Brazil *et al.*, 2004). This study characterizes the activation of NDR protein kinase by binding to coactivator protein MOB and by membrane targeting mediated hydrophobic motif phosphorylation of the kinase mediated by a STE20-like kinase.

4. THE STE20 GROUP OF PROTEIN KINASES

The STE20 group of protein kinases is related to the budding yeast Ste20p (sterile 20) protein. In mammals, this group comprises the p21-activated kinases (PAKs) and the germinal centre kinases (GCKs).

4.1. GENERAL FEATURES OF STE20-LIKE KINASES

The STE20 group of protein kinases is implicated in the regulation of apoptosis, cell shape and cell motility. The STE20 group kinases show conservation within their kinase domains, but are structurally extremely diverse in their noncatalytic domain. PAK-I contains an N-terminal SH3 domain, an autoinhibitory domain, a CDC42/RAC-1-binding domain and a Cool/Pix binding motif (a region which is important for recruitment to focal adhesions) and the C-terminal kinase domain. The N terminus of PAK-II subfamily kinases contains a CDC42/RAC-1 binding domain as well, but this shows a higher affinity to CDC42 than to RAC-1. The GCK kinases have their kinase domain at their N terminus. GCK-I kinases have several prolin-rich repeats, a citron homology domain and a conserved C-terminal extension. A C-terminal autoinhibitory sequence and a dimerisation domain are characteristic of GCK-II kinases; the C terminus of GCK-III kinases is short and does not contain defined domains. The C terminus of GCK-IV kinases contains a citron homology domain; GCK-V kinases have an AT1-46 homology domain. GCK-VI kinases have a conserved C-terminal region of unknown function; the special feature of GCK-VII kinases is a myosin light chain domain adjacent to the kinase domain, and a C-terminal calmodulin-binding domain. GCK-VIII kinases contain a long central conserved region of unknown function (Dan *et al.*, 2001). Several of the STE20 group kinases (PAK2, HPK1 (haematopoetic progenitor kinase) and MLK3 (mixed lineage kinase) are thought to function as MAP kinase kinase kinase kinases

(MAP4K). Many STE20 group kinases, such as the GCK-VIII kinases, also function as MAP kinase kinase kinases (MAP3K) (Dan *et al.*, 2001). Interestingly, STE20 group kinases are also involved in the regulation of apoptosis: several of them are cleaved by caspases. This study describes a newly discovered interaction between MST3, a STE20 group kinase subfamily kinase, and NDR kinases.

4.2. THE MAMMALIAN STE20-LIKE FAMILY OF PROTEIN KINASES

Mammalian STE20-like kinases (MST) are members of the STE20 group of serine/threonoine kinases, relatives of the yeast sterile 20 (Ste20p). Their closest relatives in budding/fission yeast, Cdc15p and Kic1p/Nak1p, are components of the mitosis exit network (MEN)/septation initiation network (SIN) or regulation of Ace2p and morphogenesis (RAM) network.

MST kinases belong to the germinal centre kinase (GCK) subfamilies II (MST1, MST2) and III (MST3, MST4 (MASK) and YSK1 (SOK1)). They share a common domain structure, a very short N terminus followed by the catalytic domain and a C-terminal regulatory domain. MST kinases (apart from YSK1) are reported have one or two caspase cleavage sites adjacent to their kinase domain. The C terminus of GCK-II kinases contains a dimerisation domain and a nuclear export sequence (NES). The C terminus of GCK-III kinases is less conserved, but at least MST3 is also known to bear a C-terminal NES. Figure 1 shows the domain structure of MST kinases.Cleavage of the kinases by caspases results in an increased activity and a nuclear translocation of the N-terminal part of the kinase (Lee *et al.*, 2001; Ura *et al.*, 2001; Dan *et al.*, 2002; Huang *et al.*, 2002).

MST kinases are implicated in the regulation of apoptosis (De Souza *et al.*, 2004) and cell migration (Preisinger *et al.*, 2004). MST1 is known to be responsible for apoptotic phosphorylation of histone 2B (Cheung *et al.*, 2003). The phosphorylation of histones is associated with a condensation of the chromatin and DNA fragmentation (Cheung *et al.*,

2003). Furthermore, MST1 activity causes cardiomyopathy by inducing cardiac myocyte apoptosis (Yamamoto *et al.*, 2003) and induces eosinophil apoptosis (De Souza *et al.*, 2002). MST1 and MST2 are both activated and cleaved in response to chemical stress or heat shock (Taylor *et al.*, 1996). However, no direct upstream activators of MST1 or MST2 are known so far, but RAS association domain family protein 1A (RASF1A), novel RAS effector 1A (NORE1A) and RAS keep MST1 kinase in a moderately active form and might be involved in recruiting the kinase to the site of activation; in agreement membrane recruitment of MST1 or NORE1A results in activation of the kinase (Praskova *et al.*, 2004). Similarly, MST2 kinase activation and dimerisation is inhibited by RAF-1 kinase and decreases the sensitivity to programmed cell death (O'Neill *et al.*, 2004).

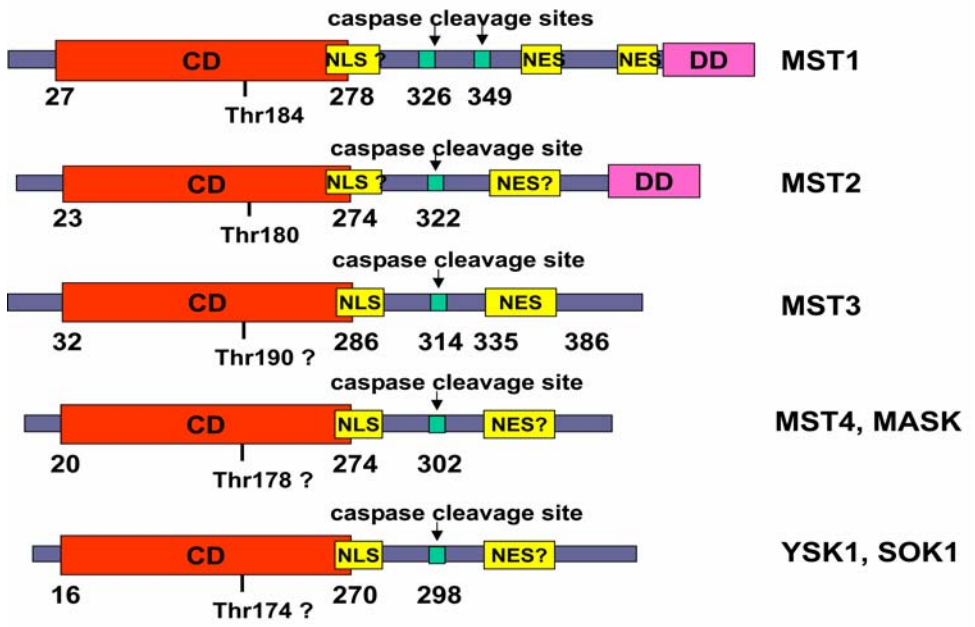

Figure 1. Domain organisation of mammalian STE20-like kinases.

The catalytic domain (CD), dimerisation domain (DD), nuclear localisation signal (NLS) and nuclear export sequence (NES) are highlighted by boxes. The amino acid number of the catalytic domain, activation segment phosphorylation site and caspase cleavage site are indicated.

Hippo, the MST1/2 orthologue in *Drosophila melanogaster*, couples cell proliferation and cell death.(Harvey *et al.*, 2003; Jia *et al.*, 2003; Pantalacci *et al.*, 2003; Udan *et al.*, 2003; Wu *et al.*, 2003).

MST3 is also thought to play a role in the induction of apoptosis (Huang *et al.*, 2002). Interestingly, MST3 shows an unusual cofactor preference and is autophosphorylated in the presence of Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} (Schinkmann and Blenis, 1997; Lu *et al.*, 2005).

MST4 and YSK1 bind to GM130, a Golgi-matrix protein, and have functions in cell motility and polarisation (Preisinger *et al.*, 2004). MST4 shows apoptosis-inducing properties, but is also thought to affect prostate cancer progression and cell transformation (Lin *et al.*, 2001; Dan *et al.*, 2002; Sung *et al.*, 2003). Activation of YSK1 requires oxidant stress and high levels of Ca^{2+} (Pombo *et al.*, 1997). However, the signalling and function of GCK-III kinases in worms, flies and mammals has been rather poorly studied, so far.

Recent studies in yeast, flies and human indicate that MST kinases are part of a conserved signalling module, together with NDR family kinases and MOB proteins (Mps one binder). This study shows for the first time that human MST3 activates an NDR kinase by hydrophobic motif phosphorylation.

5. THE AGC GROUP OF PROTEIN KINASES

The AGC group of protein kinases include the protein kinase B (PKB), glucocorticoid receptor kinase (GRK), dystrophia myotinica protein kinase (DMPK), nuclear Dbf2 related (NDR), microtubule-associated protein kinase (MAST), ribosomal protein S6 kinase (RSK), protein kinase A (PKA), phosphoinosited-dependent kinase 1 (PDK1), protein kinase C (PKC), protein kinase G (PKG), protein kinase N (PKN), RSK-like (RSKL) and yet another novel kinase (YANK) family kinases (Manning *et al.*, 2002b).

5.1 GENERAL FEATURES OF AGC-GROUP KINASES

AGC group kinases share similar structural features. Two structural lobes form the catalytic domain, with the ATP binding site in between. Phosphorylation of the activation segment

phosphorylation site bridges the α-C helix with the catalytic and Mg^{2+} positioning loops and stabilizes the active conformation. Most AGC kinases are phosphorylated by PDK1 at their activation segment (Biondi, 2004).

Another feature of all AGC kinases, except PDK1, is the C-terminal hydrophobic motif. This C-terminal region folds back onto the catalytic domain of the kinase and binds to a hydrophobic pocket in the small lobe of the kinase (Biondi, 2004). Most of the AGC kinases are regulated by phosphorylation of the hydrophobic motif, which results in an order to disorder transition of the α-C and α-B helices, stabilizing the active conformation of the kinase. However, a few kinases, such as PRK2, have constitutively ordered α-C and α-B helices, where the phosphorylation is mimicked by an aspartate mutant and, additionally, stabilizing amino acid residues (Yang *et al.*, 2002; see also results section). The N terminus of AGC group kinases also contains a regulatory domain, such as the PH domain (in the case of PKB), cAMP (in the case of PKA) or cGMP (in the case of PKG) binding domain, Ca^{2+} binding domain or phorbol ester binding sites (in the case of PKC). These N-terminal domains are often responsible for recruitment of the kinases to their sites of phosphorylation and activation. These studies describe the regulation of NDR family kinases, which differ from other AGC group kinases in several respects.

5.2 THE NDR KINASE FAMILY

The NDR kinase family comprises the fission yeast kinases SID2 and ORB6, the budding yeast kinases DBF2, DBF20 and CBK1, the *Caenorhabitis elegans* kinases SAX-1 and LATS (T20F10.1), the *Drosophila melanogaster* kinases TRC and WARTS, and the mammalian kinases NDR1, NDR2, LATS1 and LATS2. Within the AGC group of protein kinases, NDR kinase shows some special features regarding structure, function and regulation (Tamaskovic *et al.*, 2003b).

5.2.1. STRUCTURE OF NDR PROTEIN KINASES

A special feature of NDR kinases is their specific domain organisation. NDR kinases have an N-terminal regulatory S100B and MOB association (SMA) domain, which binds to S100B and/or MOB co-activator proteins (Millward *et al.*, 1998: Bichsel *et al.*, 2004). Adjacent to the SMA domain is the catalytic domain, which contains an insert between subdomains VII and VIII. This insert contains a basic sequence which resembles the peptide substrate sequence of NDR and probably acts as a pseudosubstrate or autoinhibitory sequence (AIS) (Bichsel *et al.*, 2004; see also results section). Furthermore, NDR kinases contain two major regulatory phosphorylation sites: the activation segment phosphorylation site (Ser281 in NDR1); and a C-terminal hydrophobic motif phosphorylation site (Thr444 in NDR1). Figure 2 shows the domain organization of NDR family kinases. LATS kinases have an elongated N terminus compared to NDR kinases. It contains glutamine- and proline- (flies) or proline-rich (human LATS) stretches.

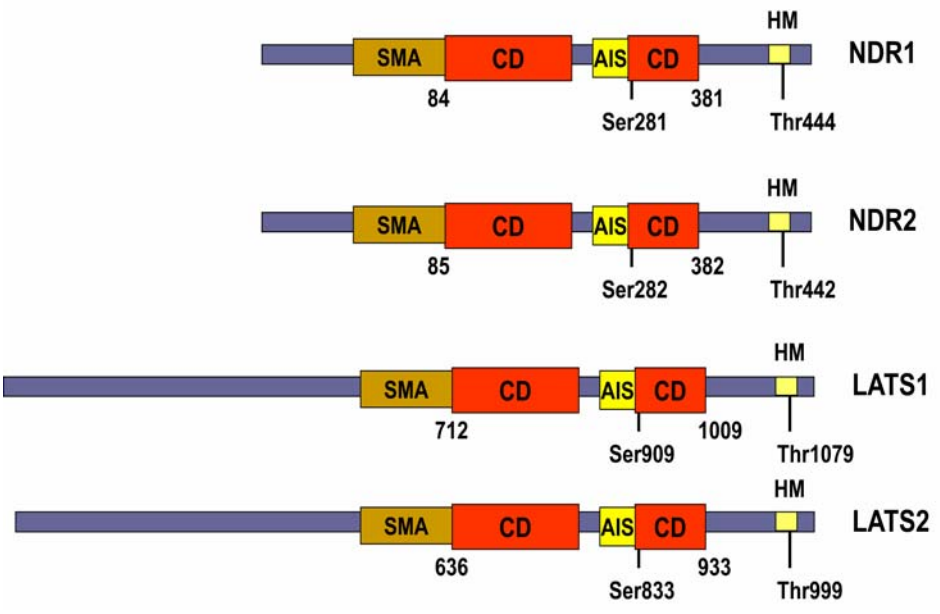

Figure 2. Domain organization of the human NDR protein kinases.

The catalytic domain (CD), S100B and MOB association (SMA) domain, autoinhibitory sequence (AIS), and the hydrophobic motif (HM) are highlighted by boxes. The amino acid number of the catalytic domain, activation segment phosphorylation site and hydrophobic motif phosphorylation site are indicated.

5.2.2. FUNCTIONS OF NDR PROTEIN KINASES

NDR protein kinases are highly conserved kinases, known in organisms ranging from plants and fungi to euplotes and animals. This section introduces some of the best-characterized NDR kinases. The conservation of NDR kinases and NDR signalling pathways is illustrated in table 1.

Table 1: NDR kinase signalling pathways

a) The LATS pathway

Organism	*Saccharomyces cerevisiae*	*Saccharomyces pombe*	*Caenorhabditis elegans*	*Drosophila melanogaster*	*Homo sapiens*
NDR kinase	**DBF2, DBF20**	**SID2**	**WARTS/T20F10.1**	**WARTS/LATS**	**LATS1/WARTS, LATS2/KPN**
Upstream kinase	**CDC15**	**SID1+CDC7**	**(F14H12.4a)**	**HIPPO**	**MST1/KRS2, MST2/KRS1**
Coactivator	**MOB1**	**MOB1**	**(F38H4.10) (F09A5.4d) (T12B3.4)**	**DMOB1/MATS**	**MOB1A,(B), (MOB3A,B,C)**
Scaffold	NUD1	CDC11+SID4	**(CBG02143)**	**SALVADOR**	**hWW45/ SAV1**
GTPase	**TEM1**	**SPG1**	**(RAB family GTPase)**	**(RAB family GTPase)**	**(RAB family GTPase)**

b) The NDR pathway

Organism	*Saccharomyces cerevisiae*	*Saccharomyces pombe*	*Caenorhabditis elegans*	*Drosophila melanogaster*	*Homo sapiens*
NDR kinase	**CBK1**	**ORB6**	**SAX-1**	**TRI-CORNERED**	**NDR1,2**
Upstream kinase	**KIC1**	**NAK1**	**(GCK-1)**	**(RE38276p)**	**MST3, (MST4, YSK1)**
Coactivator	**MOB2**	**MOB2**	**(T12B3.4) (F38H4.10) (F09A5.4d)**	**DMOB1/MATS DMOB2, DMOB3, 4**	**MOB1A,B, MOB2 (MOB3A,B,C)**
Scaffold	*PAG1/TAO3*	*MOR2/CPS15*	**SAX-2**	**FURRY**	**(AF4p12)**
Interactor	**HYM1**	**MO25**	**(Y53C12A.4)**	**(DMO25)**	**(MO25α,β)**
Methyl-transferase	**(HSL7)**	**SKB1**	**(C34E10.5)**	**(CAPSULEEN)**	**PRMT5**

The table indicates the conservation of components of the LATS and NDR signalling pathways from yeast to mammals. Orthologous proteins are in bold. However, evidence for an involvement of some of the conserved components in LATS/NDR kinase signalling is still missing, as indicated by the brackets. Slashes indicate synonymous names.

5.2.2.1.FUNGAL NDR KINASES

The budding yeast NDR family kinases DBF2 and DBF20 are components of the mitosis exit network (MEN). Mutations of DBF2 cause cells to arrest in anaphase because of an elongated spindle and a large "dumbbell" morphology (Johnston *et al*., 1990). DBF2 activity and localisation are regulated in a cell cycle-dependent manner. Active DBF2 is localised to the spindle pole bodies in anaphase (Toyn and Johnston, 1994; Visintin and Amon, 2001). The MEN plays an important role in the inactivation of mitotic CDKs and the completion of cytokinesis (Bardin and Amon, 2001).

The NDR kinase CBK1 is a central component of the regulation of ACE2 transcription and morphogenesis (RAM) network (Nelson *et al*., 2003). CBK1 mutants grow as large cell aggregates and have round cell morphology (Racki *et al*., 2000). Furthermore, the apical growth, mating projection formation and bipolar budding pattern is altered in CBK1 mutants (Bidlingmaier *et al*., 2001). Similar to DBF2, CBK1 shows a cell cycle-dependent localisation (Colman-Lerner *et al*., 2001; Weiss *et al*., 2002). CBK1 is required for the appropriate localisation of the ACE2 transcription factor, which is required for the expression of cyclin3 (CLN3), glucanase (SCW11) and chitinase (CTS1) (O'Conallain *et al.*, 1998; Colman-Lerner *et al*., 2001; Laabs *et al.*, 2004). CBK1 also controls the polarized apical growth and mating projection formation in an ACE2-independent manner (Colman-Lerner *et al*., 2001).

The MEN corresponding network in fission yeast is the septation initiation network (SIN). The NDR kinase of the SIN is SID2 (Bardin and Amon, 2001), which is involved in the coordination of mitosis exit and cytokinesis (McCollum and Gould, 2001). SID2 is cell cycle-regulated in terms of activation and localisation and is required for actinomyosin ring constriction and septum formation after chromosome separation (Balasubramanian *et al.*, 1998; Sparks *et al.*, 1999; Bardin and Amon, 2001).

The orthologous pathway of the RAM is the ORB6 signalling pathway. ORB6 regulates cell morphology and cell cycle progression. ORB6 mutants are spherical and do not have a bipolar growth pattern, which is associated with disorganised microtubule and actin cytoskeleton networks. Furthermore, entry in mitosis is accelerated (Verde *et al.*, 1998). ORB6 delays the onset of mitosis by regulating CDC2 activity. Similar to CBK1, ORB6 kinase activity and localisation is regulated in a cell cycle-dependent manner (Verde *et al.*, 1998).

5.2.2.2.WORM NDR KINASES

In *Caenorhabditis elegans*, two NDR kinases are known: a so far uncharacterised WARTS kinase, and SAX-1 protein kinase. SAX-1 mutants show morphological alterations in chemosensory and mechanosensory neurons. The neurons show reduced cell spreading and increased neurite outgrowth as well as a dentritic branching and tiling phenotype (Zallen *et al*., 2000; Gallegos and Bargmann, 2004). Usually mechanosensory PML neurons undergo phases of active growth and maintenance growth. These growth phases are tightly regulated by cell-cell signalling. The lack of growth inhibition in the SAX-1 signalling pathway causes an overlap of neighbouring dendrites (Gallegos and Bargmann, 2004).

5.2.2.3.FLY NDR KINASES

Drosophila melanogaster has two NDR family kinases, the WARTS (WTS) kinase and TRICORNERED (TRC). WARTS is an orthologue of human LATS kinase. WARTS was identified in a screen for overproliferation mutants that are lethal in early developmental stages (Justice *et al.*, 1995; Xu *et al.*, 1995) and is a tumour suppressor in flies. WARTS mutant cells have an aberrant cell shape and clones are round or spherical. Furthermore, WARTS is thought to regulate apoptosis by regulating DIAP (Drosophila inhibitor of

apoptosis) protein levels via regulating the transcriptional activity of YORKIE (YKI), a transcriptional coactivator protein (Huang *et al.*, 2005b).

TRICORNERED (TRC) kinase is about 70% identical to human NDR kinase. TRC mutations are organismal lethal. Clonal knockouts of TRC of cuticular structures such as wing hairs, bristles, lateral extensions of the arista or the larval denticle show a splitting and branching phenotype of these cellular extensions (Geng *et al.*, 2000). Furthermore, TRC is proposed to interact with the actin cytoskeleton, because cytochalasin D or latrunculin A, inhibitors of actin polymerisation, partially phenocopy the TRC mutants. However, TRC does not affect actin polymerisation or bundling itself, but is important for the fine regulation of actin bundles and is thought to be a part of a morphogenetic checkpoint (Geng *et al.*, 2000). Similar to the worm NDR kinase, TRC clonal mutants in sensory neurons show a dentritic branching and tiling phenotype. TRC mutants have excessive terminal branching, and homologous dentrites overlap due to a failure in repulsion (Emoto *et al.*, 2004).

5.2.2.4.MAMMALIAN NDR KINASE

Four NDR family kinases exist in mice and humans: LATS1(hWARTS) and LATS2(KPM), as well as NDR1 and NDR2. LATS1 kinase, as is its *Drosophila* orthologue WARTS, is a tumour suppressor (St. John *et al*., 1999). Mice lacking LATS develop soft tissue sarcomas and ovarian stromal cell tumours, mammary gland development is impaired, they are infertile and growth is retarded (St. John *et al*., 1999). Similar to their yeast relatives, the activity and localisation of LATS are regulated during the cell cycle. Interestingly, LATS1 is localised to the centrosome in interphase cells and translocates to the spindle during metaphase and anaphase and to the midbody in telophase (Nishiyama *et al.*, 1999). LATS1 is reported to restrict cell cycle progression and to promote apoptosis by regulating cyclin A and B as well as BAX levels (Yang *et al*., 2001; Xia *et al.*, 2002). Furthermore, phosphorylated LATS1

interacts directly with CDC2 and inhibits its activity (Tao *et al*., 1999). LATS1 also ensures genomic integrity and cytokinesis by regulating mitotic cell cycle progression and inhibiting LIMK1 (Iida *et al.*, 2004; Yang *et al*., 2004).

LATS2 kinase is also implicated in restriction of proliferation and promotion of apoptosis (Kamikubo *et al.*, 2003). LATS2 inhibits G1/S transition via downregulation of cyclinE/CDK2 activity (Li *et al.*, 2003). Interestingly, the localisation to centrosomes and phosphorylation of LATS2 by AURORA-A shows a cell-cycle dependency similar to what is known from NDR family kinases in lower organisms (Toji *et al*., 2004). Taken together, both LATS1 and LATS2 control cell proliferation and apoptosis, but there might also be LATS1- or LATS2-specific functions.

In contrast to the LATS kinases, very little is known about the mammalian NDR1 and NDR2 kinases. Both *Ndr1* (chromosome 6p21) and *Ndr2* (chromosome 12p11) are located in regions that have been described as cancer amplicons (Manning *et al*., 2002a). *Ndr1* mRNA is consistently upregulated in ductal carcinoma in situ, with intraductal necrosis and bad prognosis regarding progression to invasive tumours compared to DCIS without intraductal necrosis (Adeyinka *et al*., 2002). Human *Ndr2* is upregulated in the highly metastatic non-small cell lung cancer cell line NCI-H460 (Ross *et al*., 2000). The murine *Ndr2* gene was found to be interrupted in two independent B-cell lymphomas generated by retroviral insertional mutagenesis (Suzuki *et al*., 2002).

Furthermore, *Ndr2* mRNA has been found upregulated in the mouse amygdala during fear memory consolidation. It is also worth noting that NDR2 expression in PC12 cells results in decreased cell spreading and alterations in neurite outgrowth (Stork *et al*., 2004). This points to common functions of worm, fly and mammalian NDR kinases in controlling neuronal morphology.

Recently, NDR1 and NDR2 have also been identified in HIV-1 viral particles. The viral protease cleaves off the C-terminal hydrophobic motif of NDR1 and NDR2 kinases, which leads to an inhibition of NDR kinase activity, indicating that NDR kinase might affect the viral lifecycle (Devroe *et al*., 2005).

Recent data from our laboratory using NDR-deficient mouse embryo fibroblasts and stable cell lines overexpressing NDR kinase also indicate a role of NDR in the regulation of cell cycle progression, genomic integrity and cell death (Tamaskovic *et al.*, in prep.).

5.2.3. REGULATION OF NDR PROTEIN KINASES (part of a review in preparation for *Nature Reviews Molecular Cell Biology*)

A common regulatory element of kinase activity is phosphorylation. NDR kinases, like other AGC group kinases, contain two major regulatory phosphorylation sites: the activation segment (AS) phosphorylation site (Ser281 for human NDR1); and the hydrophobic motif (HM) phosphorylation site (Thr444 in human NDR1). In contrast to other AGC group kinases, where PDK1 is the AS kinase (Biondi, 2004), the NDR kinase activation segment is regulated by autophosphorylation, whereas the hydrophobic motif is targeted by an upstream kinase (Tamaskovic *et al.*, 2003a). The importance of HM phosphorylation for AGC group kinases is well illustrated by the structure of activated PKB (Yang *et al.*, 2002). Phosphorylation results in a structural ordering of the α-C helix. This enables an interaction of the helix with the activation segment phosphorylation site, which restructures the activation segment in an active conformation. Phosphorylation and activation of NDR and LATS kinase can be induced by treatment of cells with okadaic acid, a potent inhibitor of phosphatase 2A (PP2A) (Millward *et al.*, 1999). Mimicking this phosphorylation by replacing the HM of NDR with the PIFtide sequence (the HM phospho mimic derived from PRK2), results in a constitutively

active NDR kinase (Stegert *et al.*, 2004; this study). Furthermore, PP2A negatively regulates the yeast MEN network (Wang and Ng, 2005).

In the past few years, studies in yeast and flies have demonstrated that STE20-like kinases genetically interact with and phosphorylate NDR kinases. CDC15 in budding yeast is required for phosphorylating and activating the NDR kinases DBF2 (Mah *et al.*, 2001). *Drosophila* Hippo, an MST1/2 orthologue, phosphorylates WARTS, a LATS orthologue. Furthermore, human MST1 and MST2 phosphorylate LATS1 and LATS2 *in vitro* (Chan *et al.*, 2005).

MST3 is the first STE20-like kinase which has been shown to specifically phosphorylate the NDR kinase hydrophobic motif, but not the activation segment, providing a mechanism for the interaction of STE20-like kinases with NDR kinases (see results section). The localisation of NDR kinase and STE20-like kinase is critical for NDR kinase activation, and provides a tool to coordinate cellular events in a temporal and spatial manner (Visintin and Amon, 2001; Hergovich *et al.*, 2005; see also results section). Interestingly, STE20-like kinases are also activated by PP2A inhibition (Kakeya *et al.*, 1998; Praskova *et al.*, 2004; see also results section). The close collaboration between NDR family kinases and MST kinases points to a common role of these kinases in the regulation of cell cycle progression, apoptosis and morphology.

Another mode of regulation of NDR kinases is the binding of a coactivator to the N terminus of the kinase. MOB proteins were shown to genetically and physically interact with NDR kinases in yeast, flies and man. MOB proteins bind to the N-terminal S100B and MOB association domain (SMA) of NDR kinase (Bichsel *et al.*, 2004). In yeast MOB proteins are important for the activation of NDR kinases (Hou *et al.*, 2000, 2002, 2004; Mah *et al.*, 2001; Weiss *et al.*, 2002). In budding and fission yeast there are two MOB proteins, MOB1 and MOB2 proteins, which show a specific binding to DBF2/SID2 and CBK1/ORB6 respectively. In contrast, in flies and humans DMOB1(MATS) and hMOB1 proteins bind and activate both

NDR and LATS kinases (Bichsel *et al.*, 2004; Devroe *et al.*, 2004; He *et al.*, 2005a; Lai *et al.*, 2005), whereas MOB2 binds preferentially to NDR1/2 kinase (Bothos *et al.*, 2005; He *et al.*, 2005a). So far, little is known about the other MOB proteins, but genetic evidence indicates that they also play a role in NDR kinase regulation (He *et al.*, 2005a). The binding of MOB1 releases NDR kinases from autoinhibition mediated by the AIS within the kinase domain insert (Bichsel *et al.*, 2004; see also results section). The binding of MOB proteins is dependent on the interaction of its negatively charged surface with the basic-hydrophobic N terminus of NDR kinases (Stavridi *et al.*, 2003; Bichsel *et al.*, 2004; Ponchon *et al.*, 2004). However, the exact binding mode and affinity differs between different MOB proteins (Devroe *et al.*, 2004; He *et al.*, 2005a). MOB1 protein from okadaic acid-treated cells show a higher affinity to NDR kinases than MOB1 from untreated cells, indicating that posttranslational modifications of MOB proteins might also have an impact on their affinity to NDR kinase (Bichsel *et al.*, 2004). Interestingly, a MOB relative phocein is reported to interact with the PP2A B'''-subunits straitin, zinedin and SG2NA (Baillat *et al.*, 2001; Moreno *et al.*, 2001). Therefore, MOB protein binding to PP2A might explain the low NDR kinase activity in unstimulated cells. (Millward *et al.*, 1999; Stegert *et al.*, 2004; see also this study).

Furthermore, MOB proteins are thought to activate the kinase by the disruption of an inhibitory self-association of the kinase molecules (Hou *et al.*, 2004). MOB proteins are also important for NDR localisation (Luca *et al.*, 2001; Weiss *et al.*, 2002; Hou *et al.*, 2004). Fission yeast MOB1 is required for spindle pole and division site localisation of Sid2p, which is thought to enable the kinase to regulate cytokinesis (Hou *et al.*, 2004). Budding yeast MOB2 and CBK1 are interdependent in terms of localisation (Weiss *et al.*, 2002) to the nucleus and cortical sites. Interestingly, the localisation of human NDR1 and NDR2 and LATS1 and LATS2 to cellular membranes by membrane targeting results in a constitutive

activation of the kinase (Hergovich *et al.*, 2005; Hergovich unpub. results). Recruitment of MOB proteins to cellular membranes co-recruits NDR and results in full phosphorylation and activation of the kinase (Hergovich *et al.*, 2005). Inducible membrane recruitment of NDR kinase via MOB also provides evidence that HM phosphorylation is dependent on MST kinases (see results section).

However, it is as yet unclear what drives the localisation of NDR or MOB to cellular membranes *in vivo*. MOB and STE20-like kinases are common element of both NDR and LATS kinase activation. Other components of the pathway might be specific for either NDR or LATS.

S100B proteins, EF- hand calcium binding proteins, also bind to the N terminus of NDR kinases and stimulate the autophosphorylation activity of NDR kinases in a calcium (Ca^{2+})-dependent manner (Millward *et al.*, 1998; Stegert *et al.*, 2004; Tamaskovic *et al.*, 2003a). Furthermore, experiments using the calcium-chelator BAPTA-AM indicate that phosphorylation at the activation segment as well as at the hydrophobic motif are calcium dependent (Tamaskovic *et al.*, 2003a). However, it is not known whether LATS kinases are also dependent on calcium and bind to S100 proteins.

FURRY proteins are specific for the NDR kinase signalling pathway. The yeast FURRY proteins PAG1/MOR2 physically interact and are important for the activation of the yeast NDR kinases CBK1/ORB6 (Du and Novick, 2002; Hirata *et al.*, 2002). The worm and fly FURRY proteins (Sax-2 and FURRY) show a strong genetic interaction with NDR kinases SAX-1 and TRC (Cong *et al.*, 2001; Emoto *et al.*, 2004; Gallegos and Bargmann, 2004; He *et al.*, 2005b). In addition, TRC physically interacts with, and requires, FURRY for activation (Emoto *et al.*, 2004; He *et al.*, 2005b). A human homologue of FURRY, AF4p12, is fused to mixed lineage leukaemia (MLL) in leukaemia patients (Hayette *et al.*, 2005). FURRY is thought to function as a scaffolding protein (Du and Novick, 2002; He *et al.*, 2005b), but

might also exhibit some transcriptional activity (Hayette et al., 2005). The scaffolding proteins of the LATS kinases are SALVADOR proteins. SALVADOR proteins (called hWW45 in humans) contain two WW domains and bind to LATS kinases (Tapon *et al.*, 2002). However, SALVADOR is less conserved than FURRY, and there is no clear orthologue in yeast. The scaffolding proteins in the MEN and SIN networks are NUD1 and SID4, together with CDC11 (Bardin and Amon, 2001; see also table 1).

MEN and SIN networks also comprise the GTPases TEM1 or SPG1, RAS family GTPases which are most closely related to RAB GTPases. TEM1 regulates the activation of CDC15 at the spindle pole bodies (Molk *et al.*, 2004). So far, no activating GTPase interaction is known for mammalian STE20-like kinases. In contrast to the MEN and SIN networks, no GTPase has been identified for the RAM network or the ORB6 signalling pathway. However, the STE20-like kinases of these signalling module were shown to genetically and physically interact with the MO25 proteins, HYM1p and MO25 (Dorland *et al.*, 2000; Bidlingmaier *et al.*, 2001; Bogomolnaya *et al.*, 2004; Kanai *et al.*, 2005). The mammalian MO25 proteins interact with STRAD proteins, a STE20-like pseudokinase and LKB1 (Baas *et al.*, 2004; Boudeau *et al.*, 2004) and functions as a scaffold, or adaptor, protein (Milburn *et al.*, 2004). The *Drosophila* MO25 does not appear to interact genetically with the NDR kinase TRC, indicating that MO25 might not regulate NDR in flies (He *et al.*, 2005a). However, further studies, including mutants and phenotypes of the upstream kinase, are required to exclude this possibility.

In fission yeast, the NDR kinase ORB6 also directly interacts with the methyltransferase SKB1, an orthologue of human PRMT5. SKB1 influences the intracellular localisation of ORB6 to cellular tips and exacerbates the phenotype of ORB6 mutants (Wiley *et al.*, 2003). Interestingly, human NDR1 was found in a transcriptional complex together with FCP1, a

PRMT5 interactor, indicating a potential collaboration of PRMT5 with human NDR kinases (Amente *et al.*, 2005).

Several reports have suggested that there is an interaction between LATS and NDR kinase pathways, indicating that LATS might partially control NDR activation and/or localisation (Weiss *et al.*, 2002; He *et al.*, 2005a; Kanai *et al.*, 2005). A feedback mechanism, whereby active NDR induces cleavage of its upstream kinase MST3, might be used to regulate the signal intensity (see results section). Similarly, LATS kinases activate caspases and induce apoptosis (Ke *et al.*, 2004), which is likely to induce cleavage of MST1/2.

In HIV-1 NDR kinase activation itself is inhibited by proteolytic cleavage mediated by the viral protease (Devroe *et al.*, 2005). Truncation of NDR2 at the cleavage site also alters its subcellular localisation (Devroe *et al.*, 2005).

The multiple modes of regulation reflect the tight control of NDR and LATS kinase activity *in vivo* and suggest a spatial and temporal activation of these kinases.

6. AIMS OF THIS STUDY

The aim of this study was to gain further insights into the function and regulation of mammalian NDR kinases. This study reports the identification and cloning of human *Ndr2* and mouse *Ndr1* and *Ndr2*. It shows expression patterns of mouse *Ndr1* and *Ndr2* mRNA. The main part of this work covers the regulation and activation of NDR protein kinase by phosphorylation and binding of the coactivator proteins S100B and MOB1A. The study demonstrates that activation of NDR is a multi-step process involving autophosphorylation at the activation segment, hydrophobic motif phosphorylation by mammalian STE20-like kinase 3 (MST3), and release of autoinhibition by MOB1A. Furthermore, it provides evidence that NDR kinase is involved in a feedback mechanism which regulates the cleavage of MST3.

This suggests a potential role of NDR in the regulation of apoptosis. Therefore, this study proposes a general mechanism for the interaction of STE20-like kinases and NDR kinases: hydrophobic motif phosphorylation.

To address the function of NDR kinases in mammals, NDR1 knockout mice were generated. The initial characterisation of the NDR1 deficiency is described in the third part of the results section. However, NDR1 mice show only a mild or late-onset phenotype. Data from mouse embryonic fibroblasts (MEFs) provide evidence that ablation of NDR1 kinase is partly compensated by NDR2 upregulation.

II. RESULTS

7. REGULATION OF NDR2 PROTEIN KINASE BY MULTI-SITE PHOSPHORYLATION

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Summary

NDR protein kinases are a family of AGC group kinases that are involved in the regulation of cell division and cell morphology. We describe the cloning and characterisation of the human and mouse NDR2, a second mammalian isoform of NDR protein kinase. NDR1 and NDR2 share 86% amino acid identity and are highly conserved between human and mouse. However, they differ in expression pattern: mouse *Ndr1* is expressed mainly in the spleen, lungs and thymus, whereas mouse *Ndr2* shows highest expression in the gastrointestinal tract. NDR2 is potently activated in cells following treatment with the protein phosphatase 2A inhibitor okadaic acid, which also results in phosphorylation of the activation segment residue Ser-282 and the hydrophobic motif residue Thr-442. We show that Ser-282 becomes autophosphorylated *in vivo*, whereas Thr-442 is targeted by an upstream kinase. This phosphorylation can be mimicked by replacing the hydrophobic motif of NDR2 with a PRK2-derived sequence, resulting in a constitutively active kinase. Similar to NDR1, the autophosphorylation of NDR2 protein kinase was stimulated *in vitro* by S100B, an EF-hand Ca^{2+}-binding protein of the S100 family, suggesting that the two isoforms are regulated by the same mechanisms. Further, we show a predominant cytoplasmic localisation of ectopically expressed NDR2.

Introduction

The NDR protein kinase family is a member of the AGC group of serine/threonine kinases, which includes cAMP-dependent kinase, cGMP-dependent kinase, protein kinase B and protein kinase C (1). The human NDR protein kinase is highly conserved and is expressed almost ubiquitously (2). The closest members of the mammalian NDR protein kinase family which come from lower organisms, TRC, SAX-1, Cbk1p and Orb6p, are all involved in the control of cell morphology. The *Drosophila* NDR kinase TRC regulates the integrity of epidermal cell extensions, such as sensory bristles, arista and wing hairs, by affecting the actin cytoskeleton, and is thought to form a part of a putative morphogenetic checkpoint (3). SAX-1 is the *Caenorhabditis elegans* NDR protein kinase and is reported to play an important role in the regulation of neuronal cell shape and neurite initiation (4). The relatives of NDR protein kinase in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, Cbk1 and Orb6, are also essential for normal morphogenesis, cell polarity and coordinating cell morphology with the cell cycle (5-8).

NDR protein kinase and its relatives have a conserved structure consisting of an N-terminal S100B/calmodulin binding site, a catalytic kinase domain containing an insertion between subdomains VII and VIII (encompassing, in the case of NDR, a nonconsensus nuclear localisation signal and the activation loop phosphorylation site), and a C-terminal regulatory domain (2,9,10). The human NDR1 protein has been shown to become autophosphorylated on Ser-281 and activated when S100B binds in a Ca^{2+}-dependent manner. The C-terminal regulatory phosphorylation site Thr-444 is phosphorylated *in vivo* by an upstream kinase, so far unidentified (11). This phosphorylation within the hydrophobic motif, which is an event typical of the

regulation of many AGC-group kinases, mediates kinase activity and protein stability (12,13). Some kinases, such as PRK2, have an Asp residue instead of a Ser or Thr residue, and a mutation of the hydrophobic phosphorylation site to an Asp has been shown to result in a constitutively active hydrophobic motif for several kinases (14,15).

Here, we describe the characterisation of a second isoform of NDR protein kinase, termed NDR2, and show that m*Ndr1* is widely expressed, whereas m*Ndr2* is mainly expressed in the gastrointestinal tract of mice. NDR2 becomes activated *in vivo* following phosphorylation of three conserved sites, Thr-75, Ser-282 and Thr-442. Further, an NDR2-PIFtide chimera, which contains the PRK2 hydrophobic motif (PIFtide), is constitutively active. *In vitro*, the Ca^{2+} binding protein S100B stimulates activation of NDR2 and autophosphorylation on Thr-75, Ser-282 and Thr-442.

Experimental Procedures

PCR and Molecular cloning — BLAST searches of the NCBI database were performed to identify NDR-related sequences. The *Ndr2* cDNA was assembled by PCR screening using a Marathon-Ready™ human brain cDNA library (Clontech) following standard protocols (16). The mouse *Ndr1* cDNA was cloned from a mouse λZAPII (Stratagene) cDNA library; the mouse *Ndr2* cDNA was cloned using a 3' mEST (GeneBank accession number AA277870) and subcloning the 5' end from a mouse brain cDNA library (Clontech) using PCR. Sequences of all clones were obtained using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) using custom synthesised primers, and compared to the appropriate genomic databases (Ensembl and Celera). Sequence analysis was performed using Seqweb 1.2 (Genetics Computer Group, Inc.).

Plasmids — Mammalian expression vectors encoding HA and GST-epitope tagged hNDR1 described previously (2). Expression vectors for HA-hNDR2 and GST-NDR2 were constructed in a similar way. GFP-Ndr2 was constructed by amplifying the Ndr2 cDNA with primers 5'-CGGGATCCGGTACCATGGCAATG-ACGGCAGGGACTAC-3' and 5'-CGGGATCCCTTCATTCATAACTTCCCAGC-3' using Pfu polymerase (Promega). The PCR product was then digested using BamHI and cloned into pEGFP-C1 (Clontech). HA-NDR2-PIFtide was constructed by amplifying NDR2 cDNA with primers 5'-CTTCCAAGCTTAGTCGACATGGC-TTACCCATACGATGTTCCAGATTACGCTTCGGCAATGACGGCAGGGACTA-CAACAACC-3' and 5'-CGGGATCCTCACCAGTCGGCGATGTAGTCGAAGTC-GCGGAACATCTCCTGCTCCTCTTTGTAGTCCGGTTCTGTGGTTATT-3' using Pfu polymerase (Promega). The PCR product was then digested using BamHI and SalI and cloned into pCMV5. All plasmids were confirmed by sequence analysis.

RNA Extraction and Real-time RT-PCR — Tissues of three 129SvPas mice were isolated and subjected to RNA extraction using Trizol Reagent (Invitrogen) and the RNeasy 96 kit (Qiagen). Reverse transcription reactions were performed using the GeneAmpRNA PCR kit according to the manufacturer's instructions (Applied Biosystems). Real-time quantitative PCR analysis was performed using an ABI Prism 7700 Sequence Detector. Specific primers and probes for each gene were designed using Primer Express 2.0 software. Amplicon sizes were 67 bp for m*Ndr1* and 98 bp for m*Ndr2*. TaqMan PCR reactions were performed for m*Ndr1*, m*Ndr2* and 18S rRNA according to the user manual. Details of primers and probes are available on request. Relative quantifications were performed by comparing the corrected C_t value of each tissue to the corrected C_t value of the brain, as described in the ABI PRISM 7700 user bulletin number 2.

Bacterial Expression and Kinase Assay of Human GST-fused NDR2 — Expression of pGEX-2T_NDR2 species in the BL21-DE3 (pRep4) *E. coli* strain and *in vitro* kinase assays [autophosphorylation in presence or absence of 100 μM $CaCl_2$ and 10 μM bovine S100B (Sigma)] were performed, as described previously for NDR1 (11).

Cell Culture and HA-NDR2 Kinase Assay — Culture and transfection of COS-1 and COS-7 cells, and measurement of kinase activity of HA-NDR2 variants were as described previously for HA-NDR1 (9).

Western Blotting — Immunodetection of NDR2 phosphorylated on Thr-75, Ser-282 or Thr-442 was as previously described (11).

Mass Spectrometry — Analysis of the phosphorylation sites of GST-NDR2 was performed according to Tamaskovic *et al*. (11).

Immunofluorescence Microscopy — Exponentially growing cells were plated on coverslips and transfected the next day with indicated constructs using Fugene 6 (Roche) as described by the manufacturer. After 24 h of transfection, cells were washed with PBS and fixed in 3% paraformaldehyde/2% sucrose in PBS at pH 7.4 for 10 min at 37°C. They were then permeabilised using 0.2% Triton X-100 in PBS for 2 min at room temperature. All subsequent steps were carried out at room temperature. Coverslips were rinsed twice with PBS and incubated for 1 h with anti-HA Y11 (Santa Cruz) diluted in PBS containing 1% BSA/1% goat serum. After three 1-min washes in PBS, goat anti-rabbit FITC (Sigma) was used as the secondary antibody. DNA was counterstained with 4 μg/ml Hoechst (Sigma). Coverslips were then inverted into 5 μl Vectashield medium (Vector Lab). Images were obtained with an Eclipse E800 microscope using a CoolPix950 digital camera (Nikon) and processed using Adobe Photoshop 6.0 (Adobe Systems Inc.). Only cells with intact nuclei were

included in the statistical evaluation. Cells expressing GFP-NDR2 were fixed and then stained for DNA without permeabilisation and antibody incubation steps.

Results

Conservation of NDR Kinases — BLAST searches of the NCBI database identified the human KIAA0965 clone (GeneBank accession number AB023182/ hj06174s1) as a partial cDNA with significant homology to human NDR1 protein kinase. We determined the sequences of h*Ndr2*, m*Ndr1* and m*Ndr2* cDNAs, and the corresponding mouse genes. The deduced amino acids sequences were compared and aligned to the known sequences of hNDR1, *D. melanogaster* NDR TRC, *C. elegans* NDR SAX-1 and *S. cerevisiae* Cbk1 (Fig.1). The human and mouse NDR1 sequences show 99% identity, the NDR2 sequences 97%, and NDR1 and NDR2 show an identity of 86%, the identity to fly, worm and yeast NDR kinases are 68%, 67% and 47% respectively, indicating an extremely high sequence conservation during evolution. Human and mouse NDR2 are 464 amino acids long, with a predicted mass of 54 kDa. Gene mapping indicates that h*Ndr1* and m*Ndr1*, as well as h*Ndr2* and m*Ndr2*, are located on orthologous regions: h*Ndr1* maps to 6p21 (17), and h*Ndr2* to 12p12.3. The corresponding mouse genes map to 17B1 and 6G2-G3. In addition, pseudogenes of *Ndr2* were found on chromosomes 1D and 8A1.2 of the mouse genome. The human and mouse genes consist of 14 exons, with conserved intron-exon boundaries. Exon 1 is a non-coding exon containing 5' UTR, exon 2 contains the start codon, and the stop codon is located in exon 14 (data not shown).

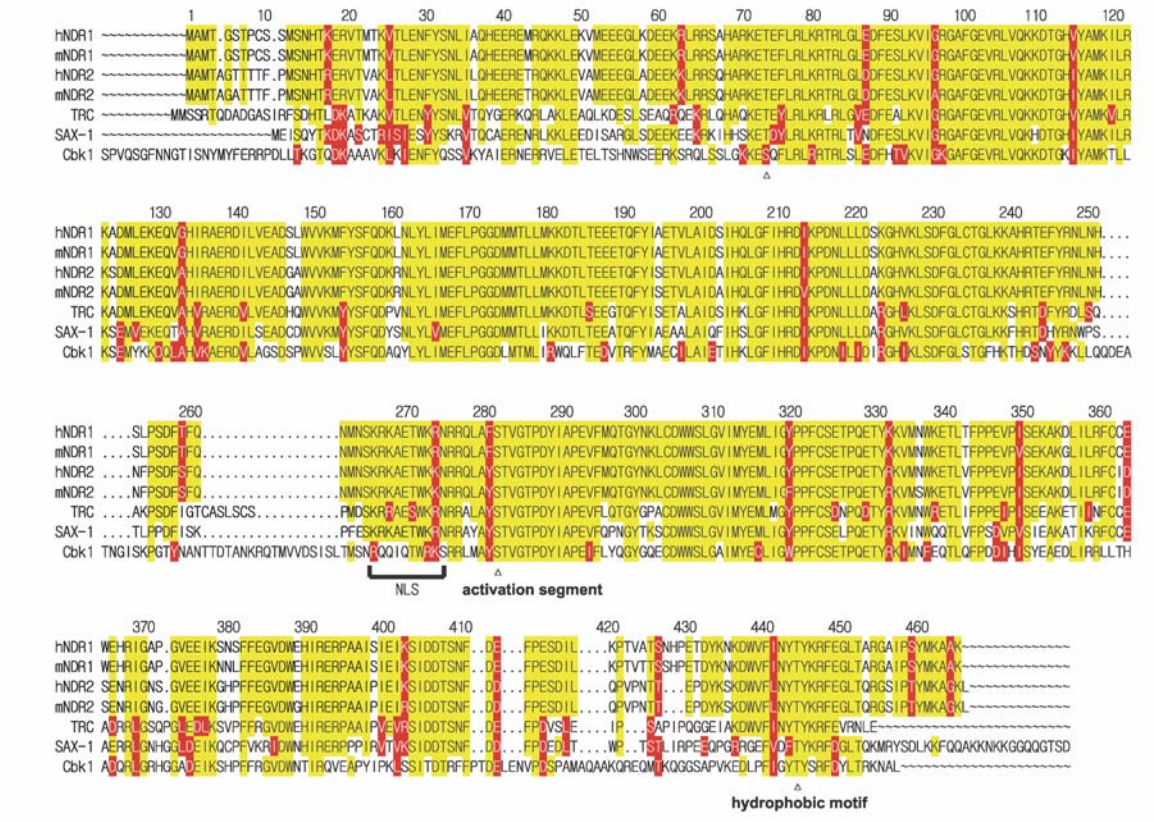
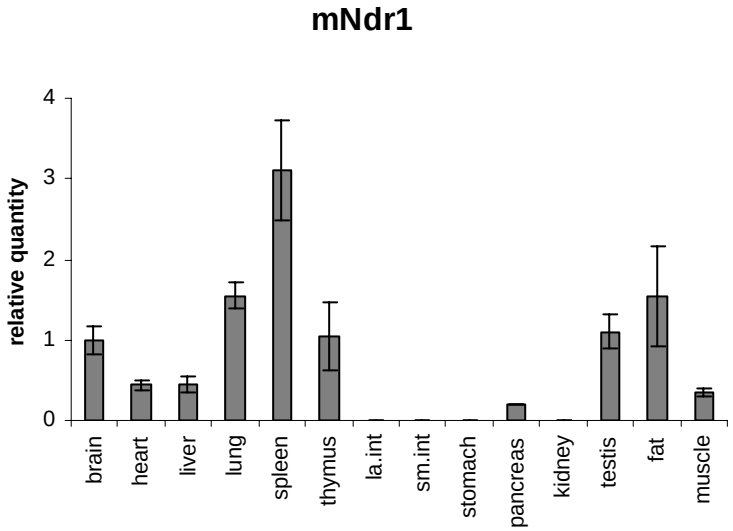


Fig. 1. Amino acid sequence alignment of *Homo sapiens, Mus musculus, Drosophila melanogaster, Caenorhabditis elegans* and *Saccharomyces cerevisiae* NDR. The NDR amino acid sequences were aligned using Seqweb 1.2. Identical amino acids are shaded in yellow and similar residues in red. Dots represent gaps inserted to bring the sequences into alignment. The NDR phosphorylation sites Thr74, Ser 281 and Thr 444 are indicated with arrows and numbered according to hNDR1. The predicted NLS is indicated.

Tissue-specific Expression of Ndr1 and 2 — TaqMan real-time PCR analysis was used to examine the differential expression of m*Ndr1* and m*Ndr2* in mouse tissues obtained from three different mice (Fig. 2). The expression of 18S rRNA was used as a reference gene to correct for RT-PCR efficiency of each sample. The highest expression levels of m*Ndr1* were observed in the spleen, lungs, thymus, brain and fat tissue, whereas m*Ndr2* expression was mainly found in the large and small intestine, as well as in the stomach and testes. Assuming similar PCR efficiency for both reactions, m*Ndr2* appears to be the predominantly expressed isoform in mice. These data indicate tissue-specific functions of NDR1 and NDR2 in mammals.

A



B

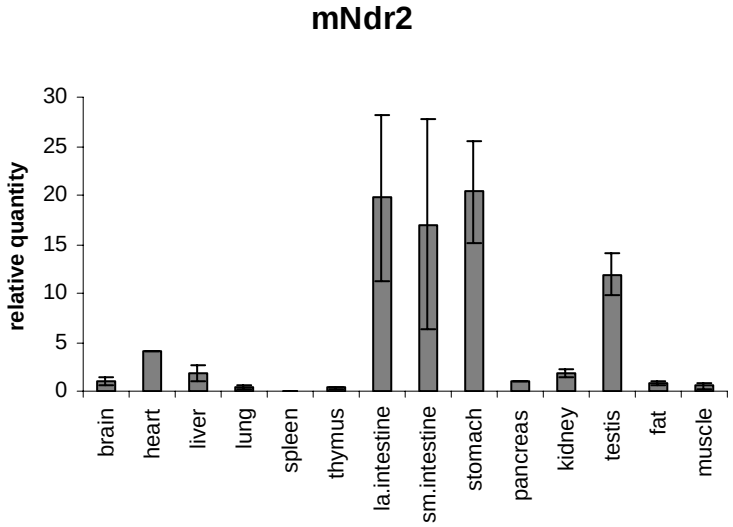


Fig. 2. Relative mRNA expression levels of m*Ndr1* (A) and m*Ndr2* (B) determined by quantitative real-time PCR. The expression levels of m*Ndr1* and m*Ndr2* are shown. RNA samples from three mice were collected and reverse transcribed. Each value was normalised against the expression of 18S RNA and compared to the relative expression of m*Ndr1* or m*Ndr2* in the brain.

Regulation of NDR2 Kinase Activity — Comparison of the NDR2 and NDR1 sequences showed that NDR2 contains three conserved phosphorylation sites corresponding to the described NDR1 phosphorylation sites Thr-75, Ser-281 and Thr-444 (10). NDR2 mutants were created, in which Thr-75, Ser-282 or Thr-442 were replaced by an alanine, and a kinase-dead mutant K119A was also made. The protein kinase activity of each of these mutants was measured following treatment of transfected Cos1 cells with 1 μM okadaic acid (OA), or solvent alone, for 1 h. HA-NDR2-WT was potently stimulated (~10-fold) by OA (Fig. 3). All three phosphorylation site mutants (T75A, S282A and T442A) displayed reduced basal activity and could not be stimulated by OA treatment. The K119A mutation reduced basal activity to almost undetectable levels and, as expected, abolished the activation by OA. Western blot analysis of the regulatory phosphorylation site mutants T75A, S282A, T442A and wild type with phospho-specific antibodies, which recognise the phospho-epitopes Ser-282P and Thr-442P, showed that NDR2 became phosphorylated on Ser-282 in wild type, T442A and T75A, and that this phosphorylation increased after OA treatment in the wild type and T442A, but not in the mutant T75A. The kinase-dead K119A mutant did not become phosphorylated on Ser-282, indicating that Ser-282 is an autophosphorylation site.

Thr-442 became phosphorylated upon OA treatment in NDR2 wild type, as well as in the S282A, T75A and K119A mutants, suggesting that Thr-442 is phosphorylated independently of NDR2 activity and is therefore targeted by an upstream kinase (Fig. 3). These results confirmed that phosphorylation on both the activation segment phosphorylation site Ser-282 and the regulatory hydrophobic motif phosphorylation site Thr-442 are required for NDR2 activation. In addition, NDR2 activity also depends on the presence of Thr75 in the N-terminal regulatory domain.

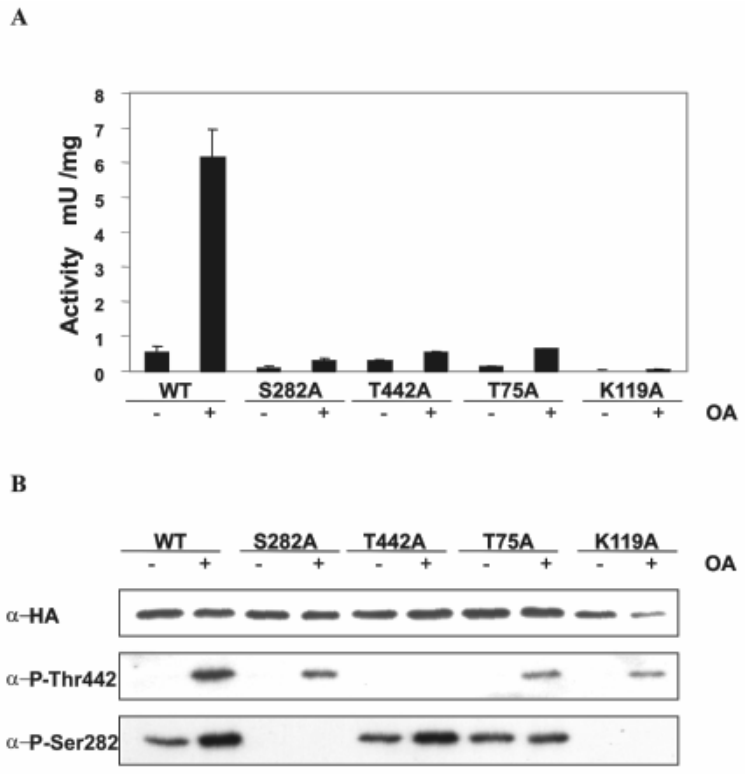


Fig. 3. NDR2 kinase activity and phosphorylation status of NDR kinase mutants. A, COS-1 cells expressing either wild-type HA-NDR2 or the indicated mutants were treated for 1 h with 1 μM okadaic acid (OA) or with solvent alone. HA-tagged NDR kinase variants were then immunoprecipitated (100 μg of cell-free protein extracts) with 12CA5 monoclonal antibody and assayed for kinase activity using a peptide substrate as described under "Experimental Procedures". Bars represent the mean ± S.D. of triplicate immunoprecipitations. **B,** An aliquot (100 μg protein) of 12CA5-immunoprecipitated proteins from transfected COS-1 cells was immunoblotted with 12CA5 to verify similar expression levels of each HA-NDR2 construct (top panel). For the analysis of phosphorylation status, 12CA5-immunoprecipitated HA-NDR2 variants (100 μg protein) were analysed by immunoblotting with phosphospecific antibodies directed against phosphorylated Ser-282 or phosphorylated Thr-442 (middle and bottom panels, respectively).

Constitutive Active NDR2 — The phosphorylation of Ser/Thr residues can be mimicked by the substitution with Asp or Glu for several kinases (14). However, we have recently shown that T444D or T444E mutations have only a very moderate effect (1.5- to 2-fold activation) on NDR1 kinase activity (10). Previous studies with PKB showed that the active state of the N-lobe hydrophobic groove, which is

dependent on an ordered αC-helix, could also be achieved by substituting the hydrophobic motif of PKB with the constitutive active hydrophobic motif of PRK2 (PIFtide) (15).

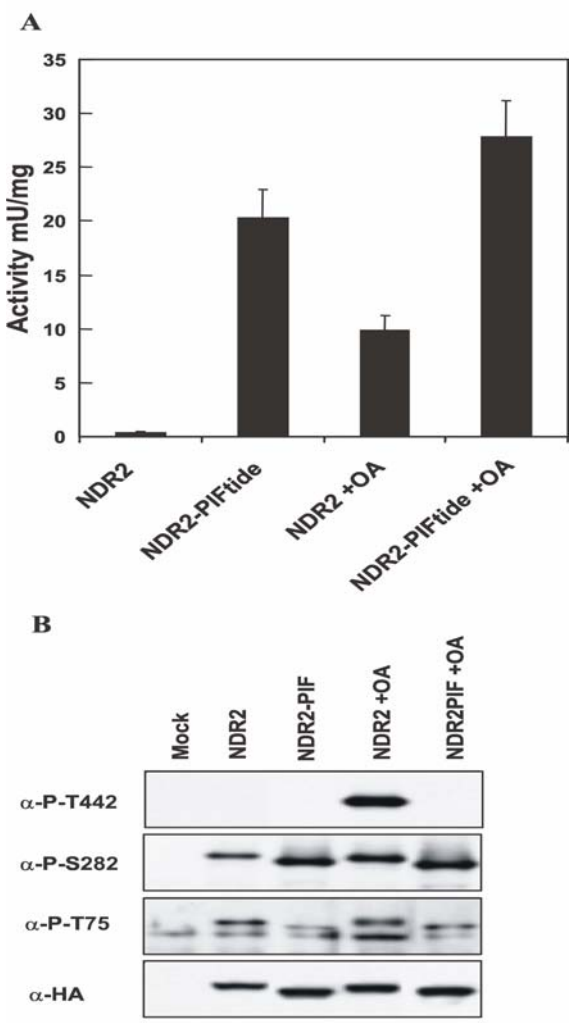


Fig. 4. NDR2-PIFtide chimera is constitutively active. A, COS-1 cells expressing either wild-type HA-NDR2 or the HA-NDR2-PIFtide chimera were treated for 1 h with 1 μM OA or with solvent alone. HA-tagged NDR kinase variants were then immunoprecipitated (100 μ g of cell-free protein extracts) with 12CA5 monoclonal antibody and assayed for kinase activity using a peptide substrate as described under "Experimental Procedures". *Bars* represent the mean ± S.D. of triplicate immunoprecipitations. **B,** For the analysis of phosphorylation status, 12CA5-immunoprecipitated HA-NDR2 variants (100 μg protein) were analysed by immunoblotting with phosphospecific antibodies directed against phosphorylated Thr-442, phosphorylated Ser-282 or phosphorylated Thr-442 (top and middle panels, respectively). An aliquot (100 μg protein) of 12CA5-immunoprecipitated proteins from transfected COS-1 cells was immunoblotted with 12CA5 to verify similar expression levels of each HA-NDR2 construct (bottom panel).

Based on the similarities between AGC group kinases, we created an NDR2-PIFtide chimera, aiming to generate an active kinase. Indeed, the NDR2-PIFtide had a more than 20-fold elevated basal kinase activity, and even increased activity compared with OA-stimulated NDR2 (Fig. 4A). Phosphorylation site analysis showed increased Ser282 phosphorylation in the NDR2-PIFtide chimera, suggesting increased autophosphorylation activity (Fig. 4B).

Therefore, we have shown that substitution of the hydrophobic motif of NDR2 with the PIFtide sequence results in a constitutively active NDR2 kinase, describing for the first time a constitutively active variant of the NDR protein kinase.

Activation of NDR2 by S100B — The sequence conservation between NDR1 and NDR2 also encompasses the previously defined S100B-binding domain of NDR1 (see Fig. 1). Therefore, the *in vitro* effect of Ca^{2+}/S100B on NDR2 activity was investigated. Ca^{2+}/S100B increased the rate of NDR2 autophosphorylation approximately 2-fold after a 4-h incubation (Fig. 5A) and stimulated specific NDR activity approximately 4-fold in a concentration-dependent manner (Fig. 5B). This indicates that NDR2 activation by Ca^{2+}/S100B also occurs by the mechanism reported for NDR1 (9,11). After *in vitro* incubation of GST-NDR2 in the presence or absence of Ca^{2+}/S100B, the proteins were digested with trypsin and the resultant mixture analysed by ESI-MS-MS in a –79 precursor scan (18). This technique measures the mass to charge ratio (*m/z*) of all peptide species liberating a single phosphate group after fragmentation. Five (NDR2-derived) phospho-peptides were identified in both samples (the GST-NDR2 and the GST-NDR2/Ca^{2+}/S100B), corresponding to the phosphorylation sites Thr-75, Ser-282 and Thr-442 (Fig. 5C). These results demonstrate that S100B proteins regulate NDR2 by a mechanism similar to that reported for NDR1 (11).

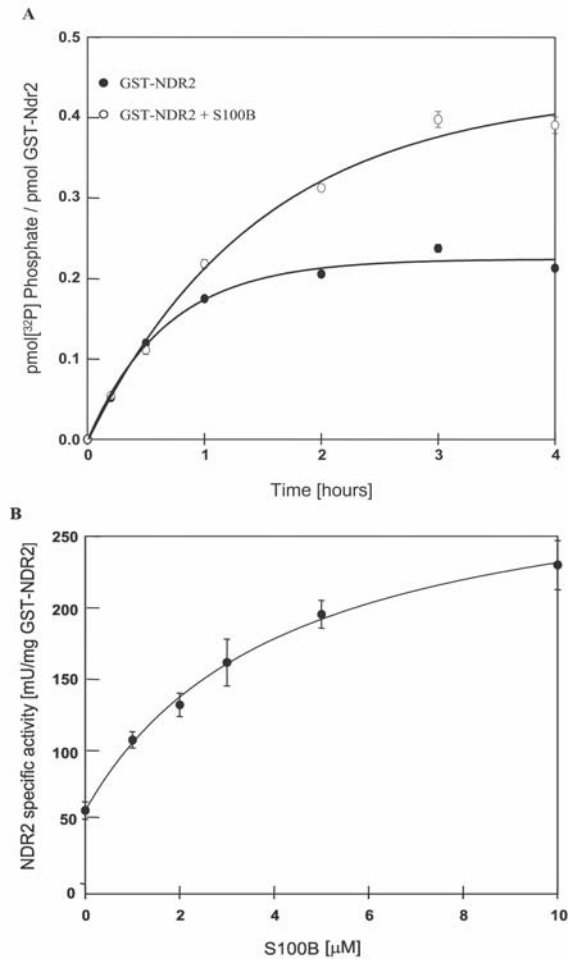


Fig. 5. Ca^{2+}/S100B promotes the auto- and transphosphorylation of GST-NDR2. A, An aliquot (1 μg) of purified GST-NDR2 wild type (~0.5 μM) was autophosphorylated for the indicated time periods *in vitro* without further additions (filled circle) or in the presence of 100 μM $CaCl_2$ and 10 μM bovine S100B (open circle) as indicated. **B,** An aliquot (1 μg) of purified GST-NDR2 was autophosphorylated at 30°C for 2 h in the absence or presence of increasing concentrations of S100B. **C,** Mapping of NDR2 *in vitro* autophosphorylation sites. An aliquot (10 μg) of GST-NDR2 was autophosphorylated for 2 h in the absence or presence of 100 μM $CaCl_2$ and 10 μM bovine S100B. After separation by SDS-PAGE, GST-NDR2 was excised and processed by tryptic cleavage for MS analysis of phosphopeptides by precursor ion scanning of *m/z* –79. Phosphopeptides whose *m/z* could be assigned to NDR2-derived phosphopeptides are labelled P1-P5. Some peptides were detected in several charged states $[M-2H]^{2-}$, $[M-3H]^{3-}$, etc. Peptide P1/P3 results presumably from a trace of chymotryptic contamination of the trypsin preparation, because it overlaps with P2 and terminates with an aromatic residue. Asterisks denote an abundant double-charged, nonphosphorylated NDR2 peptide (379-392) with *m/z* 862.

Localisation of NDR2 — To examine the subcellular localisation of NDR2, COS-7 and HeLa cells were transfected with either N-terminally GFP-tagged NDR2 (GFP-NDR2) or HA-NDR2 and processed for indirect immunofluorescence (Fig. 6A; data not shown). A statistical analysis showed that both proteins were detected mainly in the cytoplasm of both COS-7 cells and HeLa cells under these experimental settings (Fig. 6B). Similar results were also obtained when U2-OS cells were studied (data not shown). Thus, in marked contrast to the localisation pattern described for NDR1 (2), NDR2 was not predominantly localised to nuclear structures. For unknown reasons, NDR2 was found to be mainly cytoplasmic, even though it contains the same NLS as NDR1 (see Fig.1).

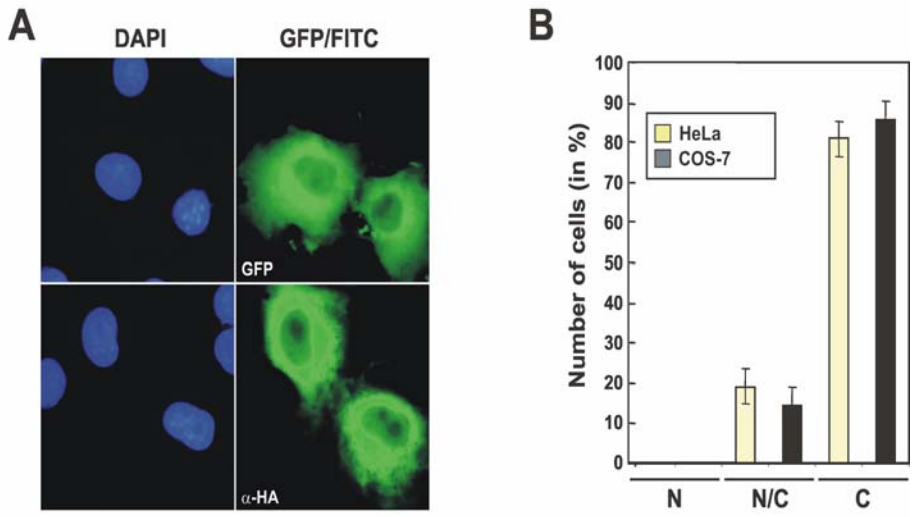


Fig. 6. NDR2 localises mainly to cytoplasmic structures. A, COS-7 cells expressing GFP-NDR2 (upper panels) or HA-NDR2 (lower panels) were processed for immunofluorescence using either no antibody (upper right) or anti-HA Y11 (lower right). Anti-HA antibody was visualised using anti-rabbit FITC (green). Corresponding DNA stains (blue; left panels). B, HeLa (light) and COS-7 (dark) cells expressing GFP-NDR2 were processed for immunofluorescence and GFP signals were scored as either mainly nuclear (N), nuclear and cytoplasmic (N/C) or predominantly cytoplasmic (C). The blotted numbers represent at least two independent experiments (±SD). A minimum of 150 cells was counted per experiment.

Discussion

In this study, we describe the cloning and characterisation of the NDR2 protein kinase, a second isoform of mammalian NDR protein kinase. The extremely high conservation of NDR protein kinases throughout the eukaryotic world indicates that this kinase is subject to a very high evolutionary pressure. Mouse and human NDR kinases also show a conserved gene organisation of 14 exons. It is likely that the kinases are components of a conserved signalling pathway (1). This has already been shown genetically for one component of the pathway, the FURRY/MOR2/PAG1 protein, the mutation of which results in similar phenotypes in *D. melanogaster*, *S. pombe* and *S. cervisiae*, as reported for NDR kinase mutations (19-21). There is no information on the function of the human FURRY protein.

Our results show that NDR2 is regulated by multi-site phosphorylation similar to many of the AGC family of protein kinases. Mutation of one or both phosphorylation site residues Ser-282 and Thr-442 of NDR2 led to an almost total loss of kinase activity, indicating that both residues are essential for kinase activity. This is not surprising, as similar observations have been made for other AGC-group kinases. Recent structural studies of PKB have delineated a mechanism by which multi-site phosphorylation brings about structural changes involving both disorder to order transitions of the alpha B and C helices, and ordering of the activation segment, concomitant with converting the kinase to a fully 1000-fold activated enzyme (13,15). Significantly, NDR2 becomes phosphorylated on three residues *in vitro.* The major site, Ser-282, which is conserved among all AGC group kinases, is an essential part of the activation segment of the kinase. The second site, Thr-75, is located within the S100B-binding domain and the third site, Thr-442, which is also conserved in the AGC-group superfamily, is located outside the kinase domain in a region enriched

with hydrophobic amino acid residues ("hydrophobic motif"). *In vivo* mutation of the phosphorylation site residues Ser-282 and Thr-442 of NDR2 ablated kinase activity and blocked activation. In wild-type NDR2, both residues were phosphorylated upon OA stimulation, whereas in the kinase-dead mutant K119A only the hydrophobic motif phosphorylation site Thr-442 was phosphorylated, indicating that the kinase activity of NDR2 is required only for phosphorylation of Ser-282. Therefore, NDR2 is phosphorylated by an upstream kinase at the C-terminal hydrophobic site in OA-stimulated COS-1 cells. However, we cannot rule out the possibility that Thr-442 autophosphorylation observed *in vitro* also contributes to the overall phosphorylation at this residue *in vivo*. Autophosphorylation on the activation segment residue has also been reported for other AGC kinases such as PKA and PKCδ (22,23). This indicates that autophosphorylation is an alternative mechanism of activation of the few AGC kinases that are not targeted by PDK1 (for review, see Ref. 24). The specificity of activation of AGC-group kinases is likely to be mediated by the phosphorylation of the hydrophobic motif residue. For the NDR kinases, some significant clues indicate that upstream kinases are members of the STE20 family. For example, the upstream kinase of Dbf2p has been identified as Cdc15p, one of the budding yeast STE20-like kinases (25), and the fission yeast STE20-like kinase Pak1p/Shk1p has been reported to interact genetically with Orb6p (8). The identification of this so far unknown upstream kinase for NDR will provide important information about the physiological regulation of the NDR protein kinase, which in turn could provide hints about the conditions under which this tightly controlled kinase is activated *in vivo*.

As demonstrated for PKB, the PIFtide sequence leads to an ordered and, therefore, fully active hydrophobic motif, concomitant with an activation of the kinase (15). The similarities within the AGC group kinases in sequence similarity and mode

of activation enabled us to create a constitutive active NDR2 by substituting the C-terminus of NDR2 with the PIFtide sequence. The NDR2-PIFtide showed an even higher activity than the OA-stimulated kinase. This is probably due to an intrinsic stimulation of the autophosphorylation activity by keeping the kinase in the active state, which is also reflected by the increased Ser-282 phosphorylation in the NDR2-PIFtide. The constitutive active kinase will probably prove to be a valuable tool for the identification of downstream targets of NDR2 protein kinase.

The *in vivo* significance of the phosphorylation at Thr-74 in NDR1 and Thr-75 in NDR2 is so far unclear. This threonine is within the identified S100B-binding domain of NDR protein kinase and its mutation is critical for NDR protein kinase activity. This might be due to a missing phosphorylation event, or this residue may be structurally important for the NDR-S100B interaction. Nevertheless, recent data show that Thr75 is not directly involved in the binding of S100B, indicating that the phosphorylation modulates the affinity between the two proteins (26). Conservation of the kinase also encompasses the S100B-binding domain, and the mechanism of *in vitro* activation by S100B appears to be identical for NDR1 and NDR2. The homology between the mammalian, fly, worm and yeast NDR kinases suggests that this mode of activation will be similar in all organisms.

The subcellular localisation of NDR2 was rather surprising, considering the high sequence similarity between NDR1 and NDR2. Whereas NDR1 was reported to be mainly nuclear (2), we detected NDR2 predominantly localised to cytoplasmic structures in our experimental settings. This might reflect different functions and/or substrate specificities of NDR1 and NDR2 within subcellular compartments.

Most importantly, the two mammalian isoforms differ mainly in their tissue- and cell type-specific expression patterns. It is striking that m*Ndr2* is mainly

expressed in highly proliferative tissues with high cellular turnover, such as the stomach, and the large and small intestines. Interestingly, h*Ndr1* has recently been found to be upregulated in highly necrotic and progressive ductal carcinoma *in situ*, as well as in some melanoma cell lines (9,27). Significantly, h*Ndr2* is upregulated in the highly metastatic non-small cell lung cancer cell line NCI-H460 (28), suggesting a potential role of NDR protein kinase in the regulation of cancer cell morphology and migration.

The major outstanding task for the future will be the full delineation of the novel highly conserved NDR signalling pathway. Of considerable importance is the question of the identification of the predicted agonist and receptor that initiate kinase activation. The answer may then help us understand how NDR contributes to the regulation of cell morphogenesis and proliferation, and how these signals are disrupted in transformed cells. Mouse knockout studies may reveal whether the observed differences in tissue-specific expression and subcellular localisation of NDR1 and NDR2 reflect differences in their functions.

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8. Regulation of NDR protein kinase by hydrophobic motif phosphorylation mediated by the mammalian STE20-like kinase MST3

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Abstract

NDR protein kinases are involved in the regulation of cell cycle progression and morphology. NDR1/NDR2 protein kinases are activated by phosphorylation of the activation loop phosphorylation site Ser281/Ser282 and the hydrophobic motif phosphorylation site Thr444/Thr442. Autophosphorylation of NDR is responsible for phosphorylation on Ser281/Ser282, whereas Thr444/Thr442 is targeted by an upstream kinase. Here we show that MST3, a mammalian STE20-like protein kinase, is able to phosphorylate NDR protein kinase at Thr444/Thr442. *In vitro*, MST3 selectively phosphorylated Thr442 of NDR2, resulting in a 10-fold stimulation of NDR activity. MOB (Mps one binder) 1A protein further increased the activity, leading to a fully active kinase. *In vivo*, Thr442 phosphorylation after OA stimulation was potently inhibited by MST3KR, a kinase-dead mutant of MST3. Knockdown of MST3 using short hairpin constructs abolished Thr442 hydrophobic motif phosphorylation of NDR in HEK293F cells. We conclude that activation of NDR is a multi-step process involving phosphorylation of the hydrophobic motif site Thr444/2 by MST3, autophosphorylation of Ser281/2 and binding of MOB1A.

Introduction

The NDR and LATS family of serine/threonine protein kinases participate in the regulation of cell cycle progression and cell morphology (14, 42, 52). These kinases share a conserved N-terminal regulatory domain that interacts with MOB (3, 6, 32, 49) and S100B proteins (30). The conserved catalytic (kinase) domain has an insertion of 30 amino acids between subdomains VII and VIII, containing the activation segment phosphorylation site Ser281/Ser282 and, in the case of mammalian

NDR1 and NDR2, an autoinhibitory sequence (3). The C-terminal regulatory domain encompasses the regulatory hydrophobic motif phosphorylation site Thr444 (31). Recent data have shown that NDR1 and NDR2 protein kinase activities are stimulated by Ca^{2+}/S100B- and MOB1-binding induced autophosphorylation on S281/S282 *in vitro* and *in vivo* (3, 30). Phosphorylation of the hydrophobic motif phosphorylation site T444/T442 is required for maximal activation, and involves an upstream kinase (41, 43).

Genetic evidence suggests that STE (Sterile) 20-like protein kinases function as upstream kinases of the NDR family. For example, one of the budding yeast STE20-like kinases, Cdc15p, phosphorylates Dbf2p (27), and the fission yeast STE20-like kinase Pak1p/Shk1p genetically interacts with Orb6p (48). Furthermore, the STE20-like kinase Kic1p functionally interacts with Cbk1p, the closest relative of NDR from *Saccharomyces cerevisiae* (32), and the *Drosophila melanogaster* STE20-like kinase HIPPO phosphorylates WARTS/LATS kinase (16, 19, 33, 44, 50). The closest mammalian homologues of Kic1p and HIPPO are the mammalian STE20-like protein kinases MST1/KRS2, MST2/KRS1, MST3, MST4/MASK and SOK/YSK, which are involved in the regulation of cell morphology, proliferation and apoptosis (7, 9, 21, 24, 37). This group of kinases contains an N-terminal kinase domain as well as an autoinhibitory C-terminal regulatory domain. Their activities are regulated by phosphorylation and proteolytic cleavage by caspases in response to stress and apoptotic stimuli (10, 18, 22, 36). Phosphorylation of MST1, for example, increases its activity several-fold and influences its subcellular localisation (22). Furthermore, the kinase-dead mutants of MST kinases are known to be inhibitors of MST kinase function *in vivo* (45).

There is evidence that several MST kinases promote apoptosis in response to stress stimuli and caspase activation. MST1, for example, can induce apoptosis and nuclear condensation in BJAB, 293T and COS-1 cells (15, 45, 46), and MST1 activity correlates with eosinophil apoptosis (8). Expression of MST3 in HEK293 cells leads to DNA fragmentation and apoptosis (18). YSK1 is activated at initial stages of necrotic cell death (34). In contrast, MST4 regulates cell growth and proliferation in HeLa and Phoenix cells (24). MST4 and YSK1 are localised to the Golgi apparatus, and the latter kinase is shown to be involved in processes such as cell migration, presumably by linking protein transport events with cellular processes such as cell adhesion or polarisation of the cytoskeleton (37).

Our recent work has revealed that recruitment of the MOB/NDR complex to the plasma membrane results in phosphorylation on Ser281 and Thr444, and activation of the kinase (17). To clarify and elucidate the role of STE20-like kinases in regulating the activity of the NDR kinases, we performed *in vitro* and *in vivo* experiments with MST3, the closest relative of Kic1p from humans. We provide evidence that MST3 can function as an NDR upstream kinase by phosphorylating the hydrophobic motif and thus stimulating kinase activity.

MATERIALS AND METHODS

Cell culture. COS-7 cells and HEK293F were maintained in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, and manipulated them as described previously (17).

Antibodies. α-P-Ser-282 and α-P-Thr-442 antibodies have been described previously (41). 12CA5 (HA) and 9E10 (myc) monoclonal antibody hybridoma supernatants were used for detection of HA-NDR, HA-MST3 and myc-MST3 variants.

Anti-GST antibodies (G7781) were purchased from Sigma. Polyclonal α-HA Y11 antibodies were purchased from Santa Cruz. α-MST3 antibodies were purchased from BD Biosciences. α-NDR-NT peptide antibody was raised against the synthetic peptide DEEKRLRRSAHARKETEF-LRLKRTRLGL, corresponding to amino acids 60 to 86 of NDR2, as described previously (17), and a rat monoclonal α-α-tubulin (YL1/2) producing hybridoma cell line was obtained from ATCC.

Plasmids. DNA constructs used for transfection were purified from *E. coli* XL1Blue using Qiafilter Maxiprep kit (Qiagen) according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) and custom-synthesized primers. Mammalian expression vector pCMV5, encoding HA-tagged NDR2 HA-MST3 and HA-MST3KR, has been described previously (18, 41). The myc-C1-MOB1A has been described previously (17). Bacterial expression constructs for pSHP-NDR2 wild type and mutants and pGEX-2T-MOB1A have been described previously (3). BamHI-flanked MST3 cDNA constructs were obtained by deleting the internal BamHI site using the QuickChange site-directed mutagenesis protocol (Stratagene) and the appropriate primers (primer sequences are available upon request) and subsequent amplification using primers 5'- CGCGGATCCATGGCTCACTCCCCG-GTG-3' and 5'-CGCGGATCCAAAGGAATTTCAGTGGGATG-3'. The resulting PCR product was subcloned as *BamHI-BamHI* into pcDNA3.0-myc (*KpnI*-MEQKLISEEDL-*BamHI*), pEGFP-C1 and pEBG2T.

A 16-amino-acid co-polymer of glutamic acid and glycine was added to the N-terminus of MST3 using PCR. The linker-MST3 fragment was amplified using primers 5'-AACTGCAGGAAGGTGAGGGCGAAGGTGAGGGCGAAGGTGAGG-GCGAAGGTGAGGGCATGGCTCACTCCCCG-3' and 5'-GCTCTAGATCAGTG-

GGATGAAGTTCC-3'. The *PstI-XbaI* fragment was subcloned into pCMV5. To create a pCMV5-HA-NDR2-MST3 fusion construct, HA-tagged NDR2 was amplified using primers 5'- CTTCCAAGCGCTTAGTCGACATGGCTTACCCATACGATGT-TCCAGATTACGCTTCGGCAATGACGGCAGGGACTACAACAACC-3' and 5'-AACTGCAGTAACTTCCCAGCTTTCATGTAGG-3' and subcloned as an *HindIII-PstI* fragment into pCMV5 (linker-MST3). The myc-C1-MOB1A has been described previously (17).

The pTER-shMST3 vectors were cloned using the HindIII-BglII digested pTER vector (47) and the following oligonucleotides: 5'–GATCCCGGCATTGACAATC-GGACTCTTCAAGAGAGAGTCCGATTGTCAATGCCTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGGCATTGACAATCGGACTCTCTCTTGAAGAGTCCGA-TTGTCAATGCCGG-3' for shMST3.

Western blotting and immunoprecipitation. Cell lysis buffer (IP buffer) contained 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 20 mM β-glycerol phosphate, 1 μM microcystin, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin and 1 mM benzamidine. To detect HA-NDR, SHP-NDR, HA-MST3 and myc-MST3, samples were resolved by 10% or 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween 20) containing 5% skimmed milk powder and then probed overnight at 4°C with the following antibodies: anti-GST 12CA5 α-HA, 9E10 α-myc, α-Thr-442P or α-Ser-282-P, α-NDR-NT, α-MST3 and α-α-tubulin YL1/2. Bound antibodies were detected using horseradish peroxidase-linked secondary antibodies and ECL. For immunoprecipitations, HEK293F or COS-7 cells transfected with HA-MST3 wild type, HA-MST3-KR or HA-NDR2 variants were harvested as described above. Cell

lysates (0.5 mg protein) were precleared with protein A- or G-Sepharose and mixed subsequently for 3 h at 4°C with α-HA 12CA5 antibody prebound to protein A-Sepharose. The beads were then washed twice with IP buffer, once with IP buffer containing 1 M NaCl, once again with IP buffer, and twice with 20 mM Tris-HCl pH 7.5 containing 4 μM leupeptin and 1 mM benzamidine. Samples were then subjected to kinase assays and/or were resolved by 10% SDS-PAGE. HA-MST3, HA-MST3KR and HA-NDR2 variants were detected by western blotting using α-HA 12CA5 monoclonal antibodies.

Bacterial expression of human GST-fused MOB1A and human SHP-fused NDR2. XL-1 Blue *E. coli* was transformed with the pGEX-2T-MOB1A plasmid. Mid-logarithmic phase cells were induced using 0.1 mM isopropyl β-D-thiogalactopyranoside overnight at 20°C. Bacterial lysis buffers contained 20 mM Tris-HCl pH 8.5, 10 mM 2-mercaptoethanol, 1% NP-40, 0.5 M NaCl and 'complete' proteinase inhibitor cocktail (Roche). Bacteria were disrupted using a French press in the presence of 1 mg/ml lysozyme and protease inhibitors, and the fusion proteins were purified on glutathione-Sepharose. SHP-NDR2 wild type and mutant plasmids were transformed into XL-1 Blue *E. coli* as described for GST-hMOB1A and protein purified on Ni-NTA-Sepharose.

NDR protein kinase assays. Transfected COS-7 cells were washed once with ice-cold phosphate-buffered saline and harvested 24 or 48 h after transfection in 1 ml of ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and 20 mM β-glycerol phosphate before lysis in 500 μl IP buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 20 mM β-glycerol phosphate, 1 μM microcystin, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, and 1 mM benzamidine). Lysates were centrifuged at 20,000 *g* for 20 min and

duplicate aliquots (250 μg) of the supernatant were precleared with protein A-sepharose for 1 h and mixed for 3 h at 4°C with α-HA 12CA5 antibody prebound to protein A-sepharose. The beads were then washed twice with IP buffer, once for 10 min with IP buffer containing 1 M NaCl, again for 10 min with IP buffer, and twice with 20 mM Tris-HCl pH 7.5 containing 4 μM leupeptin and 1 mM benzamidine. Thereafter, the beads were resuspended in 30 μl of buffer containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}P] ATP (~1,000 cpm/pmol), 1 μM cAMP-dependent protein kinase inhibitor peptide, 4 μM leupeptin, 1 mM benzamidine, 1 μM microcystin and 1 mM NDR substrate peptide (KKRNRRLSVA). After a 60-min incubation at 30°C, the reactions were processed as previously described (43).

Purified recombinant SHP-NDR2 wild type and mutants (1 μg) were pre-autophosphorylated or prephosphorylated for 60 min in the presence of HA-MST3 or HA-MST3KR (immunoprecipitated from 500 μg lysates of untreated and OA-treated HEK293F cells) in 30 μl of buffer containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM ATP, 1 μM cAMP-dependent protein kinase inhibitor peptide, 4 μM leupeptin, 1 mM benzamidine and 1 μM microcystin. Aliquots of 10 μl of the supernatant containing SHP-NDR or mutants were removed. One aliquot was assayed for activity using our standard conditions (reaction mixture of 30 μl containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}P] ATP [~1,000 cpm/pmol], 1 μM cAMP-dependent protein kinase inhibitor peptide, 4 μM leupeptin, 1 mM benzamidine, 1 μM microcystin and 1 mM NDR substrate peptide). After incubation for 30 min at 30°C, the reactions were processed and kinase activity determined as described for the HA-NDR kinase assay. The second aliquot was resolved by 10% SDS-PAGE and analysed for phosphorylation and amount of

protein using α-T-442-P and α-S-282-P antibodies by western blotting or Coomassie staining, respectively.

Time course analyses of hydrophobic motif phosphorylation and activity of SHP-NDR2 variants (1 µg) in the presence or absence of GST-MOB1A and in the presence or absence of GST-MST3 from untreated or OA-treated HEK293F cells were performed using our kinase assay standard conditions, as described above. Kinase assays were stopped after 0, 15, 30, 60, 90 and 120 min by adding 3 µl of 0.5 M EDTA. The reactions were processed and kinase activity was determined as described for the HA-NDR kinase assay. Phosphorylation of NDR was detected by western blotting, as described above.

Localisation of MST3 and NDR2. Exponentially growing cells were plated on coverslips and transfected the next day with the indicated constructs using Fugene 6 (Roche) as described by the manufacturer. After transfection for 24 h, cells were washed with PBS and fixed in 3% paraformaldehyde/2% sucrose in PBS at pH 7.4 for 10 min at 37°C. They were then permeabilised using 0.2% Triton X-100 in PBS for 2 min at room temperature. All subsequent steps were carried out at room temperature. Coverslips were rinsed twice with PBS and incubated for 1 h with α-HA Y11 (Santa Cruz) and α-myc 9E10 diluted in PBS containing 1% BSA/1% goat serum. After three 1-min washes in PBS, goat anti-rabbit FITC (Sigma) and goat anti-mouse Texas Red (Sigma) were used as secondary antibodies. DNA was counterstained with 4 µg/ml Hoechst (Sigma). Coverslips were then inverted into 5 µl Vectashield medium (Vector Lab). Images were obtained using an Eclipse E800 microscope and a CoolPix950 digital camera (Nikon) and processed using Adobe Photoshop 6.0 (Adobe Systems Inc). Only cells with intact nuclei were included in the statistical evaluation.

Cells expressing GFP-MST3 were fixed and then stained for DNA without permeabilisation and antibody incubation steps.

Cell fractionation. To separate cytosolic and membrane-associated proteins, cells were subjected to S100/P100 fractionation as follows: cells were collected in PBS and incubated for 20 min at 4°C in S100/P100 buffer (20 mM Tris, 150 mM NaCl, 2.5 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 4 μM leupeptin, 0.5 mM PMSF, 1 μM microcystin and 1 mM DTT at pH 7.5), homogenised by passage through a 26G needle (Beckton Dickinson) and nuclei were removed by centrifugation for 2 min at 1,000 g at 4°C. The supernatant was then centrifuged at 100,000 g for 60 min at 4°C. Equal amounts of protein from the supernatant (S100; cytoplasmic fraction) and the pellet (P100; membrane fraction) were analysed by SDS-PAGE followed by immunoblotting.

RESULTS

Catalytic domain conservation of STE20-like kinases. We compared the substrate-binding pockets (26) of the human MST kinases to the budding yeast relatives Kic1p and Cdc15p, which have previously been reported to be upstream regulators of Cbk1p and Dbf2p (27, 32). All of the kinases showed very high conservation of their catalytic domain. MST3, YSK1 and MST4, as well as MST1 and MST2, have identical residues in their substrate-binding pockets, indicating similar substrate specificities (Fig. 1A). We also compared the hydrophobic motifs of the budding yeast Cbk1p and Dbf2p with human NDR kinases. The hydrophobic motifs of hNDR1 and hNDR2 as well as LATS1 and LATS2 show a significant similarity to corresponding regions in the yeast kinases (Fig. 1B). To test our hypothesis that a mammalian STE20-like (MST) kinase is the hydrophobic motif kinase of NDR

protein kinases, we performed *in vitro* kinase assays using MST3, the closest relative of the yeast Kic1p-Cbk1p pathway. For this study we used NDR2 because it was easier to produce recombinant full-length protein.

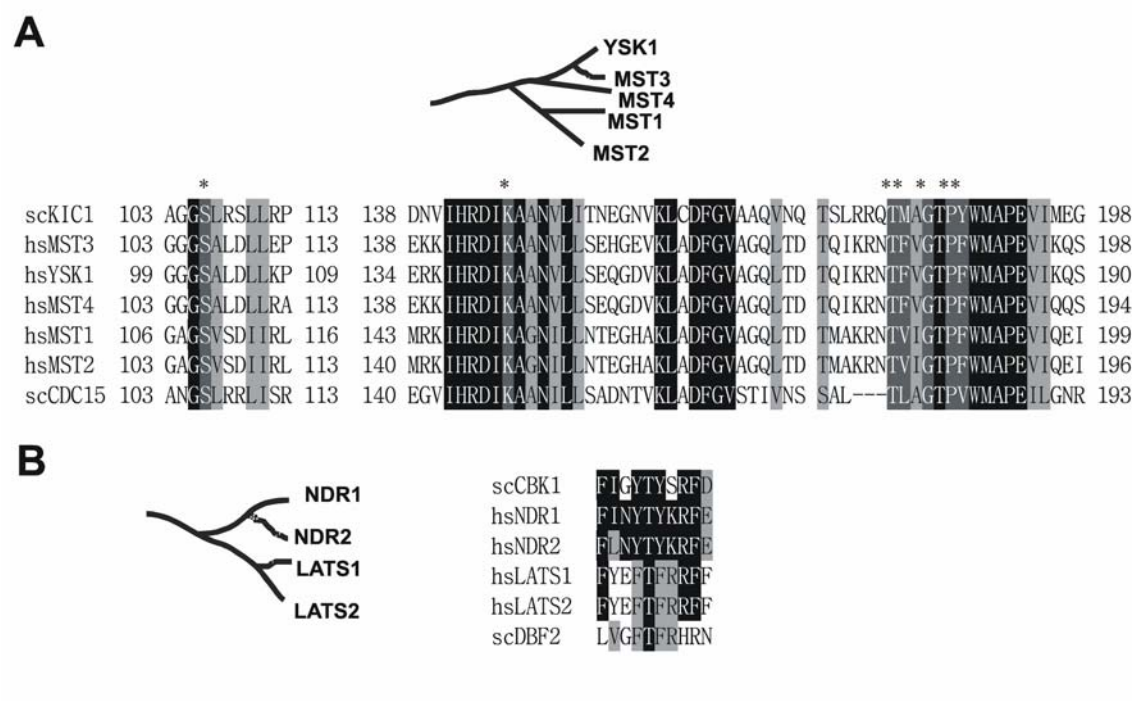

Fig. 1. Conservation of yeast and human STE20-like kinases. A. Dendrogram and sequences surrounding the substrate binding pocket of the *Saccharomyces cerevisiae* STE20-like kinases Kic1p and Cdc15p, and the human mammalian STE20-like kinases MST3, YSK1, MST4, MST1 and MST2 (28). Identical residues are shaded in black, similar residues in grey. Residues determining the substrate-binding pocket are indicated with asterisks and shaded in dark grey. **B.** Dendrogram and hydrophobic motif sequences of the human NDR kinase family. Identical and similar residues are shaded as above.

Activation and hydrophobic motif phosphorylation of NDR protein kinase by MST3. We expressed HA-MST3wt and the kinase-dead HA-MST3KR in HEK293F cells and treated them with 1 μM okadaic acid (OA) for 1 h (MST3+; MST3KR+) or solvent control (MST3; MST3KR). The HA-tagged MST3 kinase variants were immunoprecipitated with the α-HA 12CA5 monoclonal antibody and incubated with SHP-NDR2 and catalytically inactive SHP-NDR2KD for 1 h at 30°C. Subsequently,

the beads with bound MST3 were removed and the supernatants containing the SHP-NDR proteins were analysed for activity and phosphorylation status using α-Ser282-P and α-Thr442-P specific phospho-antibodies. Kinase assays were carried out for 30 min with the NDR substrate peptide (29). (We had previously established that activated MST3 does not significantly phosphorylate the NDR substrate peptide).

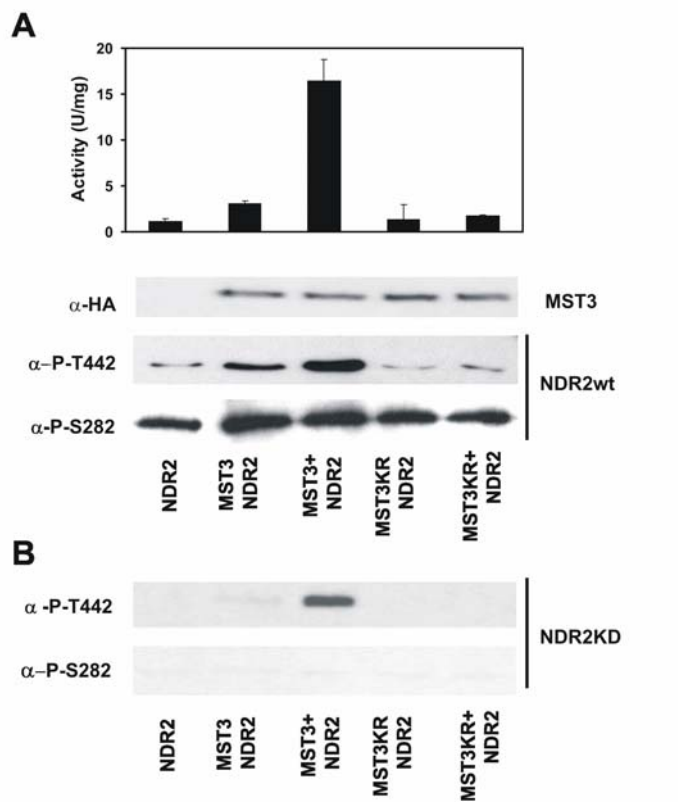

Fig. 2. Activation and hydrophobic motif phosphorylation of NDR2 by MST3. A. Wild type recombinant SHP-NDR2 was pre-incubated for 60 min with or without immunoprecipitated HA-MST3 or HA-MST3KR from untreated and OA-treated (+) HEK293F cells. The activity of SHP-NDR2 was determined using the NDR kinase substrate peptide. The results shown are means ± S.D. of assays carried out in duplicate, and are representative of two independent experiments. Samples from each pre-incubation were also analysed for phosphorylation by western blotting using α-P-T442 and α-P-S282 antibodies. HA-MST3wt and HA-MST3KR were quantified by western blot analysis using the α-HA 12CA5 monoclonal antibody. **B.** Phosphorylation of kinase-dead NDR2 by MST3. Kinase-dead $NDR2^{K119A}$ was incubated for 60 min with or without immunoprecipitated HA-MST3 or HA-MST3KR from untreated and OA-treated (+) HEK293F cells. Samples were analysed by western blotting as in **A**.

Significantly, pre-incubation with non-stimulated MST3 only led to a two-fold activation of NDR2, while pre-incubation with OA-activated MST3 resulted in a 10-fold increase in NDR2 activity. This result reflected an increase in phosphorylation of both the activation segment phosphorylation site Ser282 and the hydrophobic motif phosphorylation site Thr442 (Fig. 2A). Analysis of the phospho-status of the kinase-dead NDR2 revealed that MST3 phosphorylates NDR2 on Thr442, but not on Ser282 (Fig. 2B). Results from the same experiments performed with the kinase-dead MST3KR confirmed that phosphorylation, as well as activation of NDR2, depends on MST3 activity. Significantly, our results also show that OA treatment of HEK293F cells promotes the activation of MST3 in a similar way to that previously found for NDR (31).

Mechanism of NDR activation by MST3 and MOB1. Next, we sought to define the role of the MOB proteins in regulating NDR activity. Previously, we and others had demonstrated that NDR1 and NDR2 are activated by MOB proteins (3, 11). We performed NDR kinase assays in the presence and absence of MOB1A and MST3 using GST-MOB1A and SHP-NDR2 expressed in *E. coli*, as well as OA-stimulated (1 μM for 1 h) and non-stimulated GST-MST3 expressed in HEK293F cells. Proteins were affinity purified on glutathione-Sepharose (GST-MOB1A and GST-MST3) and Ni-NTA-Sepharose (SHP-NDR2), respectively. We performed time-course kinase assays (0-90 min) with various combinations of the proteins employing the NDR substrate peptide. NDR2 alone showed only a moderate increase in kinase activity over the time course (Fig. 3A). Addition of MOB1A resulted in a three- to four-fold increase in kinase activity compared with NDR2 alone during the time course (Fig. 3A). Addition of activated MST3 led to a rapid initial increase in kinase activity that reached a maximum after 30 min and remained approximately constant for the

remainder of the time course (Fig. 3A). Addition of activated MST3 together with MOB1A resulted in a 20-fold higher NDR activity (Fig. 3A), suggesting that both MOB1A and MST3 kinase are required for full kinase activation. Non-stimulated MST3 kinase had no significant effect on NDR2 kinase activity either in the presence or absence of MOB1A (data not shown), indicating that activation of MST3 is crucial for NDR activation. However, the Thr442 phosphorylation obtained with wild-type NDR2/MOB1A suggests a minor contribution of hydrophobic motif phosphorylation by NDR2 itself *in vitro*, as previously reported (41).

To obtain more detailed information about NDR2 activation by MST3 and MOB1A, we performed *in vitro* activation and phosphorylation time courses with kinase-dead and MOB binding-deficient NDR2 mutants (3). The SHP-NDR2 mutants were expressed and purified as described above for wild-type SHP-NDR2. As expected, the MOB1 binding-deficient mutant $NDR2^{Y32A}$ was not activated by MOB1A and showed only a basal activity similar to wild-type NDR2 (Fig. 3B). Addition of activated MST3 resulted in a five- to six-fold increase in NDR activity to levels similar to those observed with wild type NDR2 (Fig 3A, B). The activation profiles and phospho-blots of the $NDR2^{Y32A}$ mutant in the presence of MOB1A showed patterns similar to the corresponding profiles and blots of wild type NDR2 in the absence of MOB1A (Fig. 3A, B). The kinase-dead mutant showed the same degree of Thr442 phosphorylation in the presence and absence of MOB1A, indicating that MOB1A does not affect phosphorylation of NDR by MST3.

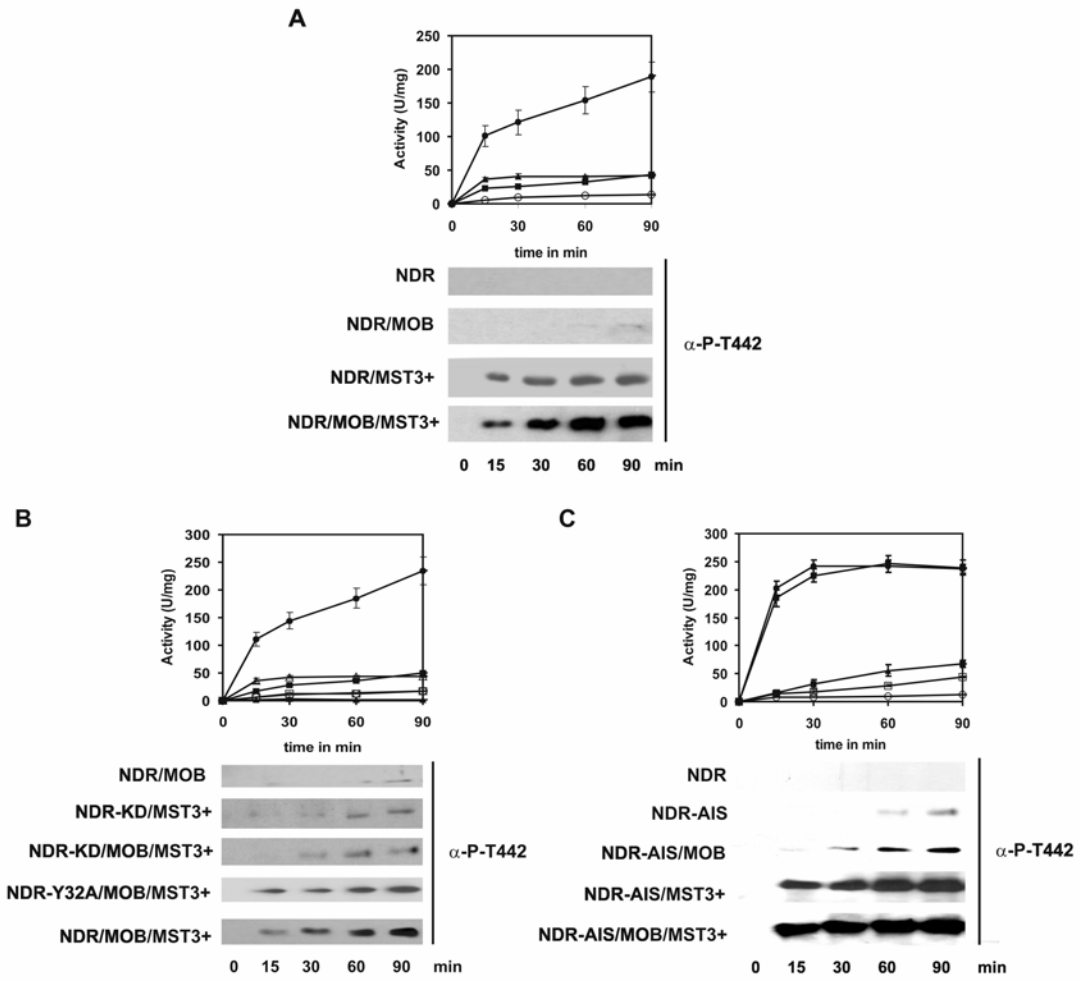

Fig. 3. Analysis of NDR2 activation and hydrophobic motif phosphorylation by MST3 and MOB1A. A. Activation and phosphorylation of wild-type NDR2. NDR2 SHP-NDR2 was incubated with GST-MOB1A (■), GST-MST3 from OA-stimulated HEK293F cells (▲), both (•) or alone (O) using our standard NDR kinase assay conditions. **B.** Activation and phosphorylation of kinase-dead and MOB binding-deficient NDR2. SHP-NDR2 (O), SHP-NDR2/GST-MOB1A (■), SHP-NDR2/MOB1A/MST3+ (•), SHP-NDR2^{K119A}/MOB1A/ MST3+ (▲), SHP-NDR2^{Y32A} (□), SHP-NDR2^{Y32A}/MOB1A/ MST3+ (Δ) were incubated using standard NDR kinase assay conditions. **C.** Activation and phosphorylation of the NDR2-AIS mutant. SHP-NDR2 (O), SHP-NDR2-AIS (□), SHP-NDR2-AIS/GST-MOB1A (▲), SHP-NDR2-AIS/MST3+ (■), SHP-NDR2-AIS/GST-MOB1A/MST3+ (•) were incubated using standard NDR kinase assay conditions. At the time points indicated, NDR kinase activity was assayed with the NDR substrate peptide; results are expressed as specific activity. Results shown are means ± S.D. of assays carried out in duplicate and representative of two independent experiments. Error bars are only shown when larger than the size of the symbols. NDR hydrophobic motif phosphorylation was determined at each time point by western blotting using the α-P-T442 antibody.

The Thr442 phosphorylation obtained with the wild type NDR2/MOB1A/MST3+ and $NDR2^{Y32A}$/MOB1A/MST3+ assays were similar, indicating that MOB1A promotes an active conformation and release of autoinhibition of the kinase. In agreement with these data, the NDR2-AIS (autoinhibitory sequence) mutant (3) that is released from autoinhibition showed an increase in basal activity; addition of MOB1A resulted in a further two-fold increase in NDR-AIS activity (Fig. 3C). Incubation of NDR2-AIS with MST3+, and NDR2-AIS with MOB1A and MST3+ resulted in very similar activation profiles, indicating that release of autoinhibition and hydrophobic motif phosphorylation are sufficient for full kinase activation (Fig. 3C). We conclude that activation of NDR2 is a multi-step process involving phosphorylation of the hydrophobic motif site Thr444/2 by MST3, autophosphorylation of Ser281/2 and binding of MOB1A.

NDR hydrophobic motif site phosphorylation by MST3 *in vivo*. To examine the role of MST3 in NDR activation *in vivo,* we performed cotransfection experiments with HA-NDR2, myc-MST3 and myc-MST3KR using COS-7 cells. Analysis of the phosphorylation of NDR revealed a small but significant increase in Thr442 phosphorylation in cells expressing MST3 and NDR2 compared with cells expressing NDR2 alone (Fig. 4A). The extent of NDR2 phosphorylation was less than that obtained after OA stimulation, as shown by the α-P-Thr442 (Fig. 4A). Cells expressing the kinase-dead variant MST3KR and NDR2 showed a reduction in Thr442 phosphorylation (Fig. 4A). Inhibition of Thr442 phosphorylation by the kinase-dead MST3KR indicates that the mutant protein acts as an inhibitor of Thr442 phosphorylation mediated by endogenous MST3 (see below). Evidence in support of this hypothesis comes from the inhibition of OA-induced phosphorylation of the NDR2 hydrophobic motif by cotransfection with MST3KR, which resulted in 70%

lower NDR activity and Thr442 phosphorylation (Fig. 4B). Ser282 phosphorylation was largely unchanged (Fig. 4B). This indicates that MST3KR acts as an inhibitor of Thr442 phosphorylation. The reduced phosphorylation was also reflected by decreased NDR activity.

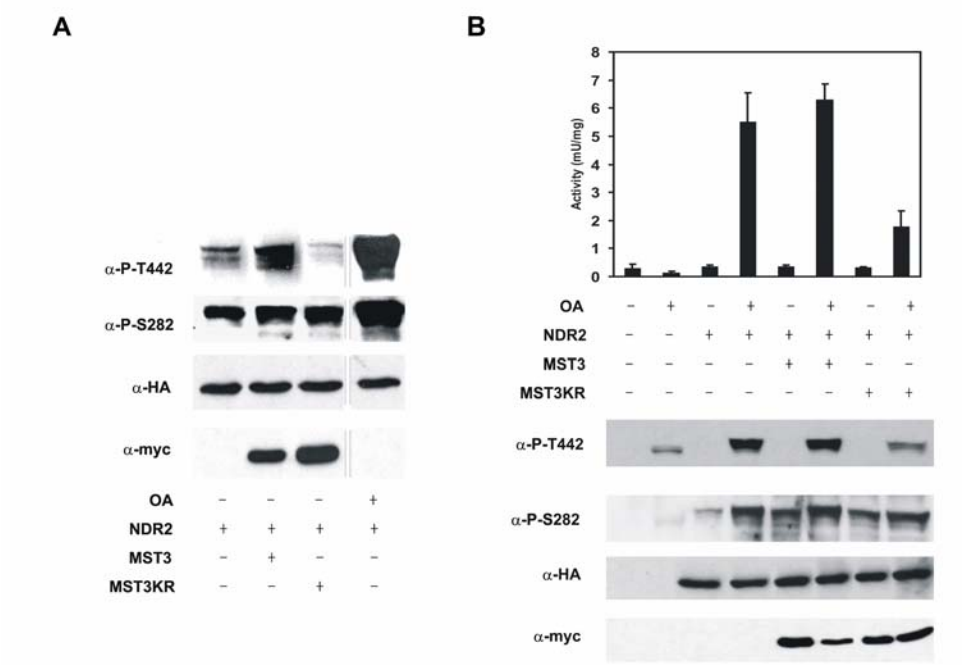

Fig. 4. NDR activation and phosphorylation by MST3 *in vivo*. A. The effect of cotransfection of MST3 on NDR2 phosphorylation. COS-7 cells were cotransfected with myc-MST3, kinase-dead myc-MST3KR and HA-NDR2. OA-treated cells transfected with HA-NDR2 were used as a control for NDR2 phosphorylation. All proteins migrated at the expected molecular mass. Cell lysates were immunoblotted with α-P-T442, α-P-S282, α-HA and α-myc. **B.** Inhibition of NDR2 activation and hydrophobic motif phosphorylation by MST3KR. COS-7 cells expressing HA-NDR2, wild-type myc-MST3 and myc-MST3KR in various combinations were treated for 1 h with 1 μM OA or with solvent alone. HA-tagged NDR kinase variants were then immunoprecipitated (100 μg of cell-free protein extracts) with α-HA 12CA5 monoclonal antibody and assayed for kinase activity using the NDR peptide substrate. Bars represent the mean ± S.D. of triplicate immunoprecipitates and are representative of two independent experiments. All cell lysates were immunoblotted with α-P-T442, α-P-S282, α-HA 12CA5 and α-myc.

Taken together, our data indicate that MST3 can act *in vivo* as an NDR hydrophobic motif kinase. We tested this hypothesis further using NDR2-MST3 fusion proteins.

To create the NDR2-MST3 fusion protein, a linker of 16 amino acids composed of an alternating copolymer of glutamate and glycine was linked to the NDR2 C-terminus and fused to MST3 in frame, as described for ERK2-MEK1 fusions (39). HA-tagged NDR2-MST3, NDR2KD-MST3 and NDR2-MST3KR fusion proteins were expressed in HEK293F cells and analyzed for kinase activity and phosphorylation (FIG. 5). The immunoprecipitated NDR2-MST3 fusion protein was 50- to 100-fold more active than NDR2 from non-stimulated HEK293F cells. The activity was even higher than the previously described constitutively active NDR2-PIF variant (FIG. 5) (41). The NDR-PIF variant contains the PRK2 hydrophobic motif (EEQEMFRDFDYIADW fused at amino acid 434), which has an Asp residue instead of a Thr or Ser residue, and is able to stabilize the kinase in an active conformation, as described for PKB (51). Control fusions with kinase-dead NDR2 were inactive in the kinase assay, indicating that MST3 did not affect the activity measurements. Analysis of the NDR variants with phospho-specific α-P-T442 antibodies showed that NDR2-MST3 and NDR2KD-MST3, but not NDR2-MST3KR, are phosphorylated on the hydrophobic motif phosphorylation site without OA stimulation, suggesting that the interaction is direct. Similar results were obtained with NDR1-MST3 fusion proteins (data not shown).

Activation and phosphorylation of NDR by endogenous MST3. Membrane targeting of NDR leads to phosphorylation and activation of the kinase (17). Constitutive membrane targeting of NDR or MOB using the Lck myristoylation/palmitoylation motif (MGCVCSSN), or inducible membrane targeting using the C1-domain derived from PKCα (amino acids 26 to 126), results in an increase of hydrophobic motif phosphorylation and NDR activity (17).

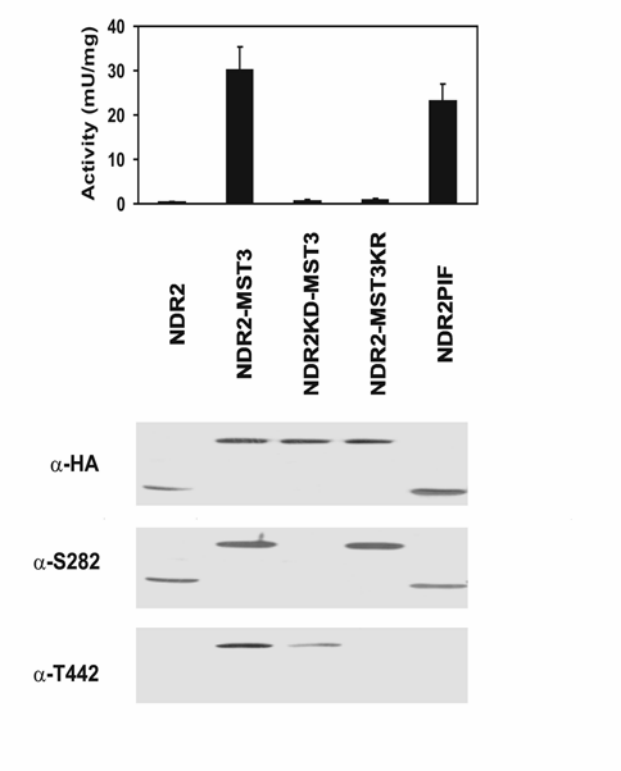

Fig. 5. Activity of NDR2-MST3 fusion proteins. HA-tagged variants of NDR2, NDR2-MST3, NDR2KD-MST3, NDR2-MST3KR and NDR2-PIF in the pcDNA3.1 vector were expressed in HEK293F. The NDR kinase variants were then immunoprecipitated (100 µg of cell-free protein extracts) with α-HA 12CA5 monoclonal antibody and kinase activity assayed using the NDR peptide substrate. *Bars* represent the mean ± S.D. of triplicate immunoprecipitates and are representative of two independent experiments. Additionally, all immunoprecipitates were subjected to SDS-PAGE and western blotting using α-HA 12CA5, α-P-S282 and α-P-T442 antibodies. All NDR variants migrated at the expected molecular weight.

To further establish the *in vivo* role of MST3 in regulating endogenous NDR, we transfected HEK293F cells with a myc-C1-MOB1A chimera and different MST3 constructs designed to manipulate the levels of the upstream kinase. The cells were stimulated, after 24 h starvation, with 100 ng/ml TPA or solvent control for 15 min, and hydrophobic motif phosphorylation of endogenous NDR was determined using α-P-Thr442 antibodies (Fig. 6A). TPA treatment promoted recruitment of NDR to the membrane and promoted phosphorylation of endogenous NDR. Overexpression of HA-MST3 increased the phosphorylation of Thr442 in unstimulated and stimulated

cells. Knockdown of MST3 protein (Fig. 6A) led to almost complete inhibition of NDR2 phosphorylation.

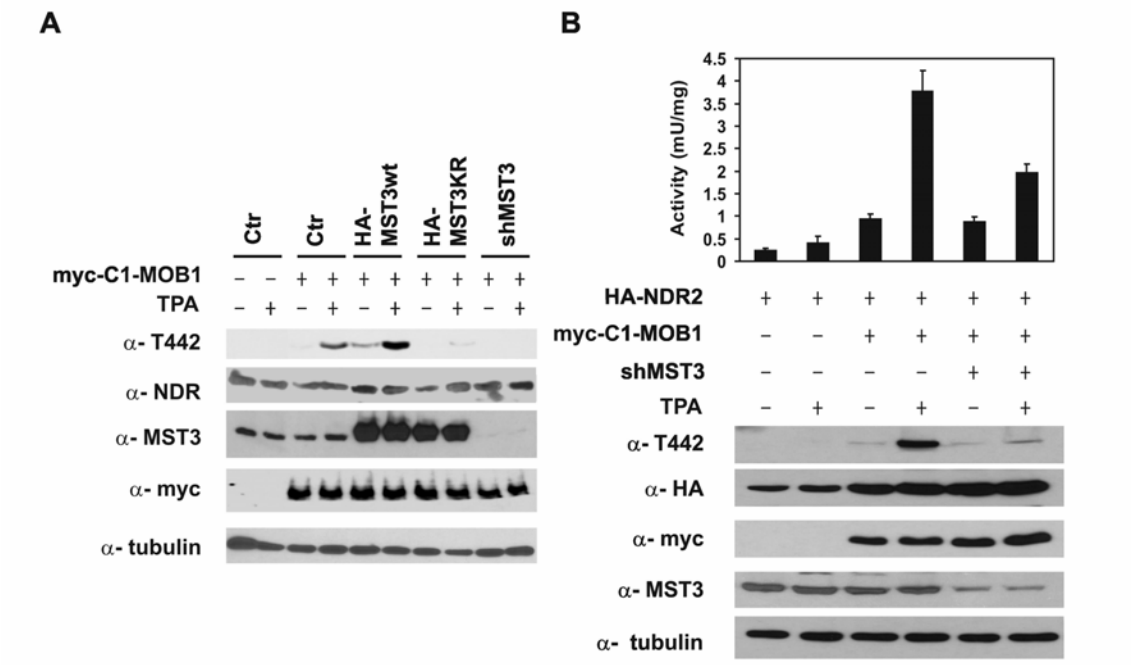

Fig. 6. Phosphorylation of endogenous NDR by endogenous MST3. A. Myc-C1-MOB1A was expressed in HEK293F cells transfected with either pcDNA3.1-HA-MST3, pcDNA3.1-HA-MST3KR or pTER-shMST3. Two days after transfection, the cells were starved for 24 h and then stimulated for 15 min with TPA (100 ng/ml) prior to harvesting. All cell lysates were subjected to SDS-PAGE and immunoblotted with α-P-T442, α-NDR-NT, α-MST3, α-myc and α-α-tubulin control. **B.** Phosphorylation and activation of NDR2 by endogenous MST3. HA-NDR2 was expressed in HEK293F cells and transfected with either pcDNA3.1 myc-C1-MOB1A and/or pTER-shMST3. Two days after transfection, the cells were starved for 24 h and subsequently stimulated for 10 min with TPA (100 ng/ml) prior to harvesting. HA-NDR2 was immunoprecipitated with α-HA 12CA5 monoclonal antibody and assayed for kinase activity using the NDR peptide substrate. Bars represent the mean ± S.D. of duplicate immunoprecipitates and are representative of two independent experiments. All cell lysates were subjected to SDS-PAGE and immunoblotted with α-P-T442, α-HA, α-MST3, α-myc and α-α-tubulin control.

Furthermore, our results show that MST3KR also inhibits phosphorylation of endogenous MST3. The results show a clear correlation between MST3 levels and hydrophobic motif phosphorylation of NDR following TPA-induced membrane

recruitment of C1-tagged-MOB1A, indicating that MST3 is indeed a physiological regulator of NDR *in vivo*. To investigate the effects of MST3 on NDR kinase activity, we used the inducible membrane targeting method described above. We transfected HEK293F cells with HA-NDR2, myc-C1-MOB1A and pTER-shMST3, and determined the activation of NDR and hydrophobic motif phosphorylation (Fig. 6B). After incubation with TPA for 10 min, the activation of NDR was significantly diminished (~50%) in samples cotransfected with pTER-shMST3. The activation was only partially inhibited because we were unable to achieve full knockdown of MST3. In agreement with our results obtained with endogenous NDR, Thr442 phosphorylation of HA-NDR2 showed a clear dependence on MST3, indicating that membrane recruitment of the NDR/MOB complex is crucial for NDR activation and phosphorylation by MST3.

Activation and phosphorylation of MOB1-binding deficient NDR2. To obtain further insights into the role of MOB1 in the activation process of NDR, we examined its activation and phosphorylation following OA treatment using the MOB1-binding deficient mutant $NDR2^{Y32A}$ (3). Despite the fact that $NDR2^{Y32A}$ was phosphorylated on Thr442 to the same extent as wild-type NDR2, activation of the kinase was only two- to three-fold compared with 30-fold for the wild-type control (Fig. 7). This result shows that MOB1 binding is required for full activation and correlates well with the results obtained *in vitro*, arguing that MST3 functions as an upstream kinase and that MOB1 releases autoinhibition.

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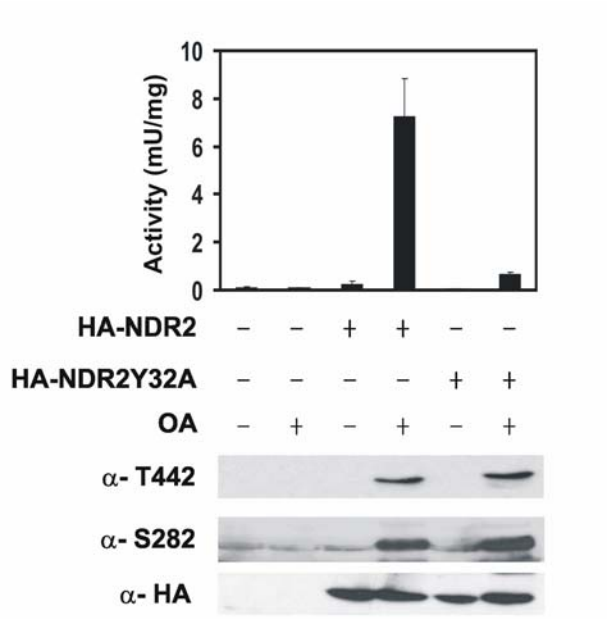

Fig. 7. Activation and phosphorylation of MOB1-binding deficient NDR2. HEK293F cells were transfected with HA-tagged NDR2 and NDR2^{Y32A} and variants and stimulated for 1 h with 1 μM OA or solvent prior to harvesting. The NDR kinase variants were then immunoprecipitated (100 μg of cell-free protein) with α-HA 12CA5 monoclonal antibody and assayed for kinase activity using the NDR peptide substrate. Bars represent the mean ± S.D. of duplicate immunoprecipitates and are representative of two independent experiments. Additionally, all lysates were subjected to SDS-PAGE and immunoblotted using α-P-T442, α-P-S282, and α-HA 12CA5 antibodies.

Colocalisation of MST3 and NDR. We examined the intracellular localisation of GFP-MST3, HA-MST, HA-MST3KR and myc-NDR2 in COS-7 cells. MST3 has previously been shown to localise predominantly to cytoplasmic structures (18), but translocates to the nucleus upon caspase cleavage. We confirmed the localisation of full-length MST3 in our experimental conditions with two different epitope tags (GFP and HA) (Fig. 8A). To evaluate whether NDR2 and MST3 localise to similar cytoplasmic structures, we co-expressed MST3 and NDR2 in COS-7 cells. Examination by confocal microscopy demonstrated a largely overlapping localisation of MST3 and NDR2 to cytoplasmic and membrane structures (Fig. 8B), indicating that these proteins are indeed able to interact *in vivo*.

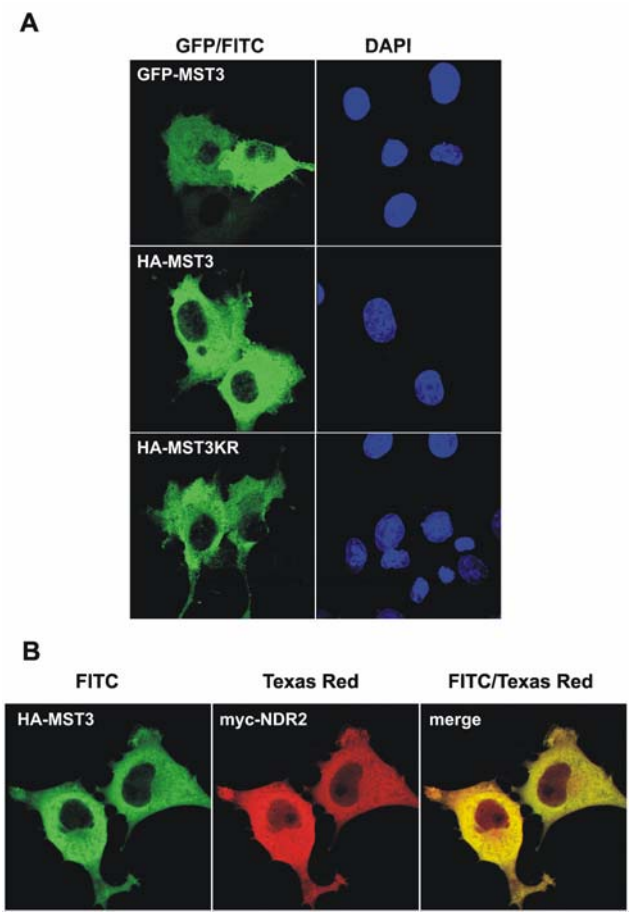

Fig. 8. Co-localisation of NDR2 and MST3 in COS7 cells. A. Localisation of MST3. COS-7 cells expressing GFP-MST3 (upper panels), HA-MST3-wt (middle panel) or HA-MST3KR (lower panels) were processed for immunofluorescence using either no antibody (upper right) or α-HA Y11 (lower right). Anti-HA antibody was visualised using anti-rabbit FITC (green) and DNA stained with 1 μM TO-PRO-3 iodide (Molecular Probes Inc) (blue; left panels). **B.** Colocalisation of NDR2 and MST3. COS-7 cells expressing HA-MST3 and myc-NDR2 were processed for immunofluorescence using α-HA Y11 and α-myc 9E10. α-HA Y11 antibody was visualised using anti-rabbit FITC (green), anti-myc antibody was visualised using anti-mouse Texas Red (red). Representative pictures of HA-MST3 and myc-NDR2 localisation are shown.

We further investigated whether the proteins form a stable complex. As expected, employing the myc-C1-MOB1A construct, TPA stimulation led to recruitment of HA-NDR2 to the membrane fraction, thereby activating and phosphorylating NDR2 at the hydrophobic motif (see also Hergovich *et al*., 2005).

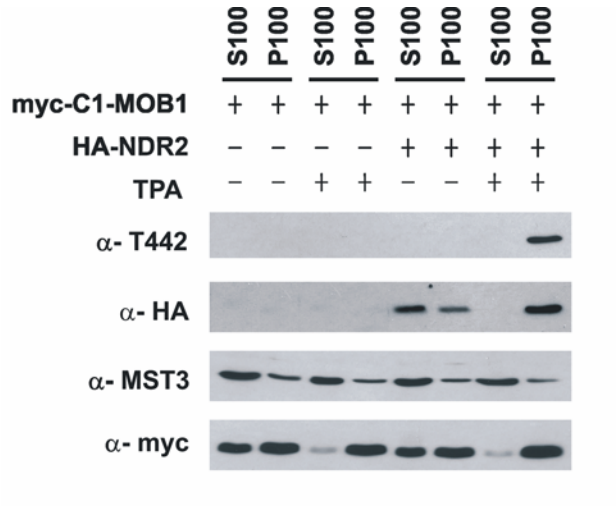

Fig. 9. Fractionation of HA-NDR and MST3 after membrane recruitment of myc-C1-MOB1A. HEK293F cells were transfected with the HA-NDR2 and myc-C1-MOB1A constructs indicated and subjected to S100/P100 fractionation (S, cytoplasm; P, membrane), before immunoblotting with α-T444-P, α-HA 12CA5, α-MST3 and α-myc antibodies.

However, the distribution of endogenous MST3 was unchanged in these conditions (Fig. 9). The major population of MST3 was cytoplasmic, but a significant portion was associated with the membrane. Further, MST3 was apparently not detected in HA-NDR2 immunoprecipitates (data not shown). This indicates that MST3 and NDR2 did not form a stable complex under our conditions, and that membrane recruitment of the NDR/MOB1 complex is crucial for NDR phosphorylation by MST3 *in vivo.*

NDR promotes cleavage of MST3. It has been reported that members of the MST family are frequently cleaved following caspase activation (10, 18, 20, 36). Based on our initial observations (data not shown) that most of the wild-type MST3 protein was efficiently cleaved but the kinase-dead MST3KR was only cleaved to a minor degree in samples harvested 36 h after transfection in COS-7 cells, we asked whether MST3 cleavage depends on the activity of full-length MST3 and whether this regulation involves NDR (Fig. 10A). We co-transfected COS-7 cells with kinase-dead MST3KR

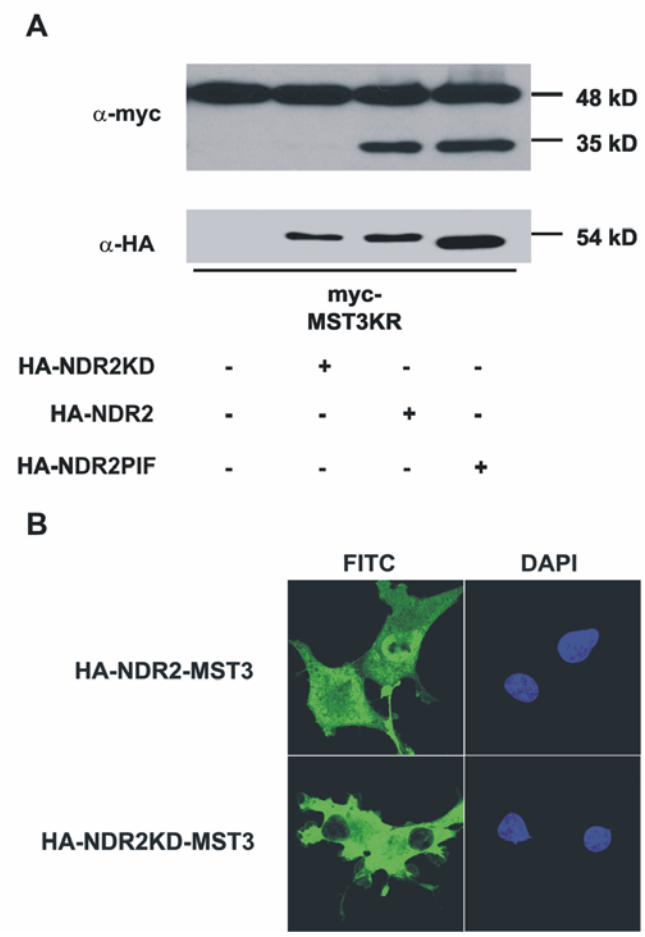

Fig. 10. NDR promotes cleavage of MST3 in COS-7 cells. A. Induction of MST3 cleavage by overexpression of catalytically active NDR2. Lysates of COS-7 cells transfected as indicated with myc-MST3KR, HA-NDR2, HA-NDR2KD and HA-NDR2-PIF were harvested after 36 h and subjected to western blotting using α-HA and α-myc antibodies. **B.** Localisation of NDR2-MST3 fusion proteins. COS-7 cells expressing HA-NDR-MST3 (upper panel) or HA-NDR2KD-MST3 (lower panel) were processed for immunofluorescence 24 h after transfection using α-HA Y11 (right panels). α-HA antibody was visualised using anti-rabbit FITC (green). Corresponding DNA stains (blue; left panels). Representative pictures of HA-NDR2-MST3 and HA-NDR2KD-MST3 localisation are shown.

alone or together with HA-tagged NDR2, NDR2KD and NDR2-PIF. Co-transfection of wild-type NDR2 and NDR2-PIF resulted in significantly higher cleavage of MST3KR than in cells transfected with only MST3KR, whereas co-transfection of MST3KR with kinase-dead NDR2 had no detectable effect on the cleavage of full-length MST3KR (Fig. 10A). This indicates that cleavage of MST3 can be induced by NDR activity. This is also clearly reflected by the localisation of NDR-MST3 fusion

proteins. MST3 was shown recently to contain both a nuclear localisation signal (NLS; amino acids 278-294) and an export signal (NES) located in the C-terminal region (amino acids 335-386) downstream of the kinase domain. The NES is cleaved off (residue 314) after caspase activation, which leads to nuclear translocation of the kinase (23). In COS-7 cells, NDR2-MST3 fusion proteins showed both nuclear and cytoplasmic localisation, whereas an NDR2KD-MST3 fusion was localised almost exclusively in the cytoplasm (Fig. 10B). Our results indicate that NDR can function in a regulatory feedback loop controlling MST3 cleavage.

Discussion

We provide evidence that MST3 functions as a hydrophobic motif kinase of NDR protein kinase and our current model for NDR regulation is summarised in Fig. 11. This is the first demonstration that a STE20-like kinase specifically phosphorylates the hydrophobic motif (Thr442), and not the activation segment phosphorylation site, of NDR. We have established that NDR2 is phosphorylated by activated MST3 (see below) specifically on the hydrophobic motif, leading to an approximately 10-fold activation of the kinase. MST3KR mutants were not able to activate or phosphorylate Thr442, demonstrating that MST3 kinase activity was necessary for NDR activation. Based on the catalytic domain conservation of mammalian STE20-like kinases and yeast STE20-like kinases, it is likely that the mechanism of NDR family kinase phosphorylation by STE20-like kinases is conserved, similar to many of the components of this signalling network (42). The mechanism of regulation of NDR kinases reported in yeast and flies, for example the Cdc15p-Dbf2p and Kic1p-Cbk1p interactions in *S. cerevisiae*, or the HIPPO-WARTS interaction in *D. melanogaster*, is probably through hydrophobic motif phosphorylation. We propose that NDR2

hydrophobic motif phosphorylation functions in a manner analogous to the reported disorder-to-order conformational transitions described for PKB (51). A similar disorder-to-order transition mechanism is probable for the other NDR-related kinases. It will be interesting to investigate whether other members of the STE20 group of protein kinases function as hydrophobic motif kinases for other AGC kinases.

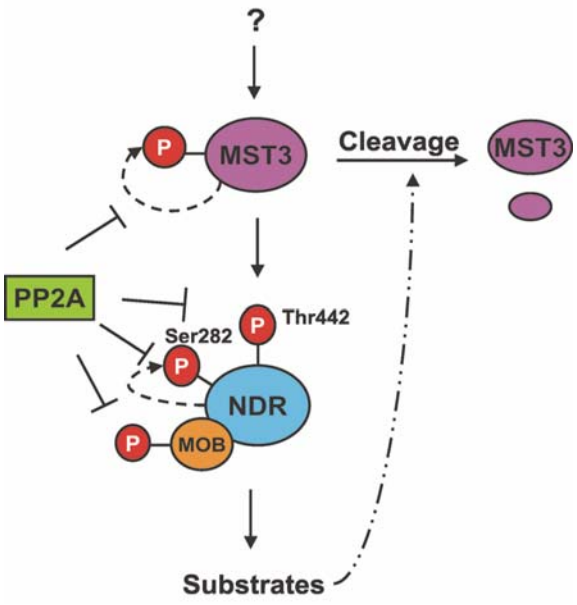

Fig. 11. Model of NDR protein kinase activation by multisite phosphorylation. MOB and activated MST are required for full phosphorylation and activation of NDR. MOB binding stimulates auto- and transphosphorylation of NDR by releasing autoinhibition, whereas MST3 phosphorylation at the hydrophobic motif results in an active conformation of the kinase. The kinase complex is negatively regulated by PP2A or PP2A-related phosphatase. Activated NDR kinase in turn promotes cleavage of MST3.

Previous results have revealed that MOB proteins play a critical role in the activation of several NDR kinase homologues. Mob1p has been shown to be required for the activation and phosphorylation of Dbf2p by Cdc15p (27), and Mob2p is required for Cbk1p function (6, 49). Recent studies have shown that human MOB proteins interact with NDR1 and NDR2 (3, 11). MOB1A binding induces the release of autoinhibition and thus stimulates autophosphorylation at the activation segment phosphorylation site of NDR kinase (3). Here we provide the first evidence for a functional interaction between mammalian STE20-like kinase, MOB1A protein and NDR2 kinase. *In vitro*,

all three proteins, MST3, MOB1A and NDR2, are required for full kinase activity. MOB1A binding is essential for the release of NDR autoinhibition, thus stimulating auto- and transphosphorylation activity. As expected, the NDR2-AIS mutant showed full activation in the presence of activated MST3, independent of MOB1A binding, supporting the proposed mechanism. Additionally, we provide evidence that the functional interaction between MST3 and NDR2 occurs *in vivo*. Cotransfection of MST3 and NDR2 in COS-7 cells resulted in an increase in Thr442 phosphorylation, whereas cotransfection of MST3KR and NDR2 reduced phosphorylation of the hydrophobic motif. Nevertheless, these *in vivo* changes were lower than those observed after activation of the kinase with OA. This indicates that activation of the pathway is tightly controlled and/or that other upstream regulatory components and proper localisation also play an important role.

Targeting of NDR to the membrane provides further evidence for the importance of sub-cellular localisation in the activation mechanism. Membrane targeting of NDR results in increased hydrophobic motif phosphorylation as well as activation of the kinase (17). Here, we show that the degree of Thr442 phosphorylation clearly depends on MST3 activity. Hydrophobic motif phosphorylation of endogenous NDR is increased by overexpression of HA-MST3 and diminished by expression of kinase-dead MST3KR or RNA interference using short hairpin constructs targeting endogenous MST3. Furthermore, activation of membrane-targeted HA-NDR2 is significantly reduced in cells transfected with pTER-shMST3. However, our results suggest that the interaction between NDR2 and MST3 is likely to be transient. Recruitment of NDR to the membrane via myc-C1-MOB1 resulted in phosphorylation of the kinase. MST3 was not co-recruited, but a significant portion of MST3 was already associated with the membrane. This indicates that the interaction between

MST3 and NDR2 is not stable, similar to the interaction reported between MST1/2 and the NDR-relative LATS1/WARTS (5, 20). However, it has also been reported for other kinase substrate interactions that the affinity for their substrate is weak (4) and below the detection limit of immunoprecipitation (34). We propose that membrane recruitment of NDR is an essential part of the NDR activation mechanism and that the phosphorylation of NDR by MST3 occurs at the membrane. However, future experiments will have to address the question of what signals are responsible for membrane recruitment of the NDR/MOB complex *in vivo.* It is also possible that NDR is targeted to the membrane to phosphorylate proteins specifically localised to this region of the cell.

MST3 purified from OA-treated cells showed significantly more activity in the cell-free assays compared with the enzyme isolated from untreated cells. This indicates that MST3 activity is regulated by phosphorylation that can be initiated by treating cells with the PP2A inhibitor OA. This indicates that activation of the kinase cascade is likely to be tightly controlled *in vivo*. The mechanism of MST3 regulation has not yet been fully elucidated, but the available results provide an explanation for the dramatic effects of OA on NDR activity. Previously, we found that MOB1A binding to NDR is apparently facilitated by treatment of cells with OA (3). We conclude that PP2 or a PP2A-type phosphatase negatively regulates both kinases and MOB1A function; the key issue in the future will be to understand the regulation of the phosphatase in this signalling network.

Significantly, we provide the first evidence that cleavage of MST3 can, at least partially, be induced by NDR in a regulatory feed back loop. NDR2 overexpression can induce cleavage of MST3 and MST3KR, whereas the kinase-dead NDR2^{K119A} had no effect on cleavage. This indicates an involvement of NDR in the promotion of

cleavage. Interestingly, caspases-3, 7, 8 and 9 are processed in cancer cells infected with an adenovirus expressing the human LATS2 kinase, a close relative of NDR. Further, it was shown that the *Drosophila melanogaster* WARTS kinase, together with the STE20-like kinase HIPPO, regulates the levels of DIAP1 (Drosophila inhibitor of apoptosis) (16, 19, 33, 44, 50). Mammalian IAP proteins are known inhibitors of caspase activation (25, 40), but also play a role in signal transduction and cell cycle regulation (1, 38).

Our results provide a molecular mechanism for the interaction and regulation of STE20-like kinases with NDR kinases, which is likely to be conserved from yeast to mammals. MST3 functions as a hydrophobic motif kinase that promotes NDR activation in a MOB protein-dependent mechanism (Fig. 11). Our earlier studies (31) also illustrate the importance of PP2A or PP2A-like protein phosphatase in negatively regulating this signalling pathway. Genetic studies with yeast, worms and flies have shown that many of the components of the NDR signalling pathway are highly conserved, such as the scaffold protein Pag1p (mor2p, sax-2, FURRY), the STE20-like kinase Kic1p (gck-1), the adapter protein Hym1p (dMO25), the activator protein Mob2p (dMOB proteins), and the transcription factor Ace2p (Krueppel-like transcription factor) (42). Based on the conserved protein components, the NDR signalling pathway is more closely related to the signalling pathway established for Cbk1p than to Dbf2p (which appears to be more closely related to LATS). Our work on the human orthologues indicates that conservation is also maintained with the mammalian counterparts of some of these proteins. However, despite the high conservation of the proteins, the processes they regulate are likely to differ due to species variation between yeast (e.g. regulation of chitinase expression) and higher organisms. Further work is required to fully elucidate the functions of these different

proteins. However, the involvement of the yeast NDR kinases in the MEN (mitotic exit network) and RAM (regulation of Ace2p transcription and morphogenesis) network (42) raises the possibility of a cell-intrinsic regulation of NDR activity, which might explain why all attempts (growth hormones, oxidative stress, UV, ceramide, fluoride, serum) to identify a ligand/hormone or endogenous stimuli have, so far, failed (2).

Significant insights into the physiological roles of NDR kinases have been obtained using yeast, worm and fly genetic screens. Early studies from yeast found that NDR family kinases play a significant role in the regulation of actin and tubulin cytoskeletal organisation during polarised growth and cytokinesis, and are central components of the mitotic exit network (42). Worm NDR regulates cell spreading and neurite outgrowth of chemosensory neurons (52). The fly NDR is reported to be important for the integrity of cellular outgrowths such as wing hairs and bristles (14). Recently, it has been shown that NDR kinase activity plays an important role in the regulation of dendritic branching and tiling in worms and flies (12, 13). These results predict that mammalian NDR also plays a significant role in the control of cell division and morphogenesis. Identification of NDR-specific substrates will help to define the physiological roles of this conserved signalling pathway.

Acknowledgements

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9. NDR1 deficiency in mice

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Abstract

NDR protein kinases are a conserved family of Ser/Thr kinases that play pivotal roles in regulating cell cycle progression and cell morphology. Genetic and biochemical data revealed that NDR kinases are part of a conserved signalling module consisting of MOB and mammalian STE20-like protein kinases. These studies address the question of functional implications of human NDR kinases using NDR1 knockout mice. We report that NDR1-knockout mice are viable and do not show any apparent morphological abnormalities. However, aged NDR1 knockout mice showed incidences of hepatis peliosis and lymphomas as well as enlarged seminal vesicles.

Introduction

The NDR family of serine/threonine kinases are members of the AGC group of serine/threonine kinase which are implicated in the regulation of cell cycle progression and cell morphology. In *Saccharomyces cerevisiae* there are three NDR kinases: Dbf2p and the redundant Dbf20p kinase are members the mitotic exit network (MEN), a signalling module which coordinates the inactivation of cyclin-dependent kinases and cytokinesis at the end of mitosis (23, 27); and Cbk1p is important for polarized growth, cell separation and daughter-cell-specific gene activation (5, 6, 35). In *Saccharomyces pombe* there are two NDR kinase family members: the Dbf2p orthologue and member of the MEN-analogous septation initiation network (SIN) Sid2p; and Orb6p, a Cbk1p orthologue, which is likewise required for the maintenance of cell polarity. In *Caenorhabditis elegans* there are two NDR kinase family members: the tumour suppressor-like protein 1K868, which has

not yet been characterised; and SAX-1, which has been reported as having an important role in regulating dendritic branching and tiling as well as cell spreading and neurite outgrowth of chemosensory neurons (12, 38). The *Drosophila melanogaster* NDR kinase family members are WARTS/LATS and TRICORNERED. Mutations of WARTS/LATS are tumour suppressors that lead to tissue overgrowth in affected tissues or clones as well as developmental lethality in embryonic, larval, pupal or pharate-adult stages in different allelic mutations. (19, 37). TRICORNERED mutations are also embryonically lethal. Clonal mutations in the fly's wing hairs, bristles or antennae result in a splitting and branching phenotype of the cellular outgrowths. Just like SAX-1 mutations in the worm, TRICORNERED mutations have also been shown to cause a branching and tiling phenotype in sensory neurons.

In humans and mice there are four NDR kinases: LATS1 (also termed WARTS) and LATS2 as well as NDR1 and NDR2 (33). LATS1 knockout mice develop soft tissue carcinomas and ovarian tumours and are highly susceptible to carcinogenic treatment (28). Additionally, expression of a LATS1 mutant has been shown to cause prolonged delay in anaphase entry, and tetraploidy (16). Furthermore, LATS1 negatively regulates cell cycle progression (36). LATS2 knockout mice are embryonically lethal due to overgrowth of the mesodermal lineage. Knockout MEFs (mouse embryonic fibroblasts) show increased proliferation and lack of contact inhibition, as well as centrosome amplification, aberrant cytokinesis and genomic instability (24). Furthermore, LATS1 and LATS2 are thought to regulate IAP proteins, known inhibitors of caspase activation (10, 22), and promote apoptosis (21, 36).

However, little is known about the function of mammalian NDR1 and NDR2 kinases. *Ndr1* mRNA is mainly expressed in the spleen, thymus and lungs, and at lower levels in the brain, heart, liver, testes and muscle, whereas *Ndr2* mRNA is ubiquitously

expressed, and especially high in the gastrointestinal tract (29). *Ndr1* mRNA is upregulated in progressive ductal carcinoma *in situ* (1), and *Ndr2* is upregulated in the highly metastatic non-small cell lung cancer cell line NCI-H460 (26). In addition, *Ndr2* mRNA is upregulated in the amygdalla of fear-conditioned mice and has been shown to affect cell spreading and neurite outgrowth of PC12 cells (30). In contrast, NDR1 and NDR2 kinases are well characterised in biochemical terms. NDR kinases are part of a conserved signalling module regulated by the release of autoinhibition by binding to MOB1A protein, hydrophobic motif phosphorylation mediated by MST3, and dephosphorylation by protein phosphatase PP2A (4, 8, 15, 25) (Stegert *et al.*, chapter 8.). Here we address the biological function of NDR protein kinase by generating NDR1 knockout mice by targeted disruption. Heterozygous matings yield offspring according to the Mendelian inheritance pattern. The NDR1 knockout mice are viable and fertile, and grow normally. Pathological analysis of 14-week-old NDR1 knockout mice has revealed no major abnormalities apart from a 14% decrease in testis weight compared to wild type mice, but there is no clear correlating microscopic finding. Microscopically, no major histomorphological differences were noted in NDR1 mice when compared to the wild type. However, 20- to 30-month-old NDR1-knockout mice showed a frequent occurrence of tumours and tumour-like lesions, especially in the liver and the lymphatic system.

Materials and methods

Targeted disruption of the Ndr1 gene by homologous recombination

An ~9 kb *BamHI-NotI* fragment containing exons 4, 5 and 6 was amplified from BAC clone 25140 (244/G01) (Incyte Genomics) using Expand Long Template Taq polymerase (Roche), and subcloned in pMCS5. A 5 kb IRES/*lacZ*/*Neo* cassette was

inserted in the XhoI site of exon 4. The targeting vector was linearised using the SalI site and electroporated into 129/Ola ES cells. An external probe was used for ES cell Southern screening following *Kpn*I digestion. An internal probe and a *lacZ*-Neo probe were used to characterize ES clones positive for homologous recombination (data not shown). Correctly targeted ES cells were used to generate chimeras. Male chimeras were mated with wild type C57BL/6 females to obtain *Ndr1* +/– mice. *Ndr1* +/– mice were backcrossed for at least four generations with pure C57BL/6 mice. The progeny of *Ndr1* +/– intercrosses were genotyped by multiplex PCR with the following three primers: (1) Ex4checkb, 5'-GTCTTCTCATCGCTGTCACAGCT-3'; (2) Neo-2, 5'-GCTGCCTCGTCCTGCAGTTCATTC-3'; and (3) 6540bk, 5'-GCTCCCGCTCAGT-TACCTGCTCC-3'.

Microarray analysis

Microarray analysis was performed using murine U74 GeneChips™ (Affymetrix). Total RNA (10 µg) was reverse-transcribed using the SuperScript Choice system for cDNA synthesis (Life Technologies) and biotin-labelled cRNA generated using the Enzo BioArray High Yield RNA transcript labelling kit (Enzo Diagnostics) following the manufacturer's protocol. cRNA fragmentation and hybridisation were performed as recommended by Affymetrix. Expression data were calculated using the RMA algorithm from BioConductor (17). A gene was considered to be significantly altered in its expression if it had an Affymetrix change *P*-value of less than 0.003 for either increase or decrease in both replicate comparisons, and it had a minimum expression value of 80 in at least one condition. A fold-change threshold of 1.5 was then applied and the resulting genes were subjected to a one-way ANOVA with a *P*-value cut-off of 0.05. A Benjamini and Hochberg multiple testing correction and a Tukey post-hoc test were applied.

Histopathology and haematology

Animals were euthanized after exposure to carbon dioxide followed by exsanguination. For calculation of organ weights relative to body weight, the terminal body weight measured before exsanguination at necropsy were used. In addition, organ weights were calculated relative to the brain weight. Brian, heart, kidneys, liver, ovaries, spleen, testes and thymus were weighed. The following tissues were processed for further examination: adrenals, bone marrow, brain, caecum, colon, duodenum, gall bladder, heart, ileum, jejunum, kidneys, knee joint, liver, lung, auxiliary and mesenteric lymph nodes, ovaries, pancreas, peripheral nerve, pituitary, rectum, spleen, sternum, skeletal muscle, skin, spinal cord, sternum, stomach, testes, thymus, thyroid with parathyroid and trachea.

All tissues were fixed in neutral phosphate-buffered formalin. Bone was demineralised with 10% formic acid. The tissues were embedded in Paraplast® (McCormick Scientific). Sectioning was at (nominally) 4 µm and all tissue samples stained with haematoxylin and eosin. All histologically processed tissues were subjected to microscopic examination. Data for all investigations were, whenever possible, recorded online, using software packages specifically designed for the purposes of the test facility/sites (PathData® v. 5.1). Where online recording was not possible (organ weights and macroscopic examinations), handwritten raw data sheets were used. The data were subsequently entered manually into the computer and the raw data sheets were archived. For organ weight data, an automated program was used to decide whether parametric or non-parametric group comparisons should be made. This program used Kolmogorov's test to examine the normality of the data, and Bartlett's test to examine the homogeneity of variances. Accordingly, either Dunnett's test or Student's *t*-test for parametric group comparisons, or Dunn's test or

Wilcoxon's test (U-test) for non-parametric group comparisons were used. No statistical analysis was performed for microscopic examinations.

For haematology, blood samples and bone marrow smears of wild type mice and NDR1 mice were taken by terminal bleeding. EDTA (ethylene-diamin-tetra-acetate) blood was used for the haematology determinations. No anticoagulant was used for the clinical biochemistry parameters. Complete blood counts (erythrocytes (RBC), haemoglobin (HGB), haematokrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), total and relative white blood cell count (WBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, LUCs, reticulocytes (including total and relative total, high, medium and low reticulocyte counts) and total platelet count) using the ADVIA 120® analyzer (Diamond Diagnostics) were performed on animals for which an adequate sample was available. Blood smears were prepared and stained with modified Wright stain. Bone marrow smears were prepared using a push smear technique and stained with modified Wright stain. Microscopic evaluation was performed on all blood and bone marrow smears.

Results

Targeting strategy and confirmation of genotype

To investigate the biological function of NDR1, NDR1-deficient mice were generated by targeted disruption of exon 4 as shown in Fig. 1A. The targeted disruption of the NDR1 gene locus was confirmed by Southern blotting and PCR using tail-tissue samples from NDR1 wild type ($NDR1^{+/+}$), heterozygous ($NDR1^{+/-}$) and mutant ($NDR1^{-/-}$) mice (Fig. 1B and C). Microarray analysis showed an ablation of the NDR1 mRNA in spleen samples of NDR1 mutant mice (see table 2). To confirm the absence

of NDR1 at the protein level, western blot analysis was performed on mouse embryonic fibroblasts (MEFs) using an anti-N-terminal NDR antibody, which recognizes both NDR1 and NDR2. In contrast to $NDR1^{+/+}$ MEFs, NDR1 was not detectable in samples derived from mutant mice (Fig. 1D), but NDR2 appeared to be expressed at higher levels in $NDR1^{-/-}$ mice, indicating a compensatory upregulation of NDR2.

NDR1-deficient mice

In order to address the biological function of NDR1, targeted ES cell clones were used to generate $NDR1^{-/-}$ mouse lines. Intercrosses of NDR1-targeted heterozygotes gave rise to $NDR1^{-/-}$ homozygotes at the expected Mendelian frequency (see table 1). We have not detected any effect of NDR1 deficiency on mouse fertility, health or survival up to 1 year of age. Additionally 14-week-old mice (9-10) of each sex and genotype were subjected to a detailed analysis, including organ weight measurements of brain, heart, kidneys, liver, ovaries, spleen, testes and thymus (see table 2) and histological examination of adrenals, bone marrow, brain, caecum, colon, duodenum, gallbladder, heart, ileum, jejunum, kidneys, knee joint, liver, lung, auxiliary and mesenteric lymph nodes, ovaries, pancreas, peripheral nerve, pituitary, rectum, spleen, sternum, skeletal muscle, skin, spinal cord, sternum, stomach, testes, thymus and thyroid with parathyroid and trachea (data not shown). The body weight of $NDR1^{-/-}$ animals was slightly (but statistically not significantly) decreased (by 6%) in both sexes. For the testes, a small weight decrease of 14% was recorded in knockout mice. However, there were no clear correlating microscopic findings. No other organs and tissues of $NDR1^{-/-}$ mice showed any major weight or histopathological differences when compared to wild type mice.

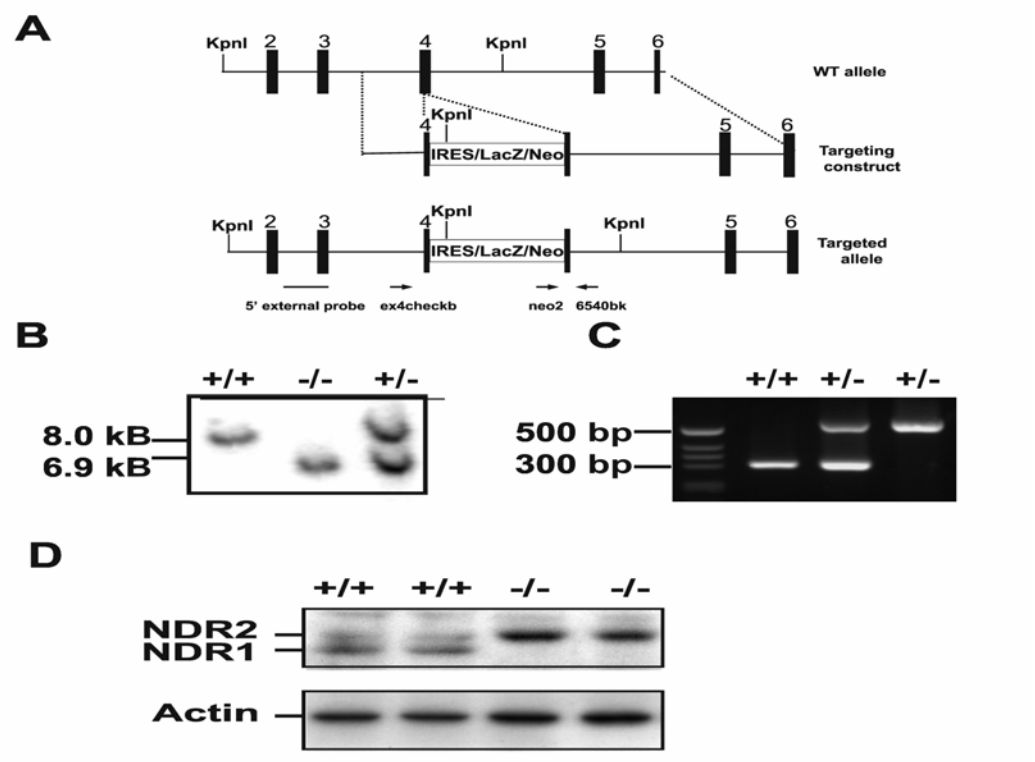

Fig. 1. Ablation of mouse NDR1 by a gene knockout approach. A, Targeting disruption of the mouse *Ndr1* gene locus by insertion of an IRES/lacZ/neo cassette into exon 4 of the kinase. **B,** Southern blot analysis of tail DNA samples obtained from a heterozygous mating. **C,** Multiplex PCR of mouse DNA tail samples. **D,** Western blot analysis of knockout and wild type MEFs using the α-NT-NDR and α-actin antibodies.

Genotype	+/+	-/+	-/-
Number of animals	63	119	60
Obtained ratio	26.03%	49.17%	24.79%
Expected ratio	25%	50%	25%

Table 1. Ratio of NDR1 wild type, heterozygous and knockout animals in comparison to the expected Mendelian ratios.

There were also no significant differences between wild type and NDR1 mice with regards to haematological evaluations.

The lack of NDR1 requirement might reflect a compensatory upregulation of NDR2, as observed in MEFs (Fig. 1D) or the upregulation of other compensatory pathways.

Microarray analyis of NDR1-deficient spleen

In order to evaluate the downstream effects of NDR signalling, we compared duplicate mRNA samples of NDR1-mutant spleen with spleen from its wild type littermate controls, because recent analysis of the expression levels of Ndr1 and Ndr2 mRNA have revealed that Ndr1 is the dominant isoform in the thymus, lung and, especially, spleen, whereas Ndr2 mRNA is ubiquitously expressed, but with very low expression in spleen (see Fig. 2) (29).

RNA was extracted from whole mouse brains, and microarray analysis was performed on three individual brains per genotype and timepoint using murine MG_U74Av2 Affymetrix GeneChips™.

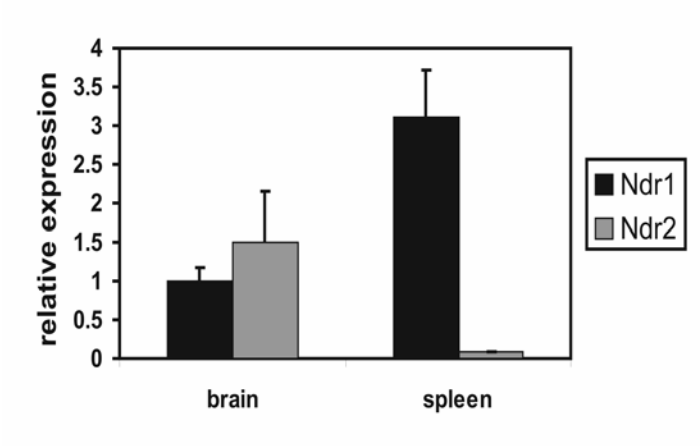

Fig. 2. Relative mRNA expression levels of m*Ndr1* and m*Ndr2* determined by quantitative real-time PCR in brain and spleen as described previously (29). The expression level were normalised to the expression of m*Ndr1* in brain.

Changes below 2.0-fold increase were considered insignificant, and differentially regulated genes were subjected to a Student's *t*-test analysis (Tables 2 and 3). Among the 9761 expressed genes, 74 genes were found to be differentially expressed in $NDR1^{-/-}$ spleens versus $NDR1^{+/+}$ spleens. Of these, 38 genes were upregulated and 36 genes were downregulated. Strikingly, several of the altered genes are associated with similar cellular processes. There are genes involved in heat shock response and folding (e.g. Hspa1a, Hsp105, Bag3), apoptosis and cytotoxicity (Fcgr3, Igh4, Plg,

Bag3, Mst2, Col18a1), protein glycosylation (Siat3, Hs3st3a), angiogenesis and vasoconstriction (Plg, Col18a1, Edn1), regulation of the cytoskeleton (Krt1-1, Dvl2, Coro2B, Tnnt1, Eln), spermatogenesis and sperm motility (Mak1, Tssk1), proteolysis

Description	Genebank	fold	Function
heat shock protein 1A, Hspa1a	M12571	8.6	heat shock, folding
cytochrome c oxidase, subunit Vic,Cox6c	AV071102	7.7	unknown
ubiquitin specific protease 14, Usp14	AV271750	7.4	synaptic transmission
gastric intrinsic factor, Gif	L24191	6.1	ion transport
alanine and arginine rich domain containing protein, Aard	AI390412	4.9	unknown
sialyltransferase 8 A, Siat8a	X84235	4.9	protein glycosylation
laminin, alpha 1	M36775	4.4	cell adhesion, migr.
cAMP responsive element binding protein 1, Creb1	X67719	4.1	transcription
male germ cell-associated kinase, Mak	X66983	4.1	sperm motility
histone 1, H1b	Z46227	3.9	transcription
Prolin rich protein HaeIII family 1, prh1	M23236	3.6	unknown
Fc receptor, IgG, low affinity III, Fcgr3	X60929	3.1	cytotoxicity, phagocytosis
heat shock protein, Hsp105	L40406	3.0	heat shock, folding
aristaless 4, Alx4	AF001465	3.0	transcription
3-phosphoglycerate dehydrogenase, Phgdh	L21027	3.0	amino acid biosynthesis
immunoglobulin heavy chain 4, Igh4	V00793	2.8	cytotoxicity, phagocytosis
protein tyrosine phosphatase non rec. substrate , Ptpns1	AV317524	2.7	unknown
5-hydroxytryptamine (serotonin) receptor 1B, Hrt1b	M85151	2.7	behaviour
pancreatic polypeptide, Ppy	M18208	2.6	hormone signalling
keratin complex 1, acidic, gene 1, Krt1-1	M27734	2.5	cyto., intermed. filaments
metallothionein 3, Mt3	M93310	2.5	neurogenesis
Rad9 homologue (S. pombe), Rad9	AF045663	2.4	cell cycle, DNA damage
plasminogen, Plg	J04766	2.4	apoptosis, angiogenesis
dishevelled 2, dsh homolog (Drosophila), Dvl2	AV312528	2.4	actin , motility
Btb (POZ) domain containing 14, Btbd14	AV369001	2.4	unknown
solute carrier family 25, member 3 Slc25a3	AW121063	2.3	unknown
coronin, actin binding protein, 2B Coro2B	AI848032	2.3	actin binding
thyrotropin releasing hormone, Trh	X59387	2.3	hormone signalling
phospholipase A2, group X, Pla2g10	AA607557	2.2	lipid catabolism
N-myc (and STAT) interactor, Nmi	AF019249	2.2	transcription
zinc finger protein, 1 (Ikaros), Znfn1a1	AI325349	2.2	transcription
cathepsin E, Ctse	M17327	2.2	proteolysis
Bcl2-associated athanogene 3, Bag3	AV373612	2.2	apoptosis, folding
heparan sulfate (glucosamine) 3-O-sulfotransferase 3A	AV341572	2.2	protein glycosylation
chitinase, acidic, Chia-pending	AI594518	2.1	chitin metabolism
ATP-binding cassette, sub-family C (CFTR/MRP), 8	AF037312	2.1	ATP-channel
potassium voltage-gated channel, beta 1, Kcnab1	AV335854	2.0	ion channel
phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A	AI850079	2.0	lipid modification

Table 2. Upregulated genes in NDR1 knockout spleen samples.

(Chia-pending, Plg, Ctse, Pcsk1), the immune response (Fcgr3, Igh4, Tacstd2, Cx3cl1, CD276) and transcription (Creb1, H1b, Alx4, Nmi1, Znfn1a, Myt1, Hes3), indicating a potential role of NDR1 in these processes.

Description	Genebank	fold	Function
HIV-1 tat interactive protein 2, homolog (human), Htatip2	AF061972	7.9	unknown
RIKEN cDNA 2410015J15 gene	AV240248	7.6	unknown
GEF (RCC1 related), Gnefr-pending	AJ243952	6.9	nuclear import
calcium/calmodulin-dependent protein kinase II, beta	X63615	6.0	cell cycle
stearoyl-Coenzyme A desaturase 1, Scd1	AW04789	5.1	lipid biosynthesis
cytochrome P450, family 4, subfamily a, polypeptide 14	Y11638	4.6	electron transport
tumour-associated calcium signal transducer 2, Tacstd2	AI563854	4.5	immune response
chemokine (C-X3-C motif) ligand 1, Cx3cl1	U92565	4.5	immune response
myelin transcription factor 1-like, Myt1l	U86338	4.1	transcription
insulin-like growth factor binding protein 6, Igfbp6	X81584	3.7	cell growth
mini chromosome maintenance deficient 7, Mcmd7	AV377480	3.6	unknown
casein kinase 1, delta, Csnk1d	AI846289	3.4	DNA repair, signalling
SPARC related modular calcium binding 1, Smoc1	AI848508	3.3	calcium binding
RIKEN cDNA E130103I17 gene	U85993	3.3	unknown
protein phosphatase 2, regulatory subunit B (B56), epsilon	AI606577	3.2	unknown
Tssk1 (spermiogenesis associated)	U01840	3.1	spermatogenesis
coiled-coil-helix-coiled-coil-helix domain containing 6	AI844709	3.1	unknown
Ndr1 protein kinase	**AA691445**	**2.8**	-
procollagen, type XVIII, alpha 1, Col18a1	U03715	2.8	angiogenesis, apoptosis
Purkinje cell protein 2 (L7), Pcp2	M21532	2.7	unknown
pyruvate carboxylase, Pcx	M97957	2.7	lipid biosynthesis
hairy and enhancer of split 3 (Drosophila), Hes3	D32200	2.5	transcription, morphology
oxysterol binding protein-like 9, Osbpl9	AI846667	2.5	unknown
Mst2 (Ste20, yeast homolog)	U28726	2.5	apoptosis
CD276 antigen	AI593640	2.4	immune response
RIKEN cDNA 3110004L20 gene	AW123347	2.3	unknown
homer homolog 3 (Drosophila), homer3, FLJ10432	AI840678	2.3	unknown
troponin T1, skeletal, slow, Tnnt1	AV213431	2.3	cytoskeleton, muscle dev.
dynein, axonemal, heavy chain 8, Dnahc8	AA144987	2.3	microtubule movement
origin recognition complex, subunit 4-like, Orc4l	AI553463	2.2	replication
RIKEN cDNA C730036B01 gene	AA690483	2.2	unknown
RIKEN cDNA 1100001I22 gene	AW121947	2.2	protein biosynthesis
elastin, Eln	U08210	2.1	actin cytoskeleton
proprotein convertase subtilisin/kexin type 4, Pcsk4	L21221	2.1	proteolysis, fertility
RIKEN cDNA 2610312E17 gene	AI853476	2.0	unknown
endothelin 1, Edn1	U07982	2.0	vasoconstriction

Table 3. Downregulated genes in NDR1 knockout spleen samples.

Phenotypes of aged NDR1 knockout mice

In aged NDR1 knockout mice we observed a frequent occurrence of peliosis hepatis and/or nodular regenerative hyperplasia (see Fig. 3 and table 4). A preliminary summary is shown in table 3. No macroscopic observations were made in four control wild type animals. However, the number of NDR1 wild type and knockout animals used for this study was rather small, and higher numbers would be required to attribute this to NDR deficiency.

Fig. 3 shows a peliosis hepatis liver, a benign vascular disorder which is characterised by blood-filled cavities and cystic hepatic sinusoidal dilatation. Notably, the microarray analysis showed a downregulation of endothelin (Edn), which is involved in vasoconsriction, an upregulation of plasminogen (Plg), an activation of angiogenesis, and a downregulation of procollagen type 18a1, an inhibitor of angiogenesis.

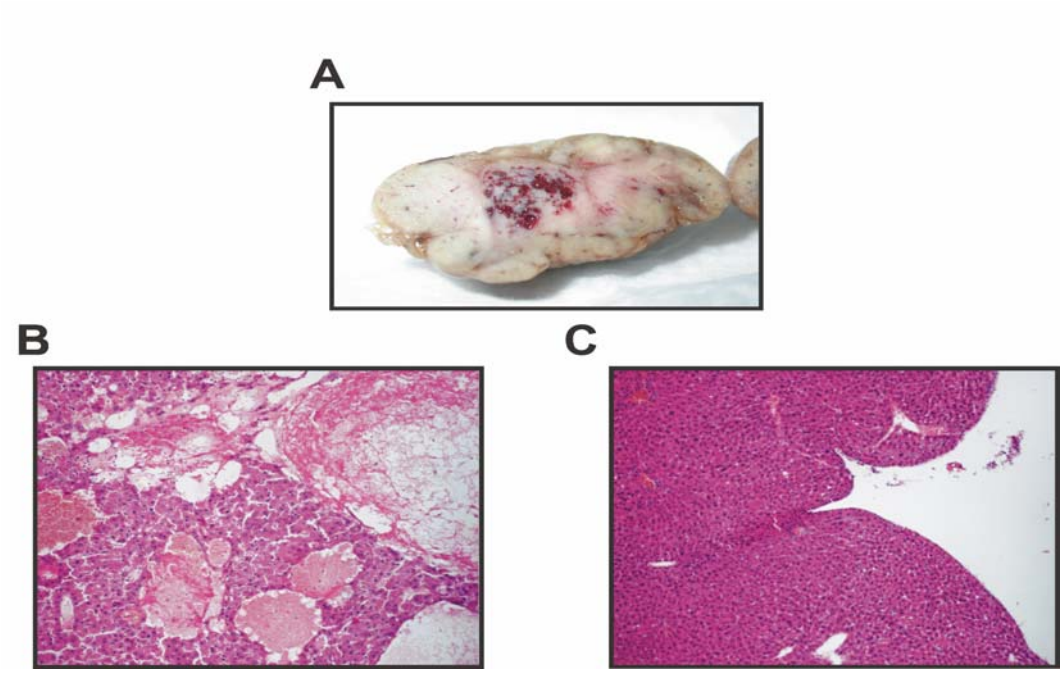

Fig. 3: Liver lesions in aged (20- to 30-month-old) NDR1 knockout mice. A, Peliosis hepatis liver characterised by blood-filled cavities in the liver parenchyma. **B,** 10x magnification of the peliosis hepatis liver with highly dilated cavities. **C,** 10x magnification of a nodular hyperplasia of the liver.

In the group of aged animals, there were also at least 2 incidences of malignant lymphomas (see table 4). None of the four wild type mice of mixed background (C57Bl/6.129SvJ background) show an apparent lesion, but wild type C57Bl/6 mice are reported to develop lymphomas during aging (3). Higher animal number are required to see whether there are significant changes between knockout and wild type animals.

An additional observation was that the size of the seminal vesicles of aged male knockout animals was heavily enlarged compared to the wild type animals (3 male mice). However, the functional implication of this is as yet unclear and requires further investigation.

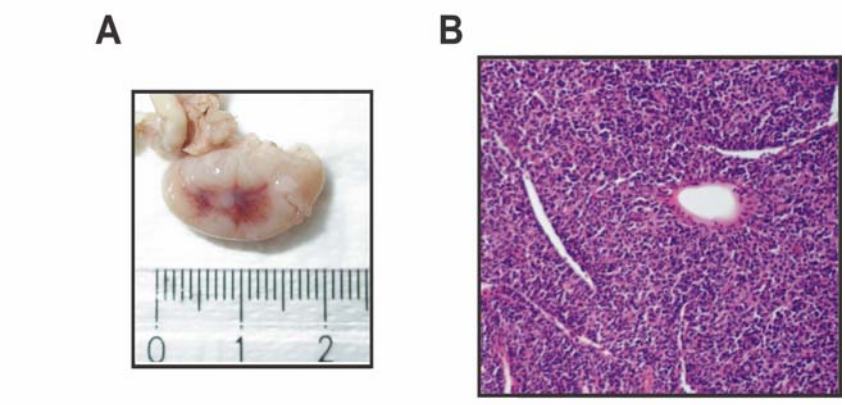

Fig. 4: Lymphoma in the abdomen. A, macroscopic view. **B,** 10x magnification of the lymphoma.

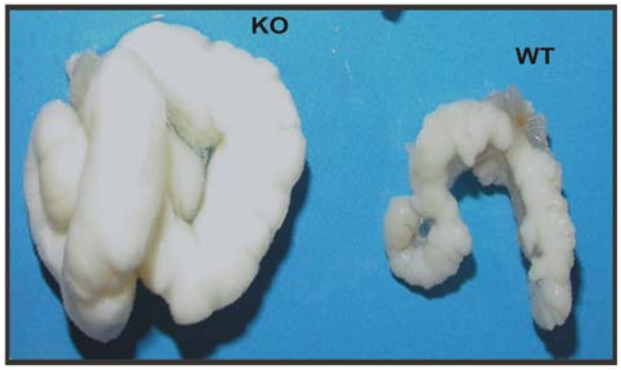

Fig. 5: Enlargement of the seminal vesicles in aged NDR1-knockout mice. Comparision of seminal vesicles from knockout and wild type animals.

Morphological abnormalities in 13 aged (8 male) knockout mice:		
Liver	2x peliosis hepatis 1x nodular hyperplasia	1 uncharacterised abnormalities
Lymphatic system	2x lymphoma	
Seminal vesicles	7x enlarged vesicles	
Not yet characterised masses	1x lung 1x abdominal mass	

Table 4. Summary of initial observation in aged NDR1 knockout animals.

Discussion

NDR protein kinases show an extremely high conservation from yeast to mammals, indicating that they have an important function. However, here we report that the phenotypic consequences of an ablation of NDR1 in mice are rather mild or of late onset.

Worms and flies only have one NDR kinase each, SAX-1 and TRICORNERED, both with a strong neuronal morphology phenotype. SAX-1 and TRICORNERED mutants show a branching and tiling phenotype of chemosensory neurons (11, 12). The TRICORNERED mutation results in a splitting and branching phenotype of bristles, wing hairs or antennae (13). The organismal knockout of TRICORNERED is lethal. In contrast, mice and humans have two isoforms, NDR1 and NDR2, indicating that the duplication occurred from a recent expansion of the kinome. Therefore, the mild and/or late onset phenotype of NDR1-knockout mice might be due to overlapping functions of NDR1 and NDR2. This idea is also supported by the increase in NDR2 protein levels in NDR1-knockout MEFs compared to wild type, which indicates a compensatory role of NDR2 in the knockout MEFs. However, an upregulation of

Ndr2 mRNA could not be detected by microarray analysis because the MG_U74Av2 Affymetrix chip used did not contain *Ndr2*. Furthermore, no differences have been found in terms of regulation of the kinases (14, 29) and both kinases were identified in the same complex using a proteomics approach (18).

The microarray analysis of spleen mRNA expression in wild type and knockout animals revealed several interesting findings. First, several genes involved in heat shock response, immune response, cellular cytotoxicity and apoptosis were changed, indicating that NDR1 might be involved in the response to cellular stress. Interestingly, NDR kinases were shown to be regulated by MST3, a STE20 group kinase, which is implicated in the regulation of apoptosis. Of note, within these genes MST2, a mammalian STE20-like kinase, which is reported to phosphorylate the NDR family kinase LATS, is downregulated. This could reflect some crosstalk between the NDR and LATS signalling modules as indicated by data from flies and yeast (14, 20). Additionally, several genes involved in the regulation of the actin cytoskeleton, microtubule network and intermediate filaments were changed. NDR in yeast, worms and flies is also implicated in the regulation of cellular morphology, especially cell spreading and cellular extensions, indicating that mammalian NDR kinase might also play a role in these processes. However, it is unclear whether these changes are primary or secondary effects of NDR1 deficiency.

In aged NDR1 knockout mice, more obvious phenotypes occur. Two out of 13 mice show hepatis peliosis, an aberrant proliferation of endothelial cells in the liver, which is also associated with an increased sinusoidal dilatation. Interestingly, endothelin, a vasoconstrictor (34), and procollagen type18 alpha1 (endostatin) (30), an angiogenesis inhibitor, are downregulated in hepatis peliosis whereas plasminogen, an angiogenic protease (2), is upregulated, providing a potential explanation for the

observed phenotype. However, further studies and increased animal numbers are required in order to make this finding more significant. Hepatis peliosis is a very rare disease in humans, but is more frequent in immunodeficient people. For example, it is more common in HIV patients. NDR kinase is also reported to be incorporated into HIV particles and proteolytically inactivated by the viral protease (9), suggesting a potential link between hepatis peliosis and NDR deficiency.

Aged NDR1 knockout animals also showed incidences of spontaneous lymphoma. NDR kinase was shown to be part of a signalling module, together with MST3, a member of the mammalian STE20-like kinases, which are implicated in the regulation of apoptosis by inducing DNA fragmentation by apoptotic histone phosphorylation in the nucleus. Furthermore, NDR has been shown to be part of a feedback regulation, which induces cleavage and nuclear translocation of MST3 (Stegert *et al.*, chapter 8.). Further research will be needed to reveal whether NDR ablation causes lymphomas by reducing programmed cell death. In humans there have been rare cases reported where hepatis peliosis is found in association with lymphomas (8). Interestingly, disruption of the murine *Ndr2* gene by retroviral insertional mutagenesis caused B-cell lymphomas (32). However, the significance of this observation is unclear, because also wild type animals of the C57Bl/6 strain are known to have a significant incidence of lymphomas (3). Therefore, studies with increased mouse numbers will be required to enable meaningful conclusions about the effect of NDR deficiency.

Furthermore, the seminal vesicles are highly enlarged in knockout animals compared to wild type. However, again further research is required in order to evaluate whether this finding correlates with defects in spermatogenesis, sperm motility or other defects. Notably, male germ cell associated kinase (*Mak*) was upregulated, and testis-specific

serine/threonine kinase (Tssk) was downregulated in the microarray experiment (see tables 2 and 3).

So far, the number of aged animals available, in combination with the late onset of the phenotype, limits the significance of this part of the study. However, an expansion of the study is underway. Further, it will be interesting to see whether the phenotype could be consolidated by crossing the mice with, for example, $p53^{-+/-}$ mice, which are more prone to the development of hepatis peliosis or lymphoma, or by treatment with carcinogens.

Furthermore, the ablation of NDR2 and the generation of $NDR1^{-/-}/NDR2^{-/-}$ mice will be critical for evaluating and understanding the role and function of NDR kinases in mammals. Indeed, studies in our laboratory using $NDR1^{-/-}$ MEFs, in which NDR2 is ablated by using stably integrated short-hairpin constructs, have indicated a role of NDR in regulating cytokinesis and genomic integrity (Tamaskovic *et al.*, in prep.).

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Supplementary data

Generation of a conditional targeting vector for mNdr1

In order to evaluate the function of NDR1 and in order to circumvent potential lethality of a disruption of the Ndr1 gene a conditional targeting vector was generated. A BamH1 –NotI fragment of the indicated region of the Ndr1 gene locus cloned into pMCS5 was used as the vector backbone. An AatII site upstream of exon 4 was used to insert a PstI flanked loxP sequence and a 'floxed' Neo cassette was insert into the PmeI site downstream of exon 4 (Fig. S1A). Exon 4 contains the catalytic lysine of the kinase. Cre recombinase induced excision of exon 4 is expected result in a catalytically inactive protein lacking 28 amino acids within the kinase domain, which might still be able to form complexes with other proteins. Therefore, this could result in a dominant negative protein. The targeting vector was linearised using the SalI site and electroporated into 129/Ola ES cells. An external probe was used for ES cell Southern screening following PstI digestion. The targeting construct was proven to integrate into the genomic locus by homologues recombination (Fig. S1B) and single integration was confirmed by Southern screening using an internal probe (Xba-Xba fragment) following a BamHI digestion (data not shown). The correctly targeted ES cell was used to generate chimeric animals. However, no germline transmission was observed using this ES cell clone. Therefore, new ES cell clones have to be screened in order to implement this strategy.

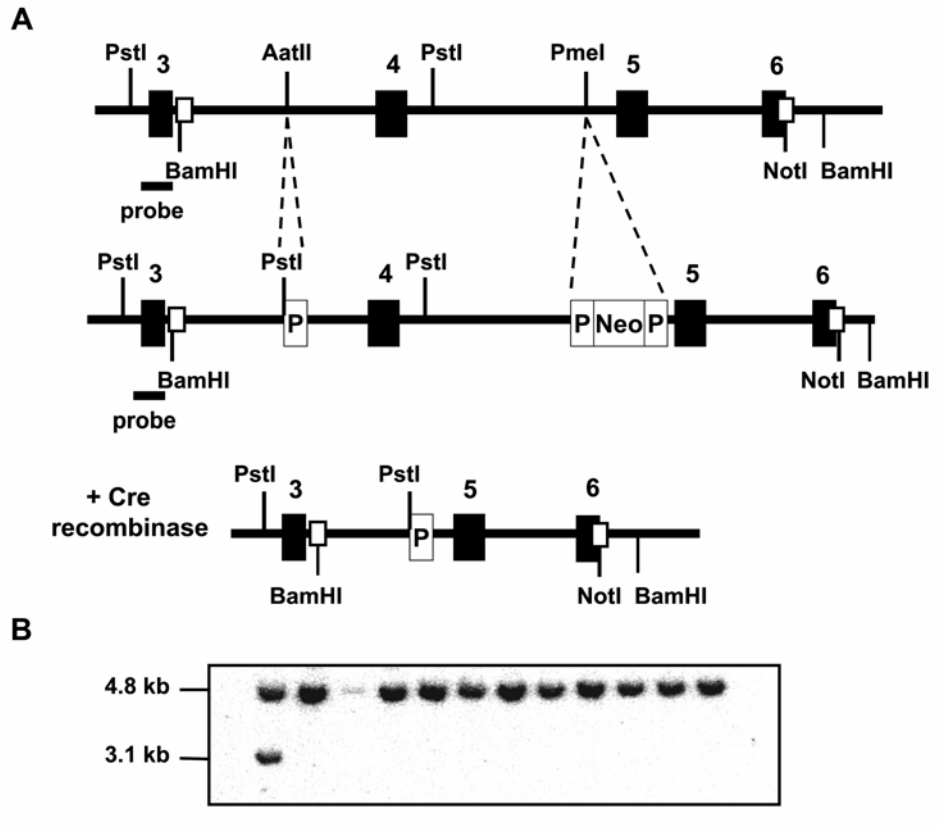

Fig. S1. Conditional targeting of the mouse NDR1 locus. A, Targeting of the mouse *Ndr1* gene locus by flanking exon 4 with loxP (P) and a 'floxed' neomycin cassette (Neo) (white squares indicate the region of the targeting construct. Expression of Cre recombinase removes exon 4, which contains the catalytic lysine of the kinase. B, Southern blot analysis of ES cell DNA clones electroporated with the targeting construct.

Generation of inducible short hairpin constructs targeting *mNdr2*

To investigate the effect of NDR deficiency we also generated short-hairpin constructs which target the mouse *Ndr2 mRNA*. The main advantage of this approach compared to generating knockout cell and animals is the reduced time required for generating the cells. However, the analysis is more complex, and as for most transgenic approaches several clones have to be used in order to ensure that the observed effect is due to the specific knockdown and not the integration of the

construct into a specific locus. Therefore, we used the pTER vector in order to generate short hairpin constructs (Wetering *et al.*, 2003). This vector contains the H1 promotor of RNA polymerase III and a TET operon which allow the doxycycline-inducible regulation of the knockdown in TET repressor expressing cells (Wetering *et al.*, 2003). The following primer pairs were used in order to subclone the targeting sequence into the BglII and HindIII cut vector backbone: pTER-shmNdr2-1 5'-GAT-CCCGTTACGTCGATCGCAGCATTTCAAGAGAATGCTGCGATCGACGTAAC-TTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGTTACGTCGATCGCAGCATT-CTCTTGAAATGCTGCGATCGACGTAACGG-3' and pTER-shmNDR2-2: 5'-GA-TCCCGAGCAGGTGGCTCATATCCTTCAAGAGAGGATATGAAGCCACCTGC-TCTTTTTGGAA-3' and 5'-AGCTTTTCCAAAAAGAGCAGGTGGCTCATATCC-TCTCTTGAAGGAAATATGAGCCACCTGCTCGG-3'. The vector is visualized in Fig. S2A.

Using this constructs in NDR1$^{-/-}$ MEFs expressing the TET repressor resulted in an inducible cell line which is NDR1 and NDR2 deficient. However, also without doxycyclin induction NDR2 was downregulated to a large degree, indicating that the system is leaking. In contrast, NDR2 was not downregulated after stable intergration of an unspecific short-hairpin construct (Tamaskovic *et al.*, in prep.).

These NDR1$^{-/-}$ NDR2-knockdown MEFs display growth defects, multinucleation, polyploidy, impaired mitotic exit, and abortive cytokinesis suggesting that NDR kinases have an important function in maintaining genomic integrity and might be part of a mammalian mitosis exit network (Tamaskovic *et al.*, in prep.)

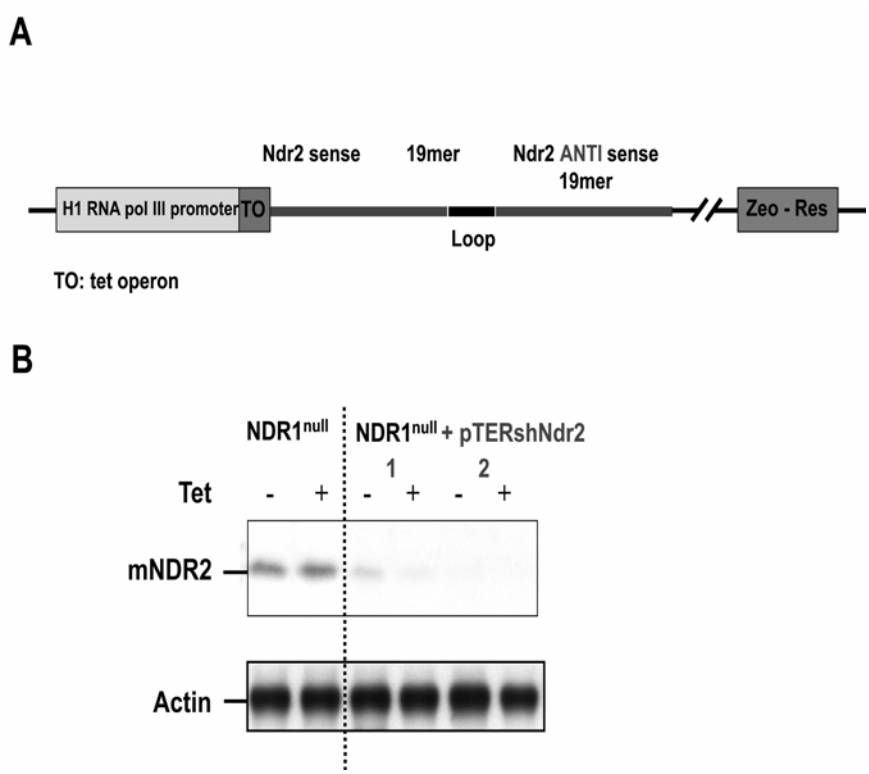

Fig. S2. Knockdown of mouse *Ndr2*. A, Schematic representation of the pTER-shmNDR2 constructs B, Downregulation of NDR2 using stably integrated pTER-shmNdr2 constructs in TET-expressing NDR1 knockout MEFs in presence and absence of doxycylin. MEF cell lysates were subjected to Western analysis using α-NT-NDR and α-actin antibodies.

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III. GENERAL DISCUSSION

10. REGULATION AND EXPRESSION OF MAMMALIAN NDR1 AND NDR2 KINASE

NDR protein kinases are tightly regulated by a number of mechanisms. Full NDR kinase activity requires autophosphorylation at the activation segment, hydrophobic motif phosphorylation by a Ste20-like kinase, and release of autoinhibition by MOB proteins. Activation of NDR is also regulated in a temporal and spatial manner. NDR hydrophobic motif phosphorylation is increased in G2/M, suggesting a role in cell cycle progression (Tamaskovic *et al.*, unpub. data). Furthermore, hydrophobic motif phosphorylation is stimulated upon localisation of NDR to cellular membranes (Hergovich *et al.*, 2005; this study). The phosphorylation at the membrane is further dependent on the levels of the Ste20-like kinase MST3 in HEK293F cells. Despite the largely overlapping localisation of MST3 and NDR2 to the cytoplasm, activation of NDR2 has additional requirements. Fractionation of cells into membranous and cytoplasmic structures indicates that a portion of the cellular MST3 is associated with cellular membranes, indicating that membrane-associated MST3 might be active. Interestingly, recruitment of Ste5-associated kinase to membrane-localised Ste20 kinase is important for the activation of the yeast pheromone response pathway (Pryciak and Huntress, 1998), and membrane recruitment is shown to activate MST1 (Praskova, *et al.*, 2004). However, additional studies are required to unravel the regulation of Ste20-like kinases.

Recent studies have further demonstrated that NDR phosphorylation and activation is dependent on the cellular level of Ca^{2+} (Tamaskovic *et al.*, 2003a). There might be

several reasons. First, NDR kinase binds to S100B proteins, EF hand proteins, which are able to bind to substrate proteins in their Ca^{2+}-bound state (Millward *et al.*, 1998). Second, upstream regulatory elements require Ca^{2+}, because BAPTA treatment of cells abolishes NDR hydrophobic motif phosphorylation. (Tamaskovic *et al.*, 2003). However, it is unclear whether this is a direct effect on the localisation of NDR, or whether Ca^{2+} affects MST3 activity. Also, other ions might affect NDR activity. MOB proteins have been shown to bind Zn^{2+} ions. MST3 also has an atypical ion preference, and autophosphorylates with Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} (Schinkmann *et al.*, 1997; Lu *et al.*, 2005). Therefore, the local ionic composition within the cell might have a crucial effect on NDR activity. Local and temporal changes in ion concentrations are a way to restrict kinase activities to their place of action. Localisation of the kinase is likely to regulate NDR function as well. Interestingly, overexpression of wild type NDR, but not kinase-dead NDR, has the same effect on MST3 cleavage as the constitutively active NDR-PIF (this study), and overexpression of NDR wild type and NDR-PIF result both in polyploidy (Hergovich *et al*., unpub. data; Tamaskovic *et al*., unpub. data). This indicates that activation itself is not sufficient to transduce the signal, but that localisation regulates activation and signal-transduction to specific substrates. NDR activity is also regulated through the binding of coactivator molecules such as MOB1A (this study) or S100B to the N-terminal regulatory SMA domain of the kinase. The binding of MOB *in vitro* stimulates phosphorylation at the activation segment as well as at the Thr75 within the SMA domain (Bichsel *et al.*, 2004). However, the *in vivo* significance of Thr75 phosphorylation still needs to be determined. MOB binding stimulates autophosphorylation by releasing the kinase from autoinhibition mediated by the AIS within the catalytic domain insert (Bichsel *et al.*, 2004; this study). The AIS sequence in the kinase domain insert resembles the

substrate peptide sequence of NDR kinases and might, therefore, act by blocking the substrate binding pocket. The crystal structures of NDR and the NDR-MOB complex will probably provide further insights into the function of the AIS.

The binding to scaffold proteins, such as the FURRY protein AF4p12, might also be important for the formation of signalling modules. Protein phosphatase 2A is a negative regulator of NDR activity, indicating that dephosphorylation is another mode of regulation of NDR activity. It will be interesting to find out what the signals are which prevent or stimulate dephosphorylation of the kinase.

Altogether, the multi-factorial regulation of NDR kinases activity and function involves phosphorylation and dephosphorylation events mediated by MST3 and PP2A respectively: binding of coactivator proteins such as MOB1A or S100B, which stimulate autophosphorylation and release the kinase from autoinhibition; and localisation of the kinase.

The intensity of NDR kinase signalling is not only regulated by altering NDR activity and localisation but also by the expression levels of the kinase. Overexpression of the kinase in COS-7 cells promotes cleavage of MST3, indicating that the levels of NDR are critical for its function and biological effects. *Ndr1* and *Ndr2* kinases show a ubiquitous expression pattern. *Ndr1* mRNA is mainly expressed in spleen, thymus, muscle and lung (Devroe *et al.*, 2004; this study). In most tissues, NDR2 shows higher expression levels and is especially high in proliferative tissue such as stomach and intestine. The high cellular turnover in these tissues, and the induction of MST3 cleavage by NDR2, which is most probably mediated by caspases, point to a potential role of NDR in the induction of apoptosis.

Interestingly, overexpression of LATS2 leads to activation of caspases and induces apoptosis (Ke *et al.*, 2004). Therefore, NDR and LATS kinases might have similar and overlapping functions.

So far, no differences are known in the regulation of NDR1 and NDR2 kinase activity, indicating that they have the same functions. In NDR1-knockout mouse embryonic fibroblasts, NDR2 levels are upregulated. This indicates that NDR2 at least partially compensates for the loss of NDR1. This might explain the mild and/or late-onset phenotype of NDR1-knockout animals. Furthermore, NDR1 and NDR2 were identified in the same protein complexes coimmunoprecipitating with 14-3-3 proteins (Jin *et al.*, 2004).

11. CONSERVATION OF THE NDR SIGNALLING NETWORK

The NDR protein kinase family is one of the most conserved kinase families throughout evolution, indicating an important function. NDR1 and NDR2 kinase are 86% identical and have a similarity of 91%. Therefore, it is not surprising that, so far, no differences have been found regarding NDR1 and NDR2 function and regulation. Also, their co-occurrence in the protein complexes points to redundant functions of mammalian NDR kinases (Jin *et al.*, 2004).

In contrast, *Caenorhabditis elegans* and *Drosophila melanogaster* only have one NDR kinase, SAX-1 and TRICORNERED, respectively, with identities of 69% and 71% to human NDR1 kinases. Both worm and fly NDR mutants show an apparent morphological phenotype in mechanosensory and chemosensory neurons: increased dendritic branching and tiling defects (Emoto *et al.*, 2004; Gallegos *et al.*, 2004). Clonal knockouts of TRICORNERED also result in a splitting phenotype of cellular

extensions such as wing hairs, bristles or arista. The organismal knockout is lethal. This points to a potential role of NDR in the regulation of cell morphology, especially neuronal morphology. Indeed, NDR2 is implicated in the regulation of neurite outgrowth and cell spreading (Stork *et al.*, 2004).

Furthermore, the budding and fission yeast NDR kinases CBK1 and ORB6 are also known to affect cell cycle progression (Verde *et al.*, 1998; Colman-Lerner *et al.*, 2001; Laabs *et al.*, 2004). The regulation of NDR hydrophobic motif phosphorylation during the cell cycle (Tamaskovic *et al.*, unpublished) indicates that mammalian NDR kinases are also involved in cell cycle progression. Therefore, it is likely that NDR kinases from yeast to humans have a common role in the regulation and coordination of cell morphology and cell cycle progression (Tamaskovic *et al.*, 2003a).

Not only NDR kinases, but also the regulatory elements of NDR kinase activation (see also table 1b of the introduction section), are highly conserved, indicating that the NDR kinase-signalling pathway is a conserved signalling module. In recent years, several proteins have been shown to genetically interact with NDR kinases in yeast, worms and flies, encompassing MOB proteins, FURRY proteins and STE20-like kinases (Zallen *et al.*, 2001; Cong *et al.*, 2002; Nelson *et al.*, 2003; Gallegos *et al.*, 2004; He *et al.*, 2005; see also table 1b of the introduction section). Similarly, the LATS kinase pathway consists of MOB proteins, SALVADOR and STE20-like kinases (see Table 1a; Bardin *et al.*, 2001; Tapon *et al.*, 2002; Harvey *et al.*, 2003; Jia *et al.*, 2003; Pantalacci *et al.*, 2003; Udan *et al.*, 2003; Wu *et al.*, 2003; Lai *et al.*, 2005). The similarity and conservation of NDR family kinase networks is likely to allow vertical and horizontal transfers of information regarding the regulation and activation of NDR kinases. This is confirmed by this and other recent studies, which showed that also human MST3, MOB1A/B and MOB2 are important for the

activation of human NDR (this study; Bichsel *et al.*, 2004; Devroe *et al.*, 2004; Hergovich *et al.*, 2005). Correspondingly, LATS kinases are activated by MOB1A, MST1 and MST2 (Lai *et al.*, 2005; Chan *et al.*, 2005). Genetic evidence indicates that MOB3A, B and C might also be able to interact with NDR and/or LATS kinases (He *et al.*, 2005b). Based on the conservation of STE20-like kinases, it is also possible that MST4 and YSK1 might be hydrophobic motif kinases of NDR, because they share identical residues in their substrate binding pocket with MST3. However, gene knockout/knockdown, and overexpression studies, are required to address the functions of these proteins in the mammal.

FURRY proteins also exist in budding and fission yeast (PAG1/TAO3 and CPS12/MOR2), worms (SAX-2) and flies (FURRY), and they are thought to function a scaffolding protein, which is important for the activation and/or function of NDR kinases (Cong *et al.*, 2001; Du *et al.*, 2002; Hirata *et al.*, 2002; Emoto *et al.*, 2004; Gallegos *et al.*, 2004). A human FURRY relative, AF4p12, was found to exhibit transcriptional activities (Hayette *et al.*, 2005). However, the interaction of AF4p12 and NDR kinases might be expected, but has not yet been described in mammals.

Apart from FURRY, MOB and STE20-like kinases, the yeast NDR kinase pathways also encompass other regulatory proteins, such as MO25 and SKB1 (Nelson *et al.*, 2003; Wiley *et al.*, 2003). MO25 proteins show a similar degree of conservation from yeast to mammals as NDR kinases. Budding as well as fission yeast MO25 (HYM1 and MO25) regulate NDR kinases by interacting with the corresponding STE20-like upstream kinases KIC1 and NAK (Dorland *et al.*, 2000; Bidlingmaier *et al.*, 2001; Nelson *et al.*, 2003; Bogomolnaya *et al.*, 2004; Kanai *et al.*, 2005). *Drosophila melanogaster* MO25 and human MO25α/β have been reported to form a signalling complex with LKB1 and the STE20 pseudokinase STRADα/β (Baas *et al.*, 2004;

Boudeau *et al.*, 2004). However, data using fly mutants of MO25 and TRICORNERED indicate that MO25 is not part of the NDR signalling pathway, at least as far as it concerns the regulation of the integrity of cellular outgrowths such as bristles and wing hairs (He *et al.*, 2005). The phenotypic differences of MO25 and TRICORNERED mutants might also be due to multiple functions of MO25 and/or the regulated upstream kinase in several pathways. Nonetheless, it is possible that not all of the conserved components are functioning in the NDR pathway.

The SKB1 relative in humans is the protein arginine methyltransferase PRMT5. Interestingly, PRMT5 and NDR1 co-immunopreciptitate with FCP1 phosphatase and 14-3-3 proteins (Jin *et al.*, 2004; Amente *et al.*, 2005) indicating that they might interact in mammals, as well.

Similarly, the conservation of the LATS signalling pathway proposes a LATS kinase signalling module consisting of MOB proteins, MST1 and/or MST2, hSALVADOR (hWW45) and RAB family GTPases. Indeed, SALVADOR and MOB1A have been shown to interact with LATS kinases (Tapon *et al.*, 2002; Lai *et al.*, 2005). Additionally, *in vitro* evidence and our results indicate that MST1 and MST2 might function as hydrophobic motif kinases of LATS1 and LATS2 (Chan *et al.*, 2005).

12. THE FUNCTIONS OF NDR PROTEIN KINASES

NDR protein kinases in yeast are implicated in the coordination of cell cycle progression and cell morphology. For example, DBF2 is a member of the mitosis network and is important for the metaphase-anaphase transition (Tamaskovic *et al.*, 2003a). Also, CBK1 is activated during the cell cycle and is important for septum degradation (Colman-Lerner *et al.*, 2001). However, despite no mammalian mitosis

exit network having been described so far, NDR protein kinase phosphorylation is also regulated during the cell cycle (Tamaskovic *et al*., unpublished), indicating a potential role of NDR kinase in a mammalian mitosis exit network and/or cytokinesis. ORB6, the fission yeast NDR kinase, also shows a cell cycle-regulated activity. Additionally, ORB6 is important for polarised growth, and has profound effects on actin and the microtubule cytoskeleton (Verde *et al.*, 1998). This effect on the cytoskeleton is also apparent in the *Caenorhabditis elegans* SAX-1 and *Drosophila melanogaster* TRC mutants. SAX1 mutants show an expanded cell body as well as additional neurite outgrowth and branching of dendrites (Zallen *et al*., 2000; Gallegos *et al.*, 2004). Similarly, TRC mutant are hypersensitive to actin cytoskeleton destabilising agents such as cytochalasin D, and show a splitting of actin bundles in clonal knockouts affecting cellular extensions such as bristles, wing hairs and arista (Geng *et al.*, 2000). Furthermore, a branching and tiling phenotype of mechanosensory neurons has been described, which is attributed to an interaction of NDR with RAC GTPase, a known modulator of the actin cytoskeleton (Emoto *et al.*, 2004). Interestingly, RAC GTPase is inhibited by caspase activation, which provides a potential link between protease activation by NDR (see results section) and inhibition RAC activity (Zhang *et al.*, 2003; Geisbrecht and Montell, 2004). Also, human NDR2 protein kinase is implicated in the regulation of neurite outgrowth and cell spreading, and interacts with actin (Stork *et al.*, 2004). This indicates a general involvement of NDR protein kinase in the regulation of the actin cytoskeleton and cell morphology. It is worth noting that the Ste20-like kinases MST3, MST4 and YSK1 are implicated in cell migration (Lai and Lai, 2004; Preisinger *et al.*, 2004).

In addition to their role in cell cycle progression and cell morphology, NDR kinases in yeast play a role in the regulation of transcription. CBK1 has been shown to

regulate daughter cell-specific transcription to determine asymmetric cell fates via the zinc finger transcription factor ACE2 (Colman-Lerner *et al.*, 2001). ACE2 regulates cyclin3 (CLN3), glucanase (SCW11) and chitinase (CTS1) expression (O'Conallain *et al.*, 1998; Colman-Lerner *et al*., 2001; Laabs *et al.*, 2004), indicating a role in cell cycle progression and sugar metabolism. Human NDR1 kinase has been identified in a coimmunoprecipitates of FCP1 phosphatase, which contained also RNA polymerase II and the protein arginine methyltransferase PRMT5 (Amente *et al.*, 2005). This indicates that NDR kinases might also have a role in the transcriptional regulation of genes. Recently, the NDR relative WARTS/LATS was shown to control the cell fate of *Drosophila melanogaster* R8 photoreceptors by repressing the expression of the antagonist MELTED, a VEPH (novel gene encoding a PH domain containing protein) homologue (Mikeladze-Dvali *et al.*, 2005).

Altogether, this indicates multiple functions of NDR kinases, ranging from the control of cell cycle progression to cell morphology and transcription.

13. POTENTIAL IMPLICATIONS OF NDR SIGNALLING IN CELLULAR PROCESSES AND DISEASES

The function of NDR protein kinases, as well as the components of the NDR signalling pathway, point to a role of NDR protein kinases in cell cycle progression and cancer. Preliminary results from aged NDR1-deficient mice (see results section 12), which show hyperplasia and/ peliosis in the liver as well as lymphomas, indicate that NDR1 protein kinases affect proliferation (this study). However, overexpression of NDR is also reported to lead to a poor prognosis in cancer (Adeyinka *et al.*, 2004). Furthermore, results from NDR-deficient MEFs show that NDR is important for

genomic integrity (Tamaskovic, unpublished). Therefore, it is not surprising that overexpression, as well as NDR deficiency, might have an impact on cancer development. Overexpression of NDR protein kinase results in the cleavage of MST3, presumably by caspase activation, which points to a possible role of NDR in the induction of apoptosis. Caspase activation is also important for cell migration and cell morphology changes, because caspases are known to cleave Rac GTPases and effector molecules such as LyGDI (Zhang *et al.*, 2003; Zhou *et al.*, 2004). Therefore, on the one hand increased NDR activity might cause cell death by induction of apoptosis, on the other hand an increased activity might also result in an enhanced migratory potential of cells and altered cell morphology. Indeed, *Ndr1* mRNA is overexpressed in aggressive forms of ductal carcinoma in situ (DICS) of the breast, and *Ndr2* mRNA levels are high in the highly metastatic non-small cell lung cancer cell line NCI-H460 (Ross *et al.*, 2000; Adeyinka *et al.*, 2004). This indicates that NDR kinase activity might be involved in cancer development and progression in different ways.

The STE20-like kinases MST4 and YSK1 are further implicated in intracellular transport mechanisms (Preisinger *et al.*, 2004). Interestingly, the yeast CBK1 was also shown to interact with proteins involved in the intracellular transport, such as SEC28 (Ho *et al.*, 2002). Furthermore, CBK1 binding protein SSD1 is a RNAse II like RNA-binding protein which is implicated in endocytosis, vesicle transport, cell wall integrity and splicing (Kurischko *et al.*, 2005). However, so far there is no evidence for an involvement of NDR protein kinases in these processes.

Another field of action for NDR kinase is neuronal processes. Human *Ndr2* mRNA is upregulated in the amygdala of fear-conditioned mice, and overexpression of NDR2 in PC12 cells affects neurite outgrowth and cell spreading (Stork *et al.*, 2004). In

worms and flies, NDR mutations result in a tiling and branching of dendrites (Emoto *et al.*, 2004; Gallegos *et al.*, 2004). Therefore, mutations of NDR in humans might have implications in a variety of neurological diseases. It is also worth noting that NDR1 protein as well as the protein arginine methyltransferase PRMT5 are co-immunoprecipitated by FCP1, 14-3-3alpha-beta and 14-3-3delta-zeta (Jin *et al.*, 2004; Amente *et al.*, 2005). PRMT5 is part of the transcriptional repressor complexes, the spliceosome and the SMN (survival of motor neuron) protein complex. NDR protein kinase has also been identified in transcriptional complexes (Amente *et al.*, 2004). Furthermore, NDR protein kinase was identified in 14-3-3alpha-beta coimmunoprecipitates together with arginine/serine-rich splicing factors (SFRS5 and 10) (Jin *et al.*, 2004). These splicing factors also contain basic sequences which resemble the NDR substrate consensus sequence. SMN1 and SMN2 proteins are mutated in spinal muscular atrophy (SMA), a disease which is characterised by the loss of motor neurons due to increased apoptosis (Kesari *et al.*, 2005). However, the cause of apoptosis is so far unknown. It will be interesting to find out whether NDR protein kinases are also involved in these processes.

14. CONCLUSIONS AND FUTURE PROSPECTS

NDR protein kinases are part of a highly conserved signalling module, indicating an important function *in vivo*. Components of the NDR signalling module indicate an involvement of NDR in a wide variety of cellular processes, ranging from transcription, cellular transport, apoptosis, cell cycle progression and cell morphology control to migration. However, little is known about the function of NDR kinases in the cellular or organismal context. $NDR1^{-/-}$ mice and upregulation of NDR in human

cancers point to an involvement of NDR protein kinase in cancer development and progression (Adeyinka *et al.*, 2004; this study). However, the phenotypic penetrance is rather low and/or characterised by a late onset. This is most probably due to a redundancy of NDR protein kinases in mammals, displayed by the upregulation of NDR2 in NDR1$^{-/-}$ MEFs (see results chapter 9.). Therefore, the generation of NDR2-deficient animals, as well as NDR1$^{-/-}$ NDR2$^{-/-}$ cells or animals, will be important for unravelling the functions of NDR kinases. A conditional approach might turn out to be the most useful strategy, because it might expand the possibilities in case of lethality of double-deficient cells and/or animals. The identification of inhibitors of NDR protein kinases would also be an extremely useful tool to characterise NDR kinases.

However, the accessibility of inhibitors to the ATP and substrate binding pockets might be blocked by the autoinhibitory insertion (AIS) of NDR kinases. In order to fully understand the mechanism of autoinhibition and NDR activation, the structures of NDR and of the NDR/MOB complex need to be determined. This might also give hints for the development of inhibitors.

An open question in NDR signalling is still the identity of *in vivo* substrates. Despite several efforts, no direct substrates are known to date. Several techniques have been described for substrate identification, ranging from bioinformatic approaches using peptide subtrate sequences, to crosslinking approaches or the use of phospho-antibodies towards peptide substrates. Other approaches include mass spectrometry analysis of protein extracts after 2D-gel electrophoresis (Johnson and Hunter, 2005). Also, the use of an ATP analogue-sensitive kinase, also described as the Shokat-method, might be useful for identifying substrates (Bishop *et al.*, 1998). Furthermore, the high conservation of the NDR signalling module might also enable the use of

information which has been obtained by using these methods in lower organisms. Substrates, especially the most conserved ones, might simply be identified by testing orthologue proteins to substrates identified in lower organisms.

The present study provides detailed insights into the activation mechanism of NDR kinase by binding MOB1A proteins, and hydrophobic motif phosphorylation by MST3 *in vitro* and *in vivo.* It will be interesting to know what effects other MOB proteins and Ste20-like kinases have on NDR activity and localisation. A key task is still the identification of signals which regulate NDR, MOB or MST3 activity and/or localisation. The high conservation of the NDR kinase pathway might indicate that, similar to the yeast ORB6 kinase, NDR kinases show a cell cycle-dependent regulation of activity (Kanai *et al.*, 2005). This is supported by the cell cycle-dependent phosphorylation of NDR on the hydrophobic motif (Tamaskovic, *et al.*, unpublished). Again, more insights might be obtained from work done in other organism, or the LATS pathway. For example, MST2 is negatively regulated by RAF-1 (Kolch *et al.*, 2004). Interestingly, NDR1 and NDR2 have been identified in 14-3-3alpha-beta and 14-3-3 complexes containing B-RAF (Jin *et al.*, 2005). Therefore, an interesting experiment would be to test whether B-RAF inhibits MST3, MST4 or YSK1 (for illustration, see table1 of the introduction section). A key question is what regulates MST3 activation and localisation at physiological conditions. The *Schizosaccharomyces pombe* NAK1 kinase regulates and cooperates with HOB1 and WSP1 to promote F-actin formation and polarized growth (Huang *et al.*, 2005a). Therefore, the HOB homologue amphiphysin and the WSP homologue Wiskott Aldrich syndrome protein (WASP) are interesting candidates for interactors of MST3. Amphiphysin is reported to play a role in vesicle formation and the sensing of membrane curvature (Peter *et al.*, 2005) and might be involved in membrane

recruitment of MST3 *in vivo*. WASP is similar to NDR kinases implicated in neurite formation and dendritic branching (Pollard and Borisy, 2003).

The human FURRY protein AF4p12 has been identified as a fusion protein with MLL, but no studies have addressed, so far, whether AF4p12 also interacts with NDR kinase in a similar way to their yeast, worm and fly orthologues, and what the molecular basis of this interaction is.

In general, the vertical (using different organisms) and horizontal transfer (using similarities within the same organism) of information has proven to be a highly valuable strategy for NDR protein kinases in order to identify interactors and modulators of the pathway, and is likely to lead to the identification of new components of the NDR signalling module. Therefore, the combination of conventional biochemical methods such as mass spectrometry, co-immunoprecipitation, immunolocalisation, microarray analysis or protein arrays, together with bioinformatics and genetics in multiple organisms, is probably the key to unveiling the functions of NDR protein kinases in future.

IV. REFERENCES

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V. APPENDIX: FURTHER PUPLICATIONS

A) Tamaskovic, R., Bichsel, S.J., Rogniaux, H., Stegert, M.R. and Hemmings, B.A. (2003). Mechanism of Ca^{2+}-mediated regulation of NDR protein kinase through autophosphorylation and phosphorylation by an upstream kinase. *J. Biol. Chem.*, **278**, 6710-18.

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Mechanism of Ca^{2+}-mediated Regulation of NDR Protein Kinase through Autophosphorylation and Phosphorylation by an Upstream Kinase*

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NDR1 (nuclear Dbf2-related) is a serine/threonine protein kinase belonging to subfamily of kinases implicated in the regulation of cell division and morphology. Previously, we demonstrated that the activity of NDR1 is controlled by phosphorylation of two regulatory residues, Ser-281 and Thr-444. Moreover, we found that NDR1 becomes activated through a direct interaction with EF-hand Ca^{2+}-binding proteins of the S100 family. In this work, we characterize this regulatory mechanism in detail. We found that NDR1 autophosphorylates *in vitro* predominantly on Ser-281 and to a lesser extent on Thr-74 and Thr-444. All of these residues proved to be crucial also for NDR1 activity *in vivo*; however, in contrast to Ser-281 and Thr-444, Thr-74 seems to be involved only in binding to S100B rather than directly regulating NDR1 activity *per se*. When we added Ca^{2+}/S100B, we observed an increased autophosphorylation on Ser-281 and Thr-444, resulting in stimulation of NDR1 activity *in vitro*. Using phosphospecific antibodies, we found that Ser-281 also becomes autophosphorylated *in vivo*, whereas Thr-444 is targeted predominantly by an as yet unidentified upstream kinase. Significantly, the Ca^{2+}-chelating agent BAPTA-AM suppressed the activity and phosphorylation of NDR1 on both Ser-281 and Thr-444, and specifically, these effects were reversed when we added the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase pump inhibitor thapsigargin.

NDR1 (nuclear Dbf2-related) is a conserved and widely expressed nuclear serine/threonine kinase that belongs to a recently identified subfamily of kinases that play a crucial role in cell division and cell morphogenesis. We originally cloned this kinase from a human fetal brain cDNA library using *Caenorhabditis elegans* expressed sequence tag clone cm11b8 (1). Later, it was mapped to chromosome 6p21 next to the major histocompatibility complex class I gene cluster (2). Recently, we isolated a second NDR isoform, termed NDR2, which is localized on chromosome 12p11 next to the K-*ras* gene.[1] The two isoforms display an identity of more than 87% at the protein and 77% at the DNA level. In addition to mammalian NDR1 and NDR2, the NDR family of protein kinases comprises orthologous proteins Tricornered (*trc* gene) from *Drosophila melanogaster*; Sax-1 from *C. elegans*; and several closely related kinases such as Warts/Lats kinases from mammals, *D. melanogaster*, and *C. elegans*; Cbk1, Dbf2, and Dbf20 from *Saccharomyces cerevisiae*; Orb6 from *Schizosaccharomyces pombe*; Ukc1 from *Ustilago maydis*; and Cot-1 from *Neurospora crassa* (for review, see Ref. 3). These kinases share 40–60% amino acid identity within their catalytic domains as well as conserved regions in their regulatory domains. The structural similarity within this family suggests that these kinases might perform related functions even in evolutionary distant organisms.

In fact, recent reports confirmed the involvement of this group of kinases in various aspects of regulation of cell division and cell morphogenesis. For instance, mutations in *trc* result in splitting of surface projections such as epidermal hairs, shafts of sensory bristles, larval denticles, and the lateral branches of arista (4). The *sax-1* mutants display expanded cell bodies and ectopic neurites in several classes of neurons (5). Mutation in *cot-1* results in excessive numbers of branched hyphal tips at restrictive temperature, but these tips fail to elongate (6). The *cbk1* mutants show profound defects in cell morphogenesis, including changes from ellipsoidal to round morphology, random budding patterns, and abnormal mating projections (7). Likewise, disruption of *ukc1* leads to a change of cell shape from elongated to rounded form, to formation of hyphal extensions, and prevents cells from forming filamentous colonies (8). The *orb6* mutants lose growth polarity and display altered, spherical morphology with disorganized microtubuli and actin filaments; and moreover, they enter mitosis prematurely (9). On the other hand, cells carrying temperature-sensitive alleles of *dbf2* arrest at the end of anaphase with uniform, large budded "dumbbell" morphology (10). Taken together, the functional and structural relatedness within this group of kinases suggests that they may be regulated by similar mechanisms, including, *e.g.* stimulatory phosphorylation and association with activating proteins, and they may also partially share the same substrates.

The NDR group kinases contain all 12 subdomains of the kinase catalytic domain described by Hanks *et al*. (11). Regarding their kinase domain sequence, they are most closely related to the members of AGC[2] family of protein kinases comprising

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[1] M. R. Stegert and B. A. Hemmings, unpublished data.

[2] For abbreviations of protein kinase names, see Manning *et al.* (48).

PKA,[3] PKB, PKCs, PRK, $p70^{S6K}$, $p90^{RSK}$, and PDK1. A unique feature of NDR group kinases is an insert of about 30 amino acids between subdomains VII and VIII, which, in the case of NDR1, accommodates a nonconsensus nuclear localization signal (1). All members of this group also contain a poorly characterized N-terminal regulatory domain of different lengths. In NDR1, this domain consists of 87 amino acids, and it encompasses a region that is predicted to form an amphiphilic α-helix responsible for binding to S100B (12).

In our previous work, we described the basic mechanisms of activation of NDR1 protein kinase (13). We found that NDR1 is potently activated by treatment of cells with the protein phosphatase 2A inhibitor okadaic acid (OA), indicating involvement of phosphorylation of serine and/or threonine residues in the regulation of NDR1 activity. Indeed, we demonstrated that activation of NDR1 involves phosphorylation of two regulatory residues, Ser-281 of the activation segment and Thr-444 from the hydrophobic motif located in the C-terminal region (13). Moreover, we described a mechanism of *in vitro* activation of NDR1 through a direct interaction with EF-hand Ca^{2+}-binding protein S100B, which augments NDR1 autophosphorylation (12). Notably, NDR1 also interacts with S100B *in vivo*, its activity is rapidly stimulated by treatment with the Ca^{2+} ionophore A23187, and this activation is dependent upon the N-terminally located S100B binding domain. Intriguingly, we observed that overexpression of S100B in several melanoma cell lines leads to hyperactivation of NDR1 and that NDR1 activity can be inhibited in those cells by W7, a cell-permeable inhibitor of CaM and S100 proteins. Altogether, these results point to the involvement of Ca^{2+} signaling in regulation of NDR1.

In this study, we have examined the mechanism of Ca^{2+}/S100B-induced autophosphorylation and activation of NDR1 by means of electrospray ionization mass spectrometry (ESI-MS) and phosphospecific antibodies raised against the regulatory phosphorylation sites Ser-281 and Thr-444. We also investigated the mechanism of NDR1 phosphorylation *in vivo* as well as the dependence of NDR1 phosphorylation events on intracellular Ca^{2+}. Our findings delineate a Ca^{2+}-dependent mechanism for activation of NDR1, involving S100B-mediated stimulation of autophosphorylation on Ser-281 and phosphorylation of Thr-444 by an unknown Ca^{2+}-dependent upstream kinase.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected at the subconfluent stage with FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. In some experiments, the cells were treated for 60 min with 1 μM OA in 0.1% *N*,*N*-dimethylformamide (Alexis Corp.), 50 μM BAPTA-AM in 0.1% dimethyl sulfoxide (Sigma), or 20 μM thapsigargin in 0.1% dimethyl sulfoxide (Alomone) 24 h after transfection.

Plasmids—Mammalian expression vector pCMV5 with HA-tagged NDR1 was constructed by PCR using primers 5′-CCC AAG CTT GCC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT TCG ATG CAA TGA CGG CAG-3′ and 5′-CGG GAT CCC TAT TTT GCT GCT TTC ATG-3′. The PCR product has been subcloned into the *Hin*dIII and *Bam*HI sites of pCMV5. pGEX-2T_NDR1 plasmid has been described previously (1). The pCMV5_HA-NDR1 and pGEX-2T_NDR1 plasmids for kinase-dead (K118A) and phosphorylation site alanine mutants (T74A, S281A, and T444A) were generated from wild-type vectors using the QuikChange site mutagenesis protocol (Stratagene) with the following primers: 5′-GGA CAT GTG TAT GCA ATG GCT ATA CTC CGT AAA GCA GAT ATG CTT GAA AAA GAG CAG G-3′ and 5′-GCA TAT CTG CTT TAC GGA GTA TAG CCA TTG CAT ACA CAT GTC CCG TAT CTT TCT TCT GAA CAA GC-3′ (K118A), 5′-GCT CGG AAG GAA GCA GAG TTT CTT CGT TTG AAG AGA ACA AGA CTT GG-3′ and 5′-CGA AGA AAC TCT GCT TCC TTC CGA GCA TGT GCT GAT CTC CG-3′ (T74A), 5′-CGT CAG CTA GCC TTC GCT ACA GTA GGC ACT CCT GAC TAC ATT GC-3′ and 5′-GGA GTG CCT ACT GTA GCG AAG GCT AGC TGA CGT CTA TTT CTT TTC C-3′ (S281A), and 5′-GGT CTT CAT CAA TTA CGC TTA CAA GCG CTT TGA GGG CCT GAC TGC-3′ and 5′-CCT CAA AGC GCT TGT AAG CGT AAT TGA TGA AGA CCC AGT CTT TGT TC-3′ (T444A). pECE_S100B plasmid has been described previously (12). Sequence of all plasmids was confirmed by DNA sequencing.

Antibodies—Anti-Ser-281P and anti-Thr-444P rabbit polyclonal antisera were raised against the synthetic peptides NRRQLAFS(PO_4)TVGTPD for the Ser-281 phosphorylation site and KDWVFINYT(PO_4)YKRFEG for the Thr-444 phosphorylation site (Neosystem, Strasbourg, France). The peptides had been conjugated to keyhole limpet hemocyanin. Rabbit injections and bleed collection were done by Strategic Biosolutions. The anti-Ser-281P antiserum was used without further purification, but the anti-Thr-444P antibody was purified on protein A-Sepharose (Amersham Biosciences) followed by antigenic peptide coupled to cyanogen bromide-activated Sepharose (Amersham Biosciences). Antibodies were eluted with 0.1 M glycine, pH 2.5. The 12CA5 HA monoclonal antibody hybridoma supernatant was used for immunodetection and immunoprecipitation of HA-NDR1 variants. A rabbit anti-NDR1_C-term polyclonal antiserum directed against a synthetic peptide TARGAIPSYMKAAK (corresponding to NDR1 amino acids 452–465) has been described previously (1). A rabbit polyclonal antiserum that recognizes S100B was raised against recombinant human S100B and was used without further purification (14).

Bacterial Expression of Human GST-fused NDR1—BL21-DE3 *Escherichia coli* strain (Novagen) was transformed with the pGEX-2T_NDR1 wild-type or mutant plasmids and the pRep4 plasmid bearing $LacI^q$ repressor (Qiagen). Mid-logarithmic phase cells were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for 4 h at 30 °C. Bacteria were lysed by a French press in presence of 1 mg/ml lysozyme, and the fusion proteins were purified on glutathione-agarose (Amersham Biosciences) as described. Recombinant proteins were assayed for kinase activity as described below, and autophosphorylation was determined after SDS-PAGE separation either by Cerenkov counting or by exposure to a PhosphorImager screen followed by analysis with ImageQuant software (Molecular Dynamics).

GST-NDR1 Kinase Assay—1 μg of purified recombinant GST-NDR1 wild-type and mutants (without further treatment or autophosphorylated for 2 h in the presence or absence of 1 mM $CaCl_2$ and 10 μM bovine S100B (Sigma)) were assayed in a 20-μl reaction containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}P]ATP (~2,000 cpm/pmol; Amersham Biosciences), and 1 mM NDR1 substrate peptide (KKRNRRLSVA). After a 30-min incubation at 30 °C, reactions were stopped with 50 mM EDTA, and 10-μl reaction solutions were spotted onto 2-cm^2 squares of P-81 phosphocellulose paper (Whatman). These were subsequently washed 4 × 5 min and 3 × 20 min in 1% phosphoric acid and once in acetone before counting in a liquid scintillation counter. One unit of NDR1 activity was defined as the amount that catalyzed the phosphorylation of 1 nmol of peptide substrate in 1 min.

HA-NDR1 Kinase Assay—Transfected and treated COS-1 cells were washed once with ice-cold phosphate-buffered saline and harvested by scraping into 1 ml of ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and 20 mM β-glycerophosphate before lysis in 500 μl of immunoprecipitation (IP) buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 μM microcystin-LR, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 1 mM benzamidine, and 1 tablet of Complete protease inhibitors/10 ml of IP buffer (Roche)). Lysates were centrifuged at 20,000 × *g* for 20 min, and triplicate aliquots (200 μg) of supernatant were precleared with protein A-Sepharose for 60 min and mixed subsequently for 3 h at 4 °C with 12CA5 antibody prebound to protein A-Sepharose (~1 μg of antibody bound to 2 μl of beads). The beads were then washed twice with IP buffer, once for 10 min with IP buffer containing 1 M NaCl, once again for 10 min with IP buffer, and finally twice with 20 mM Tris-HCl pH 7.5, containing 4 μM leupeptin and 1 mM benzamidine. Thereafter, beads were resuspended in 30 μl of

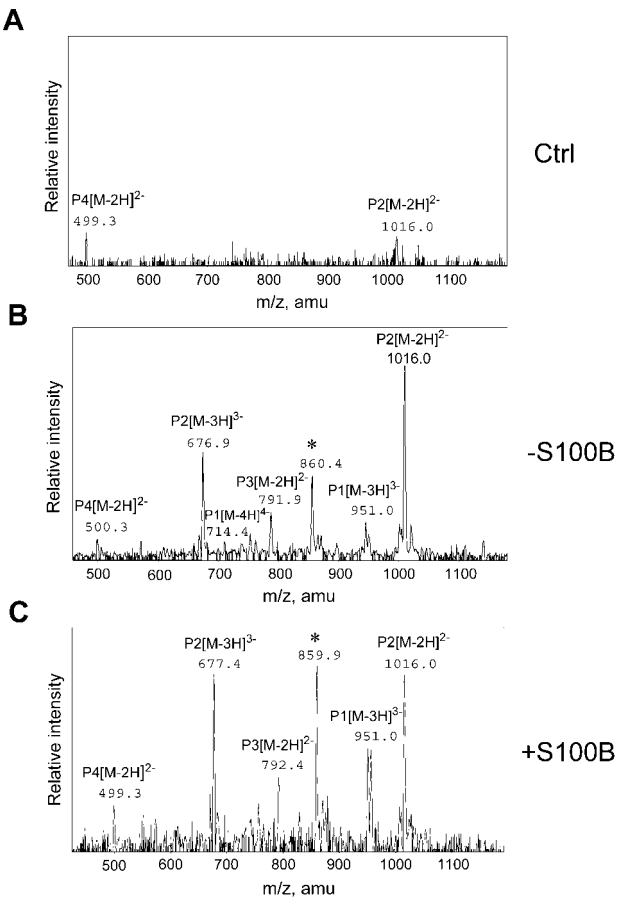
[3] The abbreviations used are: PKA, PKB, PKC, protein kinase A, B, and C, respectively; BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,N′-tetraacetic acid tetra(acetoxymethyl)ester; CaM, calmodulin; ESI-MS, electrospray ionization-mass spectrometry; GST, glutathione *S*-transferase; HA, hemagglutinin; HPLC, high performance liquid chromatography; IP, immunoprecipitation; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; OA, okadaic acid; PDK, phosphoinositide-dependent kinase.

buffer containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}P]ATP (~1,000 cpm/pmol), 1 μM cAMP-dependent protein kinase inhibitor peptide (Bachem), 4 μM leupeptin, 1 mM benzamidine, 1 μM microcystin-LR, and 1 mM NDR1 substrate peptide (KKRNRRLSVA). After a 60-min incubation at 30 °C, 15 μl of supernatant was removed, and phosphate incorporation into the substrate peptide was determined as described for GST-NDR1.

Western Blotting—To detect GST-NDR1 or HA-NDR1, samples were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were blocked in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk powder and were then probed for 2 h with 4 μg/ml anti-NDR1_C-term or 1:100 12CA5 monoclonal antibody supernatant. Bound antibodies were detected with corresponding horseradish peroxidase-linked secondary antibodies and ECL (Amersham Biosciences). For detection of phosphorylated NDR1, either 1 μg of GST-NDR1 or HA-NDR1 immunoprecipitated from 100 μg of COS-1 detergent extracts as described earlier in this paper were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in TBST containing 5% skimmed milk powder and incubated for 2 h at room temperature or overnight at 4 °C with 1:1,000 anti-Ser-281P antiserum in the presence of 50 μg/ml competing dephosphopeptide or 1:500 anti-Thr-444P purified antibody. Both antibodies were detected with horseradish peroxidase-conjugated donkey anti-rabbit Ig antibody (Amersham Biosciences) and ECL. To detect immunoprecipitated S100B, samples were separated on 18% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked for 2 h with 5% bovine serum albumin and 1% fetal calf serum in TBST and was then probed with anti-S100B antiserum diluted 1:1,000 in the same buffer. Bound antibody was then detected with corresponding horseradish peroxidase-linked secondary antibody in TBST and ECL.

Coimmunoprecipitation of NDR1 and S100B—Cytoplasmic COS-1 cell extracts were prepared by lysing the cells for 10 min in a hypotonic buffer containing 10 mM HEPES pH 7.9, 0.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 μM microcystin-LR, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 1 mM benzamidine, and 1 tablet of Complete protease inhibitors/10 ml of buffer. Afterward, the cells were homogenized in Dounce homogenizer. After centrifugation at 3,300 $\times$ *g* for 15 min, supernatants with cytoplasmic extracts were saved, and isolated nuclei found in the pellets were processed further by lysis for 30 min in a high salt buffer containing 20 mM HEPES pH 7.9, 25% glycerol, 0.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 0.5 M KCl, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 μM microcystin-LR, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 1 mM benzamidine, and 1 tablet of Complete protease inhibitors/10 ml of buffer. After centrifugation at 20,000 $\times$ *g* for 30 min, the cytoplasmic and nuclear extracts were pooled at a ratio of 4:1. 1 mg of protein extracts was precleared for 60 min with protein A-Sepharose and incubated for 3 h at 4 °C with 5 μl of anti-S100B antiserum immobilized on 10 μl of protein A-Sepharose. Afterward, the beads were washed four times in buffer containing 20 mM HEPES pH 7.9, 25 mM KCl, 5% glycerol, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 1 mM benzamidine, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 μM microcystin-LR, and 50 mM NaF. Immunoprecipitates were boiled in sample buffer and immunoblotted for S100B or HA-NDR1 as described above.

Mass Spectrometry—10 μg of untreated or autophosphorylated GST-NDR1 (2 h at 30 °C, either in presence or absence of 1 mM $CaCl_2$ and 10 μM bovine S100B homodimer) was separated by 10% SDS-PAGE, stained with Coomassie Blue R-350 (Sigma), and excised from the gel for further processing. Gel slices were sequentially washed 3 $\times$ 8 min in acetonitrile and 2 $\times$ 12 min in 25 mM NH_4HCO_3. Afterward, the gel slices were reduced for 1 h at 57 °C in 10 mM dithiothreitol followed by alkylation for 45 min at room temperature in 55 mM iodoacetamide. Upon repeated washing in acetonitrile/NH_4HCO_3, gel-bound GST-NDR1 was digested with modified trypsin (sequencing grade; Promega) overnight at 37 °C. The cleaved peptides were then extracted by two 15-min sonication steps in H_2O:acetonitrile:formic acid (20:70:10) and H_2O:methanol:formic acid (93:2:5). All experiments were performed on an API 300 triple-quadrupole mass spectrometer (PE-Sciex, Toronto, Canada) equipped with a NanoESI source (Protana, Odense, Denmark). For precursor ion scans of *m/z* −79, samples were injected by off-line nanoelectrospray according to Wilm and Mann (15). The instrument was operated in negative ion mode; a small percentage of ammonia was added in the spraying needle during sample preparation for better sensitivity in the detection of *m/z* −79. For LC-MS sequence analysis, phosphopeptides were further separated by high performance liquid chromatography (HPLC) interfaced with the API 300 mass spectrometer. The Rheos 4000 chromatograph was equipped with a 1 $\times$ 250-mm Vydac C_8 column (Hesperia, Canada). The HPLC column was equilibrated in 95% solvent A (2% CH_3CN, 0.05% trifluoroacetic acid in H_2O), 5% solvent B (80% CH_3CN, 0.045% trifluoroacetic acid in H_2O), and a linear gradient was developed from 5 to 50% of solvent B in 60 min at a flow rate of 180 μl/min After the column, the flow was split with a small percentage (about 5%) being directed to the mass spectrometer. The phosphopeptides were detected in the collected HPLC fractions by Cerenkov counting. For further sequence investigation, phosphopeptides containing fractions were dried, redissolved in a few μl of H_2O:CH_3CN:formic acid (49.5:49.5:1), individually injected into the mass spectrometer using the nanoelectrospray source and analyzed by low energy tandem MS (MS/MS) experiments in positive ion mode.



D

Peptide	Predicted mass (Da)	Detected mass (Da)	Increment
P1 QLAFS^{281}TVGTPDYLAPEVFMQTGYNK	2778,1	2858,8±1,1	(+80,7)
P2 QLAFS^{281}TVGTPDYLAPEVF	1955,2	2034,6±0,7	(+79,4)
P3 DWFINY**T**^{444}YKR	1504,7	1585,8	(+81,1)
P4 KE**T**^{74}EFLR	922,0	1001,8	(+79,8)

* nonphosphorylated

FIG. 1. **Mapping of NDR1 *in vitro* autophosphorylation sites.** *A–C*, 10 μg of GST-NDR1 was left untreated (*A*) or was autophosphorylated for 2 h in the absence (*B*) or presence of 1 mM $CaCl_2$ and 10 μM bovine S100B (*C*). After separation by SDS-PAGE, GST-NDR1 was excised and processed by tryptic cleavage for MS analysis of phosphopeptides by precursor ion scanning of *m/z* −79. Phosphopeptides whose *m/z* could be assigned to NDR1-derived phosphopeptides are labeled *P1–P4*. Some of the peptides were detected in several charged states designated $[M\text{-}2H]^{2-}$, $[M\text{-}^3H]^{3-}$, etc. Peptide P2 results presumably from trace chymotryptic contamination of the trypsin preparation because it overlaps with P1 and terminates with an aromatic residue. *Asterisks* denotes an abundant, double-charged nonphosphorylated NDR1 peptide (amino acids 378–391) with *m/z* 860. *D*, the phosphopeptides from *A–C* which derive from three regions of the NDR1 polypeptide are summarized with the phosphoacceptor residues marked in each peptide in *bold*.

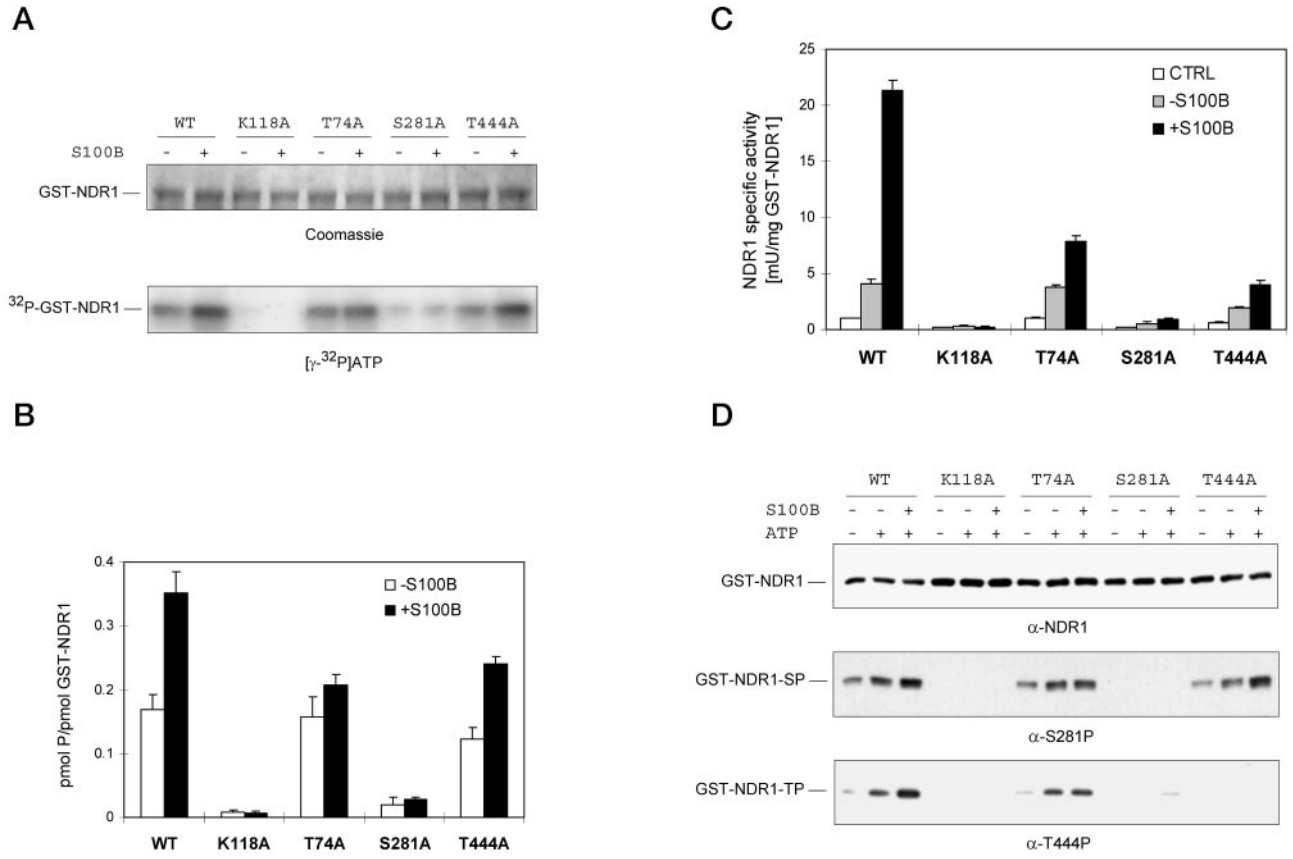


FIG. 2. **Ca^{2+}/S100B promotes the autophosphorylation of GST-NDR1 on both Ser-281 and Thr-444 through a Thr-74-dependent mechanism.** *A*, 1 μg of purified GST-NDR1 (in solution ~0.5 μM) wild-type, kinase-dead (K118A), and alanine mutants of phosphorylation residues Thr-74, Ser-281, and Thr-444 were autophosphorylated for 2 h *in vitro* without further additions or in the presence of 1 mM $CaCl_2$ and 10 μM bovine S100B as indicated. The *upper panel* represents a Coomassie Blue R-350-stained SDS-polyacrylamide gel, and the *lower panel* shows autoradiograph of identical gel developed on PhosphorImager screen for [γ-^{32}P]ATP incorporation. *B*, quantification of the experiment shown in *A*. The amount of the incorporated [γ-^{32}P]ATP was quantified by Cerenkov counting. *Bars* represent the mean ± S.D. of triplicate determinations. *C*, 1 μg of purified GST-NDR1 wild-type and alanine mutants as shown in *A* and *B* were left without further treatment or autophosphorylated for 2 h in the presence or absence of 1 mM $CaCl_2$ and 10 μM bovine S100B as indicated. The specific activity of GST-NDR1 mutants was measured by a peptide kinase assay as described under "Experimental Procedures." *Bars* represent the mean ± S.D. of triplicate determinations. *D*, GST-NDR1 mutants were treated as described in *C* and detected either with anti-NDR1_C-term antiserum or polyclonal antibodies recognizing NDR1 phosphorylated on Ser-281 or Thr-444 as indicated.

RESULTS

Mapping of in Vitro Autophosphorylation Residues of NDR1—We observed that NDR kinase becomes potently autophosphorylated on serine and threonine residues *in vitro*, and that the autophosphorylation as well as NDR1 kinase activity markedly increase upon incubation of NDR1 with the EF-hand Ca^{2+}-binding proteins of the S100 family, in particular S100B (1, 12). These results prompted us to analyze the NDR1 autophosphorylation sites by means of nanospray ECI-MS/MS analysis. 10 μg of purified GST-NDR1 was left untreated (Fig. 1*A*) or autophosphorylated either in the absence (Fig. 1*B*) or presence (Fig. 1*C*) of purified bovine S100B. After tryptic cleavage of the differently treated GST-NDR1 samples, resultant mixtures of peptides were introduced into a triple-quadrupole mass spectrometer and analyzed for phosphorylated peptides by precursor ion scanning for *m/z* −79 in negative ion mode. This procedure detects each peptide in the mixture which, upon fragmentation in the collision cell, liberates a species of *m/z* −79, representing a negative fragment ion PO_3^- characteristic of phosphorylated peptides (16, 17).

When nontreated GST-NDR1 was analyzed by precursor ion scan of *m/z* −79, only weak phosphopeptide signals corresponding to peptides P2 and P4 could been detected (Fig. 1, *A* and *D*). With the GST-NDR1 samples autophosphorylated either in the absence or presence of Ca^{2+}/S100B, however, the abundance of phosphorylated peptides increased markedly (Fig. 1, *B* and *C*). The observed *m/z* value of most of these signals could be assigned in both samples to four NDR1-derived phosphopeptides P1–P4 which, upon liberation of the phosphate group (79.97 Da), give rise to expected tryptic (or in one case chymotryptic) NDR1 dephosphopeptides (Fig. 1*D*). To confirm the identity of these peptides, we performed a LC-MS analysis of GST-NDR1 autophosphorylated with [γ-^{32}P]ATP (data not shown). After collection of the phosphopeptide-enriched radioactive fractions, we again analyzed the samples by ESI-MS using an *m/z* −79 precursor ion scanning method. Having found the same species as in the analysis of the unlabeled GST-NDR1, we subsequently performed a low energy MS/MS analysis in positive ion mode. We detected ion species corresponding to the phosphopeptides listed in Fig. 1*D*, including the phosphorylated residues, thereby confirming identity of the NDR1 *in vitro* autophosphorylation sites.

The four phosphopeptides derived from three regions of the NDR1 polypeptide. The first three phosphopeptides correspond to sites identified previously also in NDR1 activated *in vivo* by OA. P1 and P2 correspond to phosphopeptides encompassing Ser-281 of the activation segment and P3 represents a phosphopeptide containing the residue Thr-444 from hydrophobic motif located in the C-terminal region of NDR1. Significantly, the phosphopeptide P4 proved to be an as yet unidentified

TABLE I
Comparison of NDR1 substrate consensus sequence with the amino acid sequences of the identified in vitro autophosphorylation sites

NDR1 consensus sequence[a]	$^{K}/_{R}$-$^{K}/_{R}$-$^{K}/_{R}$-*X*-$^{K}/_{R}$-$^{K}/_{R}$-*X*-**S**[b]
Thr-74 sequence	L-R-R-S-A-H-A-R-K-E-**T**-E-F
Ser-181 sequence	W-K-R-N-R-R-Q-L-A-F-**S**-T-V
Thr-444 sequence	K-N-K-D-W-V-F-I-N-Y-**T**-Y-K

[a] The NDR1 consensus sequence has been identified by testing a peptide library as described previously (12).
[b] *X* stands for any amino acid. Phosphate acceptor residues are in bold. Single-letter codes used for amino acids.

phosphoresidue Thr-74 from the N-terminal part of NDR1 which has been defined previously as an S100 binding region (residues 62–83; Ref. 12).

Reduction of Autophosphorylation Rate and Kinase Activity in GST Phosphorylation Site Mutants—To confirm the *in vitro* autophosphorylation of NDR1 on the residues Thr-74, Ser-281, and Thr-444 and to estimate the influence of these residues on the autophosphorylation-induced kinase activity of NDR1, we constructed a series of GST-NDR1 alanine mutants at these positions (as well as a GST-NDR1 kinase-dead alanine mutant in the catalytic site residue Lys-118). As shown in Fig. 2, *B* and *C*, wild-type GST-NDR1 became efficiently autophosphorylated *in vitro* (up to 0.4 pmol of phosphorus/pmol of GST-NDR1). The addition of S100B homodimers brought about an approximate 2-fold increase in the autophosphorylation rate accompanied by about a 5-fold increase in kinase activity. Because the kinase-dead mutant did not show any detectable autophosphorylation, the NDR1 kinase activity must account for the observed effects. As shown in Fig. 2, *A* and *B*, the S281A mutant displayed a markedly reduced autophosphorylation rate (irrespective of the addition of S100B), thereby confirming this residue as the major *in vitro* autophosphorylation site of NDR1. As expected, the kinase activity of S281A decreased similarly and was almost undetectable (Fig. 2*C*). Although not comparable with S281A, the T444A point mutation also led to a significant decrease of both autophosphorylation rate and kinase activity, indicating that this residue is also susceptible to autophosphorylation *in vitro*. The smaller effects of T444A, compared with the S281A mutation, may be readily explained by the unfavorable, hydrophobic peptide sequence upstream of this residue with only two basic amino acids located at positions −8 and −10 (Table I). Finally, the T74A mutant displayed only a negligible reduction in autophosphorylation rate and kinase activity in absence of Ca^{2+}/S100B. Although the sequence N-terminal to this site in fact matches the basic NDR1 consensus sequence fairly well, according to the quantitative analyses by LC-MS, the phosphorylation at this site always accounted for less than 5% of the total phosphate incorporation (data not shown). After the addition of Ca^{2+}/S100B, however, the impact of this point mutation approached that of T444A, and the stimulatory effect of Ca^{2+}/S100B on autophosphorylation rate and kinase activity was diminished strongly compared with the other GST-NDR1 variants. These facts indicate that Thr-74 represents only a minor autophosphorylation site, and the low responsiveness to the addition of Ca^{2+}/S100B most likely reflects the inability of T74A mutant to interact with this protein.

Mechanism of NDR1 Activation by Ca^{2+}/S100B—To examine the effect of S100B on NDR1 phosphorylation status, we raised rabbit polyclonal antibodies directed against phosphoepitopes of the two known activation phosphorylation sites Ser-281 and Thr-444. As shown in Fig. 2*D*, these antibodies were specific for the phosphorylated NDR1 because corresponding phosphorylation point mutants S281A, T444A, and kinase-dead were not recognized. Using these reagents, we found that Ca^{2+}/S100B enhances phosphorylation on both Ser-281 and Thr-444 (similar to the general increase in autophosphorylation as presented in Fig. 2, *A* and *B*) without an apparent preference for one of the residues (Fig. 2*D*, *middle* and *bottom panels*). This synergy between the phosphorylation of both activation segment and hydrophobic motif sites may provide a simple explanation for why NDR1 activity increases more robustly than does the overall autophosphorylation after the addition of Ca^{2+}/S100B. Nevertheless, it is striking that although the S281A mutation entirely abolishes the autophosphorylation on Thr-444, the T444A mutant displayed an almost normal autophosphorylation on Ser-281. Apart from the fact that Thr-444 only was autophosphorylated to a minor extent, these data indicate that the impact of the activation segment phosphoresidue on the NDR1 kinase activity was higher than the impact of the hydrophobic motif residue or that these residues are phosphorylated in a sequential manner as is known for most of other AGC family protein kinases (18). Both alternatives may apply for an intramolecular mechanism of the NDR1 autophosphorylation as defined previously (1). Finally,

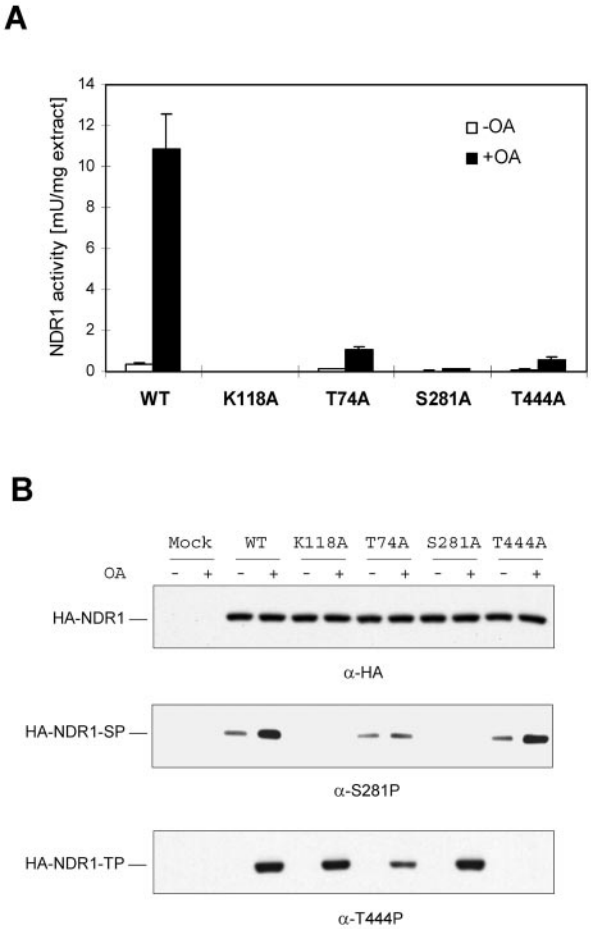


FIG. 3. **NDR1 autophosphorylates on Ser-281 and becomes phosphorylated by an upstream kinase on Thr-444 *in vivo*.** *A*, COS-1 cells expressing either wild-type HA-NDR1 or the indicated mutants were treated for 1 h with 1 μM OA (*filled bars*) or with solvent alone (*open bars*). HA-tagged NDR kinase variants were then immunoprecipitated (of 100 μg of detergent extracts) with 12CA5 monoclonal antibody and assayed for kinase activity by a peptide kinase assay as described under "Experimental Procedures." *Bars* represent the mean ± S.D. of triplicate immunoprecipitations. *B*, 1 μg of protein extracts from transfected COS-1 cells was immunoblotted with 12CA5 to verify similar expression levels of each HA-NDR1 construct (*top panel*). For the analysis of phosphorylation status, 12CA5-immunoprecipitated HA-NDR1 variants (of 100 μg of protein extract) were analyzed by immunoblotting with phosphospecific antibodies directed against phosphorylated Ser-281 or phosphorylated Thr-444 (*middle* and *bottom panels*, respectively).

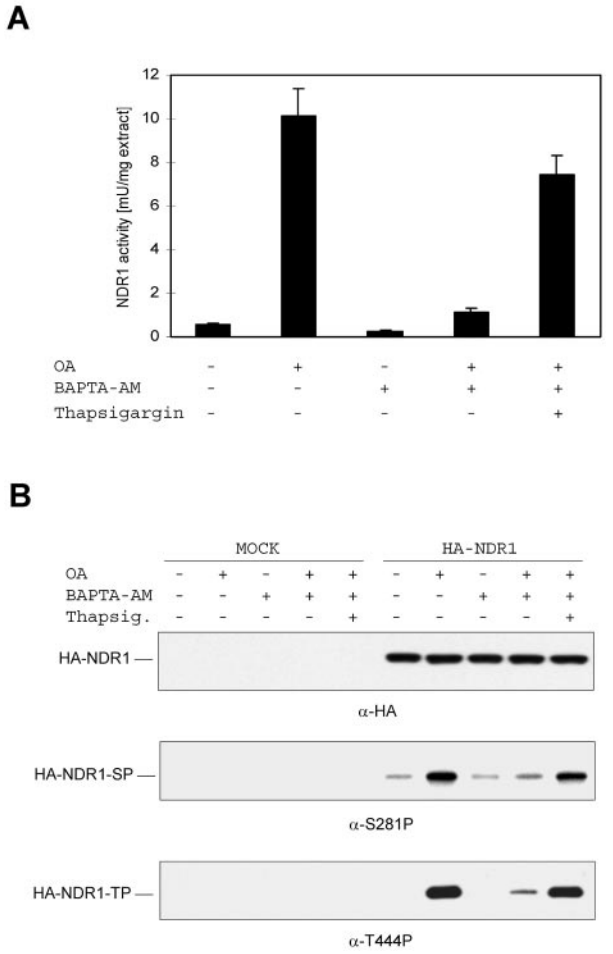


FIG. 4. **Phosphorylation of both Ser-281 and Thr-444 is dependent on intracellular Ca^{2+}.** *A*, mock-transfected or HA-NDR1-expressing COS-1 cells were treated for 1 h as indicated with 1 μM OA, 50 μM BAPTA-AM, and 20 μM thapsigargin or with solvent (0.1% *N*,*N*-dimethylformamide or 0.1% dimethyl sulfoxide) alone. 12CA5 immunoprecipitates (of 100 μg of protein extract) were then assayed by peptide kinase assay for NDR1 kinase activity. *Bars* represent the mean ± S.D. of triplicate immunoprecipitations from HA-NDR1-expressing COS-1 cells after subtraction of mean values from identically treated mock-transfected cells. *B*, Protein extracts (10 μg) from mock- and HA-NDR1-transfected COS-1 cells were immunoblotted with 12CA5 to verify equal expression levels of HA-NDR1 construct (*top panel*, *right five lanes*). For the analysis of phosphorylation status, 12CA5-immunoprecipitated HA-NDR1 (of 100 μg of protein extract) was analyzed by immunoblotting with phosphospecific antibodies directed against P-S281 or P-T444 (*middle* and *bottom panels*, *five right lanes*).

the T74A point mutation abolished the S100B-mediated increase of phosphorylation on both Ser-281 and Thr-444 residues without affecting the intrinsic NDR1 autophosphorylation, again stressing the crucial role of this residue in the interaction with S100B.

Ser-281 Is the Major Autophosphorylation Residue, whereas Thr-444 Is Targeted by an Upstream Kinase in Vivo—To evaluate the relative influence of the individual phosphorylation sites on kinase activity of NDR1 *in vivo*, we constructed a series of mammalian expression vectors with HA-tagged NDR1 wild-type, kinase-dead, and alanine mutants in the three identified phosphorylation sites. As shown in Fig. 3*A*, point mutations in the two established *in vivo* regulatory phosphorylation sites Ser-281 and Thr-444 led to an expected decline of NDR1 kinase activity in both untreated and 1 μM OA-treated COS-1 cells (again with some preference for the S281A mutant). In contrast to the GST-NDR1 kinase assays, however, we also observed a drastic reduction of HA-NDR1 kinase activity after mutating Thr-74. This indicates that the Thr-74-dependent, S100B-mediated autophosphorylation may play a crucial role *in vivo*.

To examine this possibility, we analyzed extracts from transfected COS-1 cells again with the anti-Ser-281P and anti-Thr-444P antisera. As shown in Fig. 3*B*, the Ser-281 residue was constitutively phosphorylated *in vivo* (and this phosphorylation was enhanced further after treatment with 1 μM OA), whereas the Thr-444 site only became modified after OA treatment. Intriguingly, we found that the phosphorylation of Ser-281 depends entirely on the activity of NDR1 because the kinase-dead K118A mutant did not display any phosphorylation on this position. This fact implies that Ser-281 is an autophosphorylation residue also *in vivo*. Significantly, autophosphorylation accounts for the major portion of NDR1 phosphorylation *in vivo* because wild-type NDR1 incorporated markedly higher amounts of radioactive orthophosphate than its kinase-dead counterpart in metabolically labeled COS-1 cells upon treatment with OA (data not shown). Furthermore, mutation of Thr-444 did not impair the phosphorylation of Ser-281, which again points to the low importance of this residue for NDR1 autophosphorylation (Fig. 3*B*, *middle panel*). For the phosphorylation of Thr-444, however, we found that this residue became modified also after abolishing the kinase activity of NDR1 in kinase-dead mutant K118A (Fig. 3*B*, *bottom panel*). This fact strongly suggests that, at least in OA-treated cells, this site must be targeted by an as yet unidentified upstream kinase. Finally, mutation of residue Thr-74 led to a marked reduction of both Ser-281 and Thr-444 phosphorylation (both with and without OA treatment), which explains the somewhat unexpected kinase activity data observed for this mutant. This implies that Thr-74 is not only involved in the S100B-mediated autophosphorylation of NDR1, but it probably participates also in targeting the upstream kinase to NDR1.

Phosphorylation on both Ser-281 and Thr-444 Occurs in a Ca^{2+}-dependent Manner—Because our current and previous work indicated that Ca^{2+}/S100B is essential both for NDR1 activity *in vivo* and for an efficient NDR1 autophosphorylation on Ser-281 and Thr-444 *in vitro* (Fig. 2*D*) and because the S100B-NDR1 interaction is known to be fully Ca^{2+}-dependent (12), we sought to examine the role of intracellular Ca^{2+} in the activation of NDR1. For this purpose, we treated transfected COS-1 cells with the membrane-permeable agent BAPTA-AM, which is freely taken up into cells, where it is hydrolyzed by cytosolic esterases and trapped intracellularly as active, membrane-impermeable Ca^{2+} chelator BAPTA (19). As shown in Fig. 4*A*, 50 μM BAPTA-AM dramatically reduced the OA-stimulated NDR1 activity almost to the basal activity level of the untreated cells. Likewise, examination of the phosphorylation status of NDR1 demonstrated that phosphorylation on both Ser-281 and Thr-444 declined almost to the base line (Fig. 4*B*). Notably, both NDR1 activity and the phosphorylation of Ser-281 and Thr-444 were rescued by coincubation of BAPTA-AM with 20 μM thapsigargin, a sesquiterpene lactone capable of increasing cytoplasmic Ca^{2+} by inhibition of the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase pumps, causing liberation of intracellular Ca^{2+} stores (20). These results confirm the Ca^{2+} specificity of the observed BAPTA-AM effects and combined with the experiments showed in Fig. 3, allow us to conclude that NDR1 is regulated by a Ca^{2+}-dependent, most likely S100B-mediated autophosphorylation on Ser-281 and by phosphorylation by an as yet unidentified Ca^{2+}-dependent upstream kinase on Thr-444.

Thr-74 Is Required for the Association of HA-NDR1 with Ca^{2+}/S100B—We have demonstrated previously that NDR1 forms functional complexes with S100B *in vivo* and that this interaction depends on an intact N-terminal domain of NDR1.

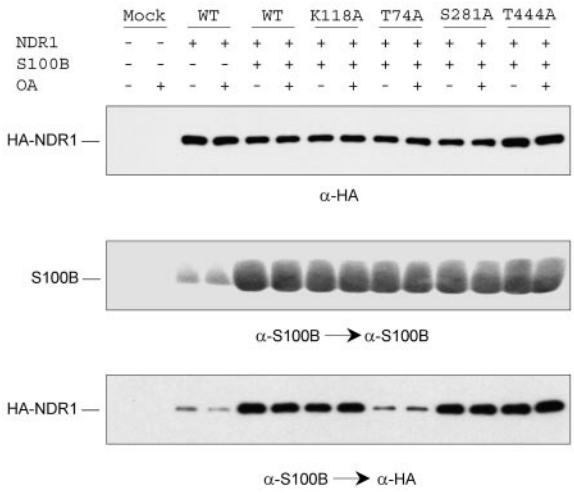


FIG. 5. **Thr-74 is required for association of HA-NDR1 with Ca^{2+}/S100B in COS-1 cells.** COS-1 cells were transfected with HA-NDR1 wild-type, kinase-dead (K118A), and alanine mutants of phosphorylation residues Thr-74, Ser-281, or Thr-444, and S100B or corresponding empty vectors as indicated. 48 h later, nondetergent nuclear and cytoplasmic cell lysates were prepared, pooled, and analyzed for expression of HA-NDR1 variants (*top panel*). 1 mg of protein extracts was immunoprecipitated further with anti-S100B-Sepharose and analyzed for S100B expression (*middle panel*) and association of NDR1 with anti-S100B immunoprecipitates (*bottom panel*) as described under "Experimental Procedures."

Now, we asked whether this association depends on phosphorylation status of NDR1 (12). Therefore, we transfected COS-1 cells with S100B and NDR1 wild-type or alanine-mutant expression plasmids and monitored the NDR1-S100B interaction by coimmunoprecipitation of NDR1 with S100B. As shown in Fig. 5, we found that the formation of NDR1·S100B complexes was independent of NDR1 kinase activity or the phosphorylation status of the regulatory residues Ser-281 and Thr-444, and thus the NDR1-S100B interaction appears to be constitutive. However, mutation of Thr-74 (the minor *in vitro* autophosphorylation site) markedly impaired interaction between these two proteins, and therefore this residue seems to be essential for NDR1 to undergo the interaction with Ca^{2+}/S100B. In the context of the results gained with the recombinant GST-NDR1 (Fig. 2) and the fact that Thr-74 is a part of a putative, S100-binding α-helix formed by amino acids 65–81, it would not be surprising if Thr-74 was one of the crucial residues forming the contact interface between NDR1 and S100B. We are currently addressing these questions by generating a phosphoepitope-specific antibody against this residue, and the results will be presented elsewhere.

DISCUSSION

We found that NDR1 autophosphorylates on three residues *in vitro*. The first of them, Thr-74, is located in the N-terminal S100B binding domain of NDR1. The second, major site Ser-281, which is well conserved among all AGC group kinases, constitutes an essential part of the activation segment in subdomain VIII of the kinase catalytic domain, immediately after nuclear localization signal and kinase domain insert of NDR1. The third site, Thr-444, also conserved in the AGC superfamily, is located outside of the kinase catalytic domain in a region enriched with hydrophobic amino acid residues (therefore "hydrophobic motif"). Mutational analysis showed, however, that the residue Ser-281 alone was responsible for the major part of phosphate incorporation into the autophosphorylated NDR1, whereas Thr-74 and Thr-444 could merely account for a minor percentage of the incorporated phosphate. Moreover, mutation of Ser-281 led to most dramatic decrease of NDR1 autophosphorylation-induced kinase activity, thereby confirming this residue as the major NDR1 autophosphorylation site.

The addition of Ca^{2+}/S100B led to a 2-fold increase of NDR1 autophosphorylation (on both Ser-281 and Thr-444) accompanied by about a 5-fold increased kinase activity. As expected, mutation of Ser-281 again led to an almost total decline of both autophosphorylation and kinase activity. Nevertheless, we also observed a marked, about 5-fold reduction of NDR1 kinase activity for the T444A mutant. These facts suggest that both residues are necessary for the full-active NDR1 and point to a synergy between these two residues in activation of NDR1. The outstanding importance of these residues is not surprising because similar observations were made also for other AGC group kinases (for review, see Refs. 21 and 22). Based upon structural analysis, the PKA activation segment residue Thr-197 was found in its phosphorylated form to align the catalytic site of that enzyme, thereby generating an active kinase conformation (23). On the other hand, the recently resolved structure of PKBβ confirmed the crucial role of the hydrophobic motif with its residue Ser-474 which, upon phosphorylation, undergoes a series of interactions with αB and αC helices of the catalytic domain, thereby promoting the disorder to order transition of this part of molecule with concomitant restructuring of the activation segment and reconfiguration of the kinase bilobal structure (24, 47).

Notably, we also observed a strongly compromised capability of the NDR1 T74A mutant to be significantly autophosphorylated and activated by Ca^{2+}/S100B. As mentioned above, this residue is a part of the S100B binding domain of NDR1 (amino acids 62–86), and it is broadly conserved among protein kinases of the NDR group from different origin (Fig. 6*A*). However, we cannot unambiguously distinguish between the possibility that solely a presence of a threonine or serine residue at this position is necessary for activation of NDR1 by Ca^{2+}/S100B and the possibility that their phosphorylation is required as well. Nevertheless, the N-terminal domain of NDR1, which is highly conserved in its S100B binding region within the whole NDR subgroup of AGC kinases, seems to exert an autoinhibitory effect on the kinase catalytic domain which can be relieved by binding S100B or other potential interacting proteins. Analogous intramolecular domain-domain interactions are well known for a number of AGC and other kinases, such as PKB (pleckstrin homology domain), PKCs (C1 domain), PRK (HR1 domain), or calmodulin (CaM)-dependent kinases (AID domain). Intriguingly, the mechanism of NDR1 regulation by Ca^{2+}/S100B is reminiscent of the regulatory features of CaM kinase II, another Ca^{2+}-controlled kinase, which is known to undergo an intramolecular (intersubunit) autophosphorylation on two threonine residues within its autoinhibitory and a nearby CaM binding domain upon binding of CaM (25). We have demonstrated previously that NDR1 also possesses the capacity to interact with CaM, although to a lesser extent than S100B, but CaM failed to activate NDR1 (12). Notably, the S100B relative S100A1 and, in part, S100B itself, are known to activate through a direct interaction the invertebrate giant sarcomeric kinase twitchin and its vertebrate counterpart titin; however, the exact molecular mechanism of how this activation occurs has not been elucidated so far (26, 27).

As we reported earlier, the Ser-281 and Thr-444 residues become phosphorylated also *in vivo* (13). However, it was not known how these two regulatory sites are targeted in living cells. Here, we report that the Ser-281 site is an autophosphorylation residue *in vivo* because the kinase-inactive K118A mutant did not get phosphorylated on this position, *i.e.* the kinase activity of NDR1 is indispensable for the phosphoryla-

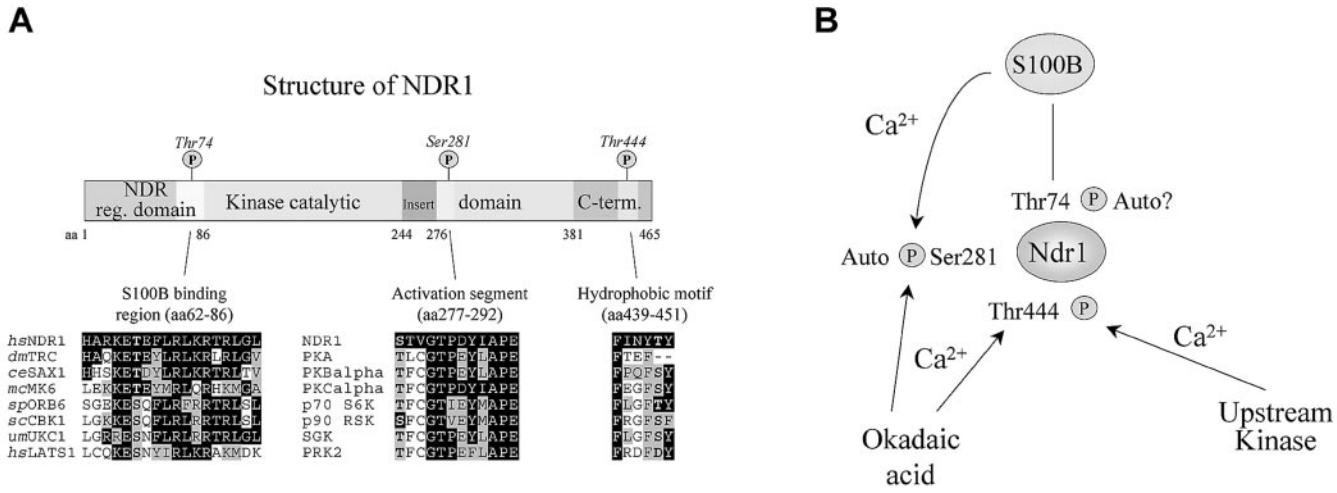


FIG. 6. **Structure of NDR1 and model of its regulation.** *A*, NDR1 domain structure is shown including the N-terminal regulatory domain containing S100 binding region and residue Thr-74; the catalytic domain spliced by a 30-amino acid insert (encompassing a nonconsensus NLS sequence) and containing the activation segment residue Ser-281; and a C-terminal part comprising the hydrophobic motif with Thr-444. Below, the conservation of S100B binding region is shown among several NDR-related kinases from different origin as well as conservation of parts of activation segment and hydrophobic motif residues within the AGC family. The identical residues are *boxed* in *black*, and similar residues are in *gray*. The phosphorylation sites are in *bold*. *B*, model of NDR1 regulation. NDR1 becomes autophosphorylated on the three indicated residues, Thr-74, Ser-281, and Thr-444 *in vitro*. However, only Ser-281 is an autophosphorylation site *in vivo*. In contrast, Thr-444 is targeted mainly by an as yet unidentified upstream kinase. Nevertheless, both phosphorylation events are Ca^{2+}-dependent and essential for NDR1 activity *in vivo*. Although Thr-74 is also a minor autophosphorylation residue *in vitro*, it is currently not clear whether Thr-74 becomes (auto)phosphorylated also *in vivo*. Nonetheless, this residue appears to be a crucial component of the S100B binding domain located in N terminus of NDR1 (for details, see "Discussion").

tion of Ser-281. Nevertheless, we cannot entirely exclude the possibility that this mutation in the substrate binding site leads, for instance, to conformational changes in the NDR1 molecule which do not permit phosphorylation action by a putative upstream kinase. In fact, in most other AGC kinases including PKB, $p70^{S6K}$, serum and glucocorticoid-regulated kinase (SGK), $p90^{RSK}$, and PKC isoforms, the activation segment residue (mostly threonine) has been reported to be a target of PDK1 (for review, see Refs. 28 and 29). The pivotal role of PDK1 in phosphorylation of AGC kinases is supported by high conservation of activation segment residues within the AGC family (Fig. 6*A*) and by the fact that a number of AGC kinases are known to undergo a direct interaction with PDK1. However, more recent studies employing $PDK1^{-/-}$ embryonic stem cells confirmed that there are several exemptions to this general concept. Some AGC kinases such as PKA, PKCδ, AMP-activated protein kinase (AMPK), MSK1, and PRK2 become efficiently phosphorylated at their activation segment residues also in the absence of PDK1 (30, 31), and at least for the two former kinases, it has been proven that this may occur through autophosphorylation (32, 33). Our earlier observation on NDR1 also corroborated that this kinase is not modified by PDK1 either, inasmuch as cotransfection of PDK1 with NDR1 did not significantly change the NDR1 activity *in vivo*, and immunoprecipitated PDK1 failed to transfer phosphate to the GST-NDR1 K118A mutant *in vitro* (13). Most likely, this can be explained by the fact that the NDR1 sequence around Ser-281 displays apparent variations in P+1 and +2 positions to the highly conserved PDK1 consensus target site (*Ser*/*Thr*-Phe-Cys-Gly-Thr-Xaa-Asp/Glu-Tyr-Xaa-Ala-Pro-Glu, where *Ser*/*Thr* is the phosphoacceptor site, and Xaa stands for a hydrophobic residue; Fig. 6*A*). Taken together, our findings suggest that NDR1, in contrast to most AGC group kinases, is not targeted by PDK1 on its activation segment residue Ser-281 but instead, becomes efficiently autophosphorylated at this residue both *in vivo* and *in vitro*.

Because the kinase-inactive K118A as well as the S281A mutant became phosphorylated at Thr-444 virtually to the same extent as wild-type NDR1 *in vivo*, we surmise that this residue is targeted by an upstream kinase in OA-treated COS-1 cells. However, we cannot rule out the possibility that Thr-444 autophosphorylation also might contribute to the overall phosphorylation rate at this residue because the vigorous treatment by OA is known to lead to a stoichiometric phosphorylation of Thr-444 (13) and may, perhaps, favor an upstream kinase at the expense of autophosphorylation (thereby being sufficient to achieve full phosphorylation also for the K118A and S281A mutants). Hence, it remains to be elucidated whether NDR1 becomes exclusively targeted by the putative upstream kinase also if stimulated by comparably mild, as yet unknown physiological stimuli. Remarkably, Thr-444 is flanked by hydrophobic amino acids, which in this region are also conserved among all AGC group kinases (consensus Phe-Xaa-Xaa-Phe-*Ser*/*Thr*-Phe/Tyr; except NDR1 has a tyrosine instead of phenylalanine at the P−1 residue, and PKA is truncated at this position). There is an ongoing dispute in the field concerning the mechanism of hydrophobic motif phosphorylation of several AGC protein kinases. We have reported recently that the corresponding residue in PKBα (Ser-473) becomes phosphorylated independently of PKBα activity status (34); and furthermore, we have characterized a constitutively active, staurosporine-insensitive Ser-473 kinase activity enriched in buoyant, detergent-insoluble plasma membrane rafts (35). Because this activity also depends on phosphoinositide 3-kinase, it is sometimes referred to as PDK2. Although few candidates such as integrin-linked kinase or mitogen-activated protein kinase-activated protein kinase-2 were originally reported to be capable of *in vitro* phosphorylation of PKBα at Ser-473 (36, 37), these proteins turned out later to be physiologically irrelevant hydrophobic motif kinases (38, 39). Thus, despite considerable efforts of several laboratories, Ser-473 kinase resisted identification and cloning so far. Moreover, because the sequence similarity between PKBα and NDR1 is low in this region (except the three conserved hydrophobic residues) and because NDR1 is

not sensitive to inhibitors of phosphoinositide 3-kinase,[4] it is rather unlikely that Ser-473 kinase will be the same protein as the hydrophobic motif kinase phosphorylating Thr-444 in NDR1. For similar reasons, it is also improbable that NDR1 could be targeted by atypical PKCs such as PKCζ/ι (which curiously possess a phosphate-mimicking, negative-charge glutamate residue in place of *Ser/Thr* at their hydrophobic motif, making them independent of an upstream kinase), as was postulated for conventional and novel PKCs such as PKCα, PKCδ, or PKCϵ, and $p70^{S6K}$ (40–42). Finally, although precedents for the autophosphorylation at hydrophobic site also exist (*e.g.* PKCβII, Ref. 43), as mentioned above, we have unambiguously ruled out this alternative for NDR1.

We observed that both autophosphorylation of NDR1 on Ser-281 as well as phosphorylation by an upstream kinase on Thr-444 are Ca^{2+}-dependent processes. In this context, it should be noted that OA, which induces phosphorylation on both sites, was reported, in addition, or because of its inhibitory effect on protein phosphatase 2A, also to increase the intracellular Ca^{2+} concentration. This occurs by at least two mechanisms: activation of L-type Ca^{2+} voltage-dependent channels (44) or release of intracellular Ca^{2+} stores (45). This fact implies that OA not only stimulates NDR1 directly through relieving the protein phosphatase 2A-mediated inhibition of phosphorylation of NDR1, but it also may activate NDR1 indirectly, through some Ca^{2+}-dependent signaling pathways. Combining the *in vitro* and *in vivo* data we gathered in this work, the Ca^{2+} dependence of autophosphorylation of NDR1 on Ser-281 can be explained readily by requirement of NDR1 for Ca^{2+}/S100B to autophosphorylate efficiently. Indeed, the T74A mutant deficient in binding to S100B also displayed a strongly diminished autophosphorylation rate on Ser-281 *in vivo*. On the other hand, the Ca^{2+} dependence of NDR1 phosphorylation on Thr-444 appears to be mediated by a Ca^{2+}-dependent kinase. However, because the T74A mutant showed a compromised ability to become phosphorylated on Thr-444 as well, it might also be possible that the N-terminal domain of NDR1, together with Ca^{2+}/S100B, can in part be responsible for the Ca^{2+} dependence of Thr-444 phosphorylation, *e.g.* through targeting an upstream kinase to NDR1.

In this report, we defined NDR1 as a new member of a broad group of Ca^{2+}-regulated protein kinases which currently comprises proteins as different as CaM and CaM kinases, twitchin/titin giant kinases, conventional PKCs, and to some extent also mitogen-activated protein kinases, PKA, PKB, and PKG (46). We characterized the molecular mechanism for activation of NDR1 by Ca^{2+} consisting of Ca^{2+}/S100B-induced autophosphorylation of NDR1 on the activation segment residue Ser-281 and phosphorylation of hydrophobic motif site Thr-444 by a Ca^{2+}-dependent upstream kinase. The exact nature of the activatory Ca^{2+} signals as well as the identity of upstream kinase and downstream targets of NDR1 still awaits elucidation but will eventually provide insights into the physiological function of this enzyme and the corresponding signaling pathway.

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[4] S. J. Bichsel, unpublished results.

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Mechanism of Activation of NDR (Nuclear Dbf2-related) Protein Kinase by the hMOB1 Protein*

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NDR (nuclear Dbf2-related) kinase belongs to a family of kinases that is highly conserved throughout the eukaryotic world. We showed previously that NDR is regulated by phosphorylation and by the Ca^{2+}-binding protein, S100B. The budding yeast relatives of *Homo sapiens* NDR, Cbk1, and Dbf2, were shown to interact with Mob2 (Mps one binder 2) and Mob1, respectively. This interaction is required for the activity and biological function of these kinases. In this study, we show that hMOB1, the closest relative of yeast Mob1 and Mob2, stimulates NDR kinase activity and interacts with NDR both *in vivo* and *in vitro*. The point mutations of highly conserved residues within the N-terminal domain of NDR reduced NDR kinase activity as well as human MOB1 binding. A novel feature of NDR kinases is an insert within the catalytic domain between subdomains VII and VIII. The amino acid sequence within this insert shows a high basic amino acid content in all of the kinases of the NDR family known to interact with MOB proteins. We show that this sequence is autoinhibitory, and our data indicate that the binding of human MOB1 to the N-terminal domain of NDR induces the release of this autoinhibition.

NDR[1] kinase belongs to a highly conserved family of kinases, a subclass of the AGC family of protein kinases (1, 2). The NDR family consists of the mammalian protein kinases NDR1 and NDR2, *Drosophila melanogaster* NDR, *Caenorhabditis elegans* SAX1, mammalian, *D. melanogaster* and *C. elegans* large tumor suppressor kinases, *Neurospora crassa* COT1, *Ustilago maydis* UKC1, *Saccharomyces cerevisiae* Cbk1, Dbf2, and Dbf20, *Schizosaccharomyces pombe* Orb6 and Sid2, and several plant kinases (1, 2). These kinases share a high sequence conservation, and some possess conserved functions, mainly involving regulation of cell morphology and the cell cycle (2–15).

The kinase domain sequence of NDR is related to that of other members of the AGC group of kinases, *e.g.* protein kinases A, B, C, and G, PRK, $p70^{S6K}$, $p90^{RSK}$, and phosphoinositide-dependent kinase 1 (1). NDR contains all 12 subdomains of the kinase catalytic domain as described by Hanks and Hunter (16). However, the catalytic domains of all of the members of the NDR family are interrupted by an insert of 30–60 amino acids between subdomains VII and VIII. This inserted sequence is not well conserved but is always rich in the basic amino acids, arginine and lysine. The catalytic domain insert has been shown to act as a non-consensus nuclear localization signal in the case of NDR1. NDR1 localizes predominantly to the nucleus in COS-1 cells, whereas mutant NDR1 with a deletion in the insert is localized to the cytosol (17). An additional special feature of the NDR family of kinases is a highly conserved N-terminal domain. In the case of NDR1, this domain consists of 81 amino acids and encompasses a region predicted to form an amphiphilic α-helix that binds to the EF-hand Ca^{2+}-binding protein, S100B (18). Finally, the C-terminal extension of NDR kinase contains a broadly conserved hydrophobic motif phosphorylation site that is an important regulatory site within the AGC group of kinases (19).

NDR kinase is efficiently (20–100-fold) activated upon treatment of cells with the protein phosphatase 2A inhibitor, okadaic acid (OA). OA treatment induces the phosphorylation of the activation segment site, Ser-281, and the hydrophobic motif site, Thr-444 (20). We have shown that Ser-281 is autophosphorylated, whereas Thr-444 is targeted by an as yet unidentified upstream kinase. Both sites are crucial for NDR activity *in vivo* and *in vitro*. NDR activation is Ca^{2+}-dependent as shown by the treatment of COS-1 cells with the Ca^{2+} chelator, BAPTA-AM, which abolishes NDR activation. It has been shown that the EF-hand Ca^{2+}-binding protein, S100B, binds to the N-terminal domain of NDR *in vivo* and *in vitro* and that Ca^{2+}/S100B activates NDR *in vitro*. S100B induces increased autophosphorylation on Ser-281. During investigations of the mechanism of S100B-induced autophosphorylation, a third autophosphorylation site, Thr-74, in the N-terminal domain was discovered (21). This site is also crucial for NDR activation, because its mutation to alanine affected NDR activity *in vivo*.

The results of several recent studies indicate a novel conserved signaling pathway involving NDR kinase family members. It has been shown in *D. melanogaster* that NDR genetically interacts with FURRY, a 300-kDa protein of unknown function (22). In *S. cerevisiae*, the FURRY relative, Tao3/Pag1, lies on the Cbk1 pathway. Furthermore, Tao3/Pag1 and Cbk1 interact physically and their localization is interdependent (23). In *S. pombe*, the FURRY-like protein, Mor2/Cps12, interacts physically with Orb6, the *S. pombe* NDR orthologue (24). The FURRY-like proteins are conserved in mammals, and thus, it is likely that other proteins interacting genetically and/or physically with *S. cerevisiae* Cbk1 or Dbf2 also play a role in the NDR kinase family pathway in higher eukaryotes. Most of

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[1] The abbreviations used are: NDR, nuclear Dbf2-related (for abbreviations of other kinases see Ref. 1); BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester; h, human; GST, glutathione *S*-transferase; HA, hemagglutinin; OA, okadaic acid; SMA, S100B and MOB association; AIS, autoinhibitory sequence.

these proteins are fairly well conserved throughout evolution.

S. cerevisiae Mob1 is a member of the mitotic exit network (25, 26). Dbf2 associates with Mob1, and Mob1 is required for phosphorylation and activation of Dbf2 (27). *S. cerevisiae* Mob2, a close relative of Mob1, is a member of the Cbk1 pathway. Mob2 is required for the biological function of Cbk1 in the mother/daughter separation after cytokinesis and maintenance of polarized cell growth. Mob2 associates physically with Cbk1, and Cbk1 kinase activity is dependent on Mob2. Furthermore, Mob2 and Cbk1 show interdependent localization (28, 29). Similarly, *S. pombe* Mob2 interacts physically with the protein kinase Orb6 and is required for Orb6 function in the coordination of cell polarity with the cell cycle (30). Multicellular organisms possess highly conserved MOB proteins. hMOB1α shares a sequence identity/similarity of 50/65% with *S. cerevisiae* Mob2 and of 57/78% with *S. pombe* Mob1. The human MOB protein family consists of two almost identical proteins, hMOB1α and hMOB1β (NCBI accession numbers Gi8922671 and 27735029), sharing a sequence identity/similarity of 95/97%; a more distantly related protein, hMOB2 (NCBI accession numbers Gi38091156), that is 41/60% identical/similar to hMOB1α; three other related proteins, hMOB3 α, β, and γ (Gi18677731, 41350330, and 3809115), with an identity/similarity of ~50/73% to hMOB1α; and the weakly similar protein, phocein (Gi41349451), that is 24/45% identical/similar to hMOB1α. Because the nomenclature of MOB proteins in the data bases is rather confusing, we use the above terminology based on homology as also proposed recently by Stavridi *et al.* (31). To date, no functional domains have been identified in the MOB proteins and the hMOB proteins have no known functions. It has been shown that the MOB relative, phocein, interacts with the protein phosphatase 2A regulatory subunit, striatin, and with proteins involved in vesicular traffic (32, 33).

Here, we characterize the interaction of hMOB1α, the closest relative of yeast Mob1 and Mob2, with human NDR kinase. We show that hMOB1 binding is dependent on the N-terminal domain of NDR and that hMOB1 stimulates NDR kinase activity both *in vivo* and *in vitro*. Furthermore, we show that a basic sequence within the insert in the catalytic domain of NDR has an autoinhibitory function and that hMOB1 may stimulate NDR activity by releasing the autoinhibitory effect of this sequence.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected at the subconfluent stage with FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. In some experiments, the cells were treated for 60 min with 1 μM OA in 0.1% *N,N*-dimethylformamide or 50 μM BAPTA-AM in 0.1% Me_2SO 48 h after transfection.

Plasmids—Mammalian expression vector pCMV5 encoding HA-tagged NDR1 was described previously (21). pCMV5-hMOB1α was constructed by PCR using the Image clone 4854541 (BG 754693) as template and the primers 5′-GGG GTA CCA CCA TGG AAC AGA AAC TCA TCT CTG AAG AGG ATC TGA GCT TCC TCT TCA GCA GCC GCT C-3′ and 5′-GCT CTA GAC ATT TAT CTG TCT TTT GAT CCA AGT TTC TCT ATT AAT TCT TGA AGA GG-3′ and subcloned into the KpnI and XbaI sites of the vector. pGex2T-hMOB1 was constructed by PCR using the primers 5′-CGG GAT CCA GCT TCC TCT TCA GCA GCC GCT C-3′ and 5′-CCG CTC GAG CAT TTA TCT GTC TTT TGA TCC AAG TTT CTC TAT TAA TTC TTG AAG AGG-3′ and subcloned into the BamH1 and XhoI sites of the vector. For the bacterial production of the NDR protein kinase, NDR2 was fused to a capsid-stabilizing protein of lambdoid phage 21 (SHP).[2] The cloning details and vector maps are available upon request. pCMV5 HA-NDR1 and pSHP-NDR2 point mutations were generated from wild-type vectors using the QuikChange site mutagenesis protocol (Stratagene) and the appropriate primers (primer sequences are available upon request). The sequences of all of the plasmids were confirmed by DNA sequencing.

Antibodies—Phosphorylated anti-Ser-281 and anti-Thr-444 antibodies were as described previously (21). Phosphorylated anti-Thr-74 rabbit polyclonal antiserum was raised against the synthetic peptide, AHARKET(PO4)EFLRLK. The 12CA5 (HA) and the 9E10 (Myc) monoclonal antibody hybridoma supernatants were used for detection of HA-NDR and Myc-hMOB1. Anti-GST-NDR polyclonal antibody was as described previously (17).

Western Blotting—To detect HA-NDR, SHP-NDR, and Myc-hMOB1, samples were resolved by 10 or 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk powder and then probed overnight at 4 °C with anti-GST NDR rabbit polyclonal antibody, 12CA5 (HA) monoclonal antibody supernatant, 9E10 anti-Myc monoclonal antibody supernatant, anti-Thr-444P, anti-Ser-281P, or anti Thr-74P. Bound antibodies were detected with horseradish peroxidase-linked secondary antibodies, or Myc-hMOB1 in HA immunoprecipitations were detected with horseradish peroxidase-conjugated protein A/G and ECL.

Bacterial Expression of Human GST-fused hMOB1 and Human SHP-fused NDR2—XL-1 Blue *Escherichia coli* was transformed with the pGEX-2T-hMOB1 plasmid. Mid-logarithmic phase cells were induced with 0.1 mM isopropyl β-D-thiogalactopyranoside overnight at 20 °C. Bacteria were disrupted using a French press in the presence of 1 mg/ml lysozyme, and the fusion proteins were purified on glutathione-agarose. SHP-NDR2 wild-type and mutant plasmids were transformed into XL-1 Blue *E. coli*, and the protein was produced as described for GST-hMOB1 and purified on nickel-nitrilotriacetic acid-Sepharose.

HA-NDR Kinase Assay—Transfected COS-1 cells were washed once with ice-cold phosphate-buffered saline and harvested in 1 ml of ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and 20 mM β-glycerol phosphate before lysis in 500 μl of IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 20 mM β-glycerol phosphate, 1 μM microcystin, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, and 1 mM benzamidine). Lysates were centrifuged at 20,000 $\times$ *g* for 20 min, and duplicate aliquots (250 μg) of the supernatant were precleared with protein A-Sepharose for 60 min and mixed subsequently for 3 h at 4 °C with 12CA5 antibody prebound to protein A-Sepharose. The beads were then washed twice with IP buffer, once for 10 min with IP buffer containing 1 M NaCl, again for 10 min with IP buffer, and finally twice with 20 mM Tris-HCl, pH 7.5, containing 4 μM leupeptin and 1 mM benzamidine. Thereafter, the beads were resuspended in 30 μl of buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}P]ATP (~1,000 cpm/pmol), 1 μM cAMP-dependent protein kinase inhibitor peptide, 4 μM leupeptin, 1 mM benzamidine, 1 μM microcystin, and 1 mM NDR1 substrate peptide (KKRNRRLSVA). After a 60-min incubation at 30 °C, the reactions were processed as described previously (21).

SHP-NDR Kinase Assay—1 μg of purified recombinant SHP-NDR wild type and mutants (without further treatment or pre-autophosphorylated in the presence of 10 μM GST-hMOB1 or GST) were assayed in a 30-μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}P]ATP (~1,000 cpm/pmol), and 1 mM NDR1 substrate peptide (KKRNRRLSVA). After incubation at 30 °C, the reactions were processed and kinase activity was determined as described for the HA-NDR kinase assay.

Immunoprecipitations—COS-1 cells transfected with HA-NDR wild type or mutants and Myc-hMOB1 were harvested as described above. Cell lysate protein (0.5 mg) was precleared with protein A- or G-Sepharose and mixed subsequently for 3 h at 4 °C with 12CA5 antibody prebound to protein A-Sepharose or with 9E10 antibody prebound to protein G-Sepharose. The beads then were washed twice with IP buffer, once with IP buffer containing 1 M NaCl, once again with IP buffer, and finally twice with 20 mM Tris-HCl, pH 7.5, containing 4 μM leupeptin and 1 mM benzamidine. Samples were resolved by 12% SDS-PAGE, and Myc-hMOB1 and HA-NDR were detected by Western blotting.

GST Pull-down Assay—25-μg aliquots of GST or GST-hMOB1 were incubated with glutathione-Sepharose for 2 h at 4 °C. The beads were washed three times with Tris-buffered saline, and then 5-μg aliquots of SHP-NDR wild type or mutants were added and incubated for 3 h at 4 °C. The beads were washed five times with Tris-buffered saline and resuspended in 30 μl of 1$\times$ SDS sample buffer, and the samples were resolved by 12% SDS-PAGE. NDR bound to GST-hMOB1 was detected by Western blotting.

[2] P. Forrer, C. Chang, D. Ott, A. Wlodawer, and A. Plückthun, submitted for publication.

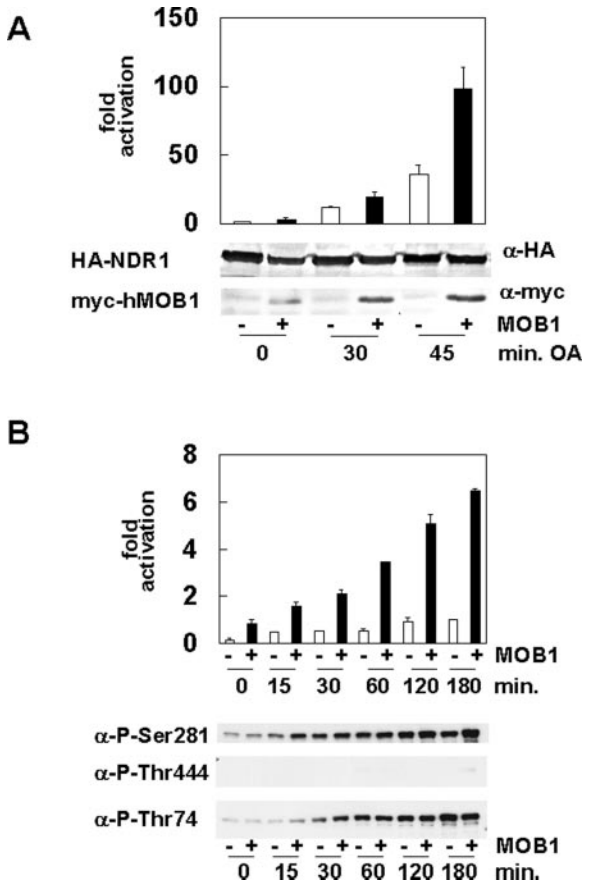

FIG. 1. **hMOB1 stimulates NDR kinase activity.** *A*, HA-NDR was cotransfected with Myc-hMOB1 or the empty vector and treated for the indicated times with 1 μM OA. 12CA5 immunoprecipitates were then assayed for kinase activity. Also shown is the expression of HA-NDR and Myc-hMOB1 in the total cell lysates. *B*, GST-hMOB1 or GST was added to bacterially expressed SHP-NDR2, and autophosphorylation reactions were performed for the indicated times. After autophosphorylation, half of the reaction was added to a mixture containing [^{32}P]ATP and NDR substrate peptide to perform kinase assays. The other half was stopped with SDS sample buffer and resolved on a 10% SDS gel for Western blots with phosphospecific antibodies. *wt*, wild type; *IP*, immunoprecipitate.

RESULTS

hMOB1 Activates NDR—Previous work by other groups has shown in yeast that Mob1 and Mob2 are required for the activity of the NDR-related kinases Dbf2 and Cbk1, respectively (26, 27). To test whether hMOB1 also plays a role in NDR activation, we co-transfected Myc-hMOB1 or the empty vector with HA-NDR1 into COS-1 cells. The cells were treated with the protein phosphatase 2A inhibitor, OA. To date, OA is the only known potent activator of NDR *in vivo* (20, 21) and it has been shown that yeast Mob1 becomes phosphorylated after OA treatment (27). The kinase activity of immunoprecipitated HA-NDR1 was stimulated 35-fold by a 45-min treatment of 1 μM OA (Fig. 1*A*). Co-expression of Myc-hMOB1 induced an additional 2–3-fold increase in NDR kinase activity (Fig. 1*A*). At the 45-min OA time point, HA-NDR1 co-expressed with Myc-hMOB1 was stimulated 100-fold compared with the control. This indicates a role for hMOB1 in NDR activation. To test whether hMOB1 acts directly on NDR, we performed *in vitro* kinase assays of bacterially expressed SHP-NDR2 in the presence of GST-hMOB1 or GST. We used NDR2 for *in vitro* experiments, because it can be readily produced in sufficient amounts. NDR2, which is 86% identical to NDR1, has been shown to be regulated in the same way as NDR1 (34). Furthermore, we confirmed with NDR1 produced in Sf9 cells that wild-type NDR1 behaves *in vitro* similar to NDR2 with respect to hMOB1 (data not shown). GST-hMOB1 stimulated SHP-NDR2 autophosphorylation ~2-fold (data not shown). Furthermore, SHP-NDR2, which was pre-autophosphorylated in the presence of GST-hMOB1, has up to a 6-fold higher kinase activity against the NDR substrate peptide than SHP-NDR pre-autophosphorylated in the presence of GST (Fig. 1*B*). These results show that hMOB1 has a direct positive effect on NDR kinase activity. We tested the effect of hMOB1 on the phosphorylation state of the NDR phosphorylation sites Thr-74, Ser-281, and Thr-444 (Thr-75, Ser-282, and Thr-442 in the case of NDR2; the phosphorylation sites of NDR2 were recognized by phosphospecific antibodies generated against the corresponding phosphorylation sites of NDR1). SHP-NDR2 phosphorylation on the autophosphorylation sites Ser-282 and Thr-75 was slightly increased, whereas Thr-442 of SHP-NDR2, which is known to be targeted by an upstream kinase *in vivo*, showed no autophosphorylation (Fig. 1*B*).

hNDR1 Interacts with hMOB1—To investigate whether hMOB1, the closest relative of yeast MOB2 and MOB1 in mammals, is able to interact with NDR1, epitope-tagged NDR1 and hMOB1 were co-transfected into COS-1 cells. In co-immunoprecipitation experiments of Myc-hMOB1 with HA-NDR1 (Fig. 2*A*) as well as those of HA-NDR with Myc-hMOB1 (Fig. 2*B*), hMOB1 associated with NDR1. The protein level of Myc-hMOB1 was dramatically increased upon OA stimulation, whereas Myc-hMOB1 transfected without NDR1 was present at similar levels in OA-treated and OA-untreated cells. We did not address the molecular basis of this phenomenon. The kinase-dead mutant with the mutated catalytic lysine in the ATP binding site, K118A, was still able to interact with hMOB1 after OA stimulation (Fig. 2*A*). Thus, the interaction was not dependent on NDR kinase activity. Furthermore, we tested whether the two important *in vivo* phosphorylation sites of NDR, Ser-281 and Thr-444, as well as a recently identified *in vitro* autophosphorylation site, Thr-74, play a role in NDR-MOB interaction. The NDR mutants S281A and T444A still interacted with hMOB1 after OA stimulation, whereas the T74A mutant showed almost complete absence of interaction with hMOB1 (data not shown).

NDR-hMOB1 Interaction Depends on OA-induced Modification on hMOB1, whereas Phosphorylation of NDR Is Not Required—Myc-hMOB1 interacted with HA-NDR1 in OA-treated COS-1 cells, but it was not clear whether OA acted just by increasing the Myc-hMOB1 protein level or whether OA-induced modification of NDR1, hMOB1, or both promoted the interaction. To address this question, we transfected HA-NDR1 and Myc-hMOB1 separately into COS-1 cells and stimulated them with 1 μM OA or left them unstimulated. We then pooled the lysates (NDR1 ± OA with hMOB1 ± OA) and immunoprecipitated with α-HA antibody. In the two combinations containing Myc-hMOB1 of OA-treated cells, Myc-hMOB1 strongly associated with HA-NDR, regardless of whether the HA-NDR was expressed in OA-treated or unstimulated cells. Myc-hMOB1 from unstimulated cells showed only a weak interaction with HA-NDR from both unstimulated and OA-stimulated cells (Fig. 2*C*). Thus, OA-induced phosphorylation of NDR is not required for interaction but hMOB1 modification is necessary. Furthermore, GST-hMOB1 was phosphorylated by immunoprecipitated HA-NDR. The phosphorylation of GST-hMOB1 increased 3–4-fold when HA-NDR was immunoprecipitated from OA-stimulated COS-1 cells. However, the phosphorylation of GST-hMOB1 was also observed when kinase-dead HA-NDR was immunoprecipitated (Fig. 2*D*). We conclude that a kinase that co-immunoprecipitates with NDR is able to phosphorylate hMOB1.

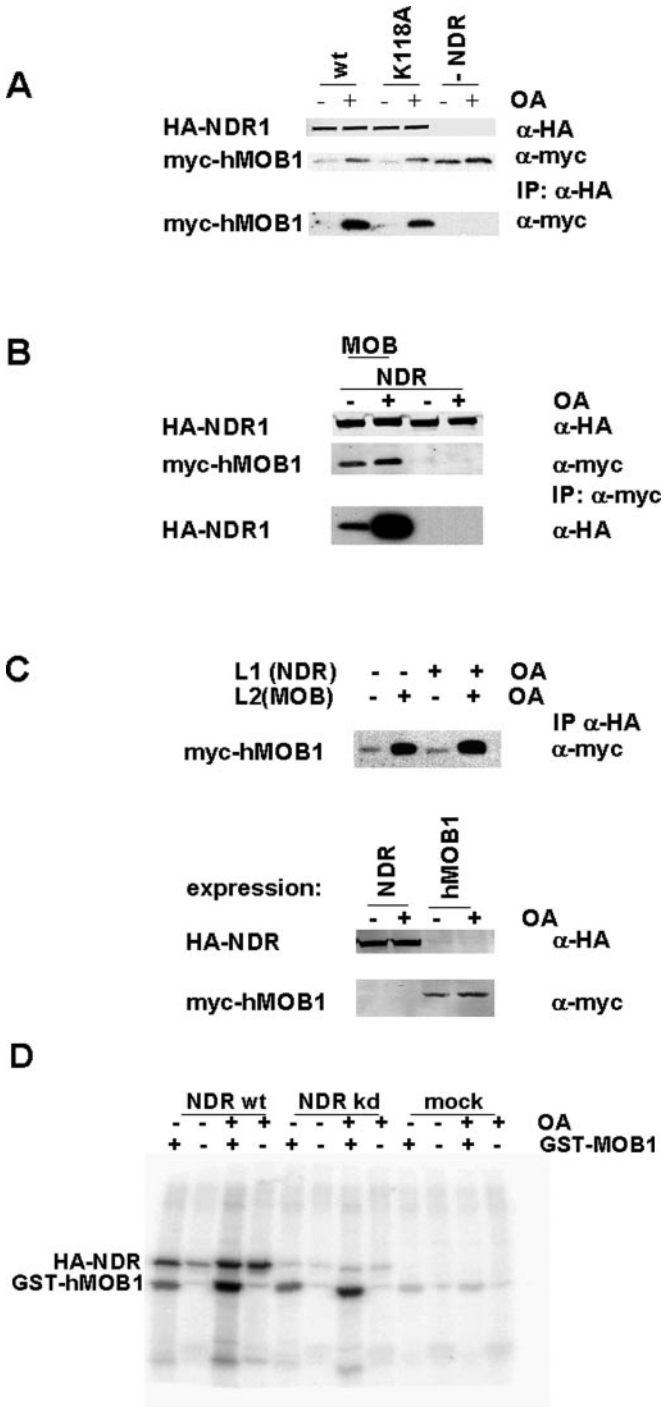

FIG. 2. **NDR interacts with hMOB1.** *A* and *B*, wild type (*wt*) and kinase-dead (*kd*) HA-NDR and Myc-hMOB1 were co-transfected into COS-1 cells, and the cells were treated with OA prior to lysis. *A*, HA-NDR was immunoprecipitated, and the co-immunoprecipitated Myc-hMOB1 was detected. *B*, Myc-hMOB1 was immunoprecipitated, and the co-immunoprecipitated HA-NDR was detected. *C*, HA-NDR and Myc-hMOB1 were transfected separately into COS-1 cells, and the cells were treated with OA or solvent alone prior to lysis. Lysates of OA-stimulated and OA-unstimulated cells containing HA-NDR1 and lysates of OA-stimulated and OA-unstimulated cells containing Myc-hMOB1 were combined. HA-NDR was immunoprecipitated from these combined lysates, and co-immunoprecipitated Myc-hMOB1 was detected. *D*, HA-NDR wild-type and the kinase-dead mutant K118A were transfected into COS-1 cells, and the cells were treated for 60 min with 1 μM OA or the solvent alone prior to lysis. HA-NDR was immunoprecipitated from 1 mg of lysate protein. HA-NDR kinase reactions were performed for 2 h with 10 μM GST-hMOB1 or GST instead of the NDR substrate peptide. The reactions were resolved on a 10% SDS-PAGE, and the incorporated ^{32}P was visualized with a PhosphorImager.

The Highly Conserved N-terminal Domain of NDR Is Required for Kinase Activation—The N-terminal regulatory domain of NDR kinase is highly conserved in the closest relatives of NDR throughout the eukaryotic world (Fig. 3*A*). Several residues are completely invariant throughout evolution from single cell organisms to humans. This prompted us to test the functional significance of these residues with respect to NDR kinase activity. Mutations of the highly conserved residues induced strong inhibition of OA-stimulated kinase activity (Fig. 3*B*). The first part of the N-terminal domain covering amino acids 1–33 and containing a predicted β-sheet in hNDR proved to be important for kinase activation. The deletion of the first 30 amino acids completely abolished kinase activation (data not shown). The point mutations in this region strongly reduced kinase activity. The mutation of Thr-16, Glu-18, and Glu-28 reduced activity to ~40%, whereas the mutation of Lys-24 and Tyr-31 reduced activity to ~20%. Mutation to alanine of Arg-41, Arg-44, or Leu-48, all of which lie in a predicted first α-helix covering the amino acids 40–55 and are situated close together on the same side of the predicted α-helix, reduced kinase activity to below 20% of wild-type activity. Mutation of the residues in a predicted second α-helix situated in the previously described S100B binding region of NDR and covering amino acids 60–80 also led to the inhibition of kinase activity. Mutation of Lys-72, Glu-73, Thr-74, Arg-78, and Leu-79 to alanine reduced kinase activity to 20% or lower. Taken together, the results imply that the high conservation of the N-terminal domain in the following termed SMA (S100B and MOB Association) domain is due to an absolute requirement of the conserved residues for proper kinase function, either by ensuring the correct structural conformation of the protein or being directly involved in binding to interacting proteins.

The SMA Domain Is Required for NDR-MOB Interaction—Thr-74, which was previously shown to be important for NDR kinase activity and interaction with S100B, also turned out to be required for NDR-hMOB1 interaction in COS-1 cells. Therefore, we investigated the involvement of the conserved residues of the SMA domain in NDR-hMOB1 interaction. Co-transfection of HA-tagged NDR mutants and Myc-hMOB1 and subsequent co-immunoprecipitation of Myc-MOB with HA-NDR revealed that several of the conserved residues important for kinase activation are also required for NDR-hMOB1 interaction (Fig. 4, *A–C*). Tyr-31, Arg-41, Thr-74, and Arg-78 seem to be absolutely required for interaction, whereas the Lys-24, Arg-44, and Leu-79 mutants displayed reduced interaction. Only three of the mutants with a strongly reduced kinase activity (Arg-48, Lys-72, and Glu-73) showed no decrease in the ability to bind hMOB1.

Tyr-32 of NDR2 Kinase Is Essential for Activation by hMOB1 in Vitro—The residues Tyr-31, Arg-41, Arg-44, Thr-74, and Arg-78 of NDR1 were shown to be important for kinase activation and interaction with hMOB1 in COS-1 cells. We examined whether these residues are involved directly in the interaction of NDR and hMOB1 or indirectly by influencing interactions with other proteins in the cells. We used bacterially produced SHP-NDR2, wild type, and the mutants Y32A, R42A/R45A, E74A, T75A, and R79A (corresponding to the NDR1 mutants Y31A, R41A/R42A, E73A, T74A, and R78A) together with GST-hMOB1 protein to perform *in vitro* kinase assays to measure the direct effect of hMOB1 on NDR kinase activity (Fig. 5*A*). The mutation of Glu-74, a residue that affects kinase activity but not interaction with hMOB1 in COS-1 cells, led to a reduction in kinase basal activity *in vitro*. This very highly conserved Glu-74 seems to have an important intrinsic role for the function of NDR kinase, although it is not involved in the binding to

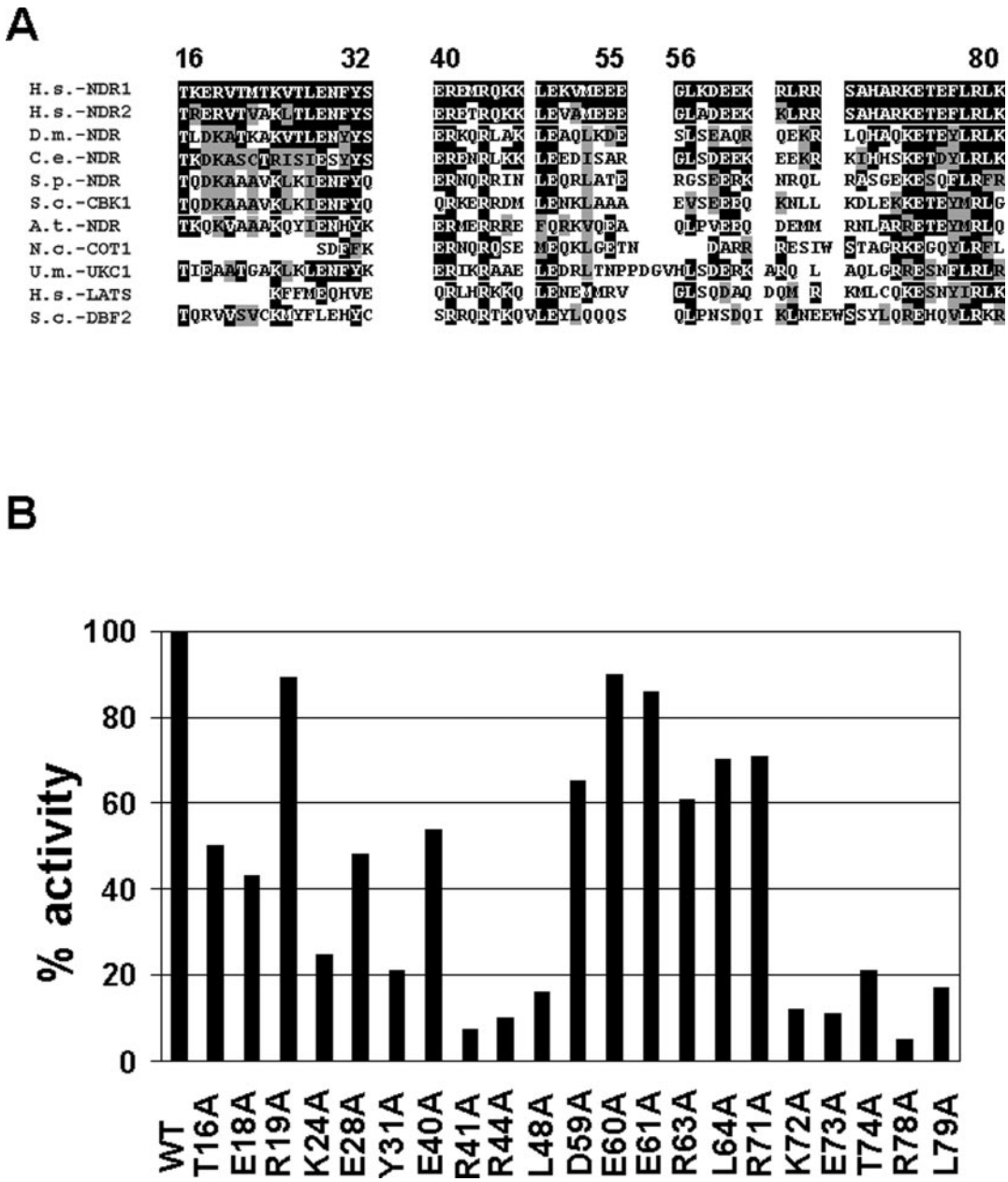

FIG. 3. **The N-terminal SMA domain of NDR is essential for kinase activity.** *A*, alignment of the N-terminal domain of NDR and its relatives. *Numbering* is based on NDR1. *B*, relative kinase activities of HA-NDR1 point mutants from OA-stimulated COS-1 cells. The kinase activities of HA-NDR and the indicated mutants were measured. The data are a summary of three experiments (mutant amino acids 16–31, 40–60, and 61–79). NDR wild type was stimulated 20–30-fold by OA in these experiments. The activities of the NDR mutants were compared with the activity of wild-type NDR in each experiment. Similar expression levels of NDR wild type (*WT*) and mutants were confirmed in each experiment by HA-Western blotting. *H.s*, *H. sapiens*; *D.m.*, *D. melanogaster*; *C.e.*, *C. elegans*; *S.p.*, *S. pombe*; *S.c.*, *S. cerevisae*, *A.t.*, *A. thaliana*; *N.c.*, *N. crassa*; *U.m.*, *U. maydis*.

hMOB1. The mutation of residues Arg-42 and Arg-45, which lie on the same side of a predicted α-helix and whose mutation affects NDR-hMOB1 interaction as well as kinase activation in COS-1 cells, led to a reduction in kinase activity *in vitro* but did not completely abolish the activation by hMOB1. NDR with mutated Tyr-32 showed an intact basal kinase activity but was not activated by hMOB1 *in vitro* at all, pointing to an important role for this residue in the direct interaction with hMOB1. The activation of the T75A and the R79A mutants by hMOB1 was not different to wild-type NDR, suggesting that Thr-75 and Arg-79 are not directly involved in binding to hMOB1 and do not affect the kinase activity of NDR *per se*. Furthermore, the pull-down assays showed that NDR2 with mutated Tyr-32 does not bind to hMOB1, whereas the binding of NDR2 with mutated Arg-42 and Arg-45 is not abolished (Fig. 5*B*).

The Insert in the Catalytic Domain Acts as an Autoinhibitory Sequence—A common feature of the NDR family of kinases is an insert in the catalytic domain of 30–60 amino acids between subdomains VII and VIII. The insert sequence is not highly conserved but contains in all cases many positively charged amino acids. Therefore, we tested the importance of these positive residues for NDR activity and NDR-hMOB1 interaction. Mutating the amino acid sequence ^{265}KRKAETWKRNRR^{276} to amino acid sequence ^{265}AAAAETWAANRR^{276} increased both the kinase activity and phosphorylation of Thr-74, Ser-281, and Thr-444 in COS-1 cells (Fig. 6, *A* and *B*) but did not affect NDR-MOB binding (data not shown). To test whether this insert sequence has an autoinhibitory function, we produced recombinant SHP-NDR2 insert mutant and tested its *in vitro* activity. NDR with the mutated insert sequence had a 4–6-fold

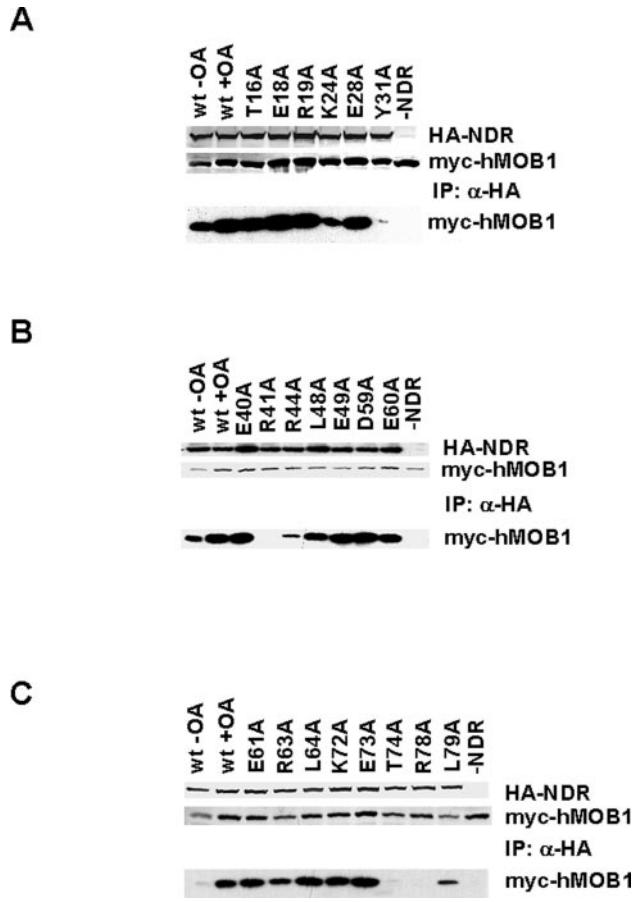

FIG. 4. **The SMA domain of NDR is required for NDR-hMOB1 interaction.** HA-NDR N-terminal point mutants and Myc-hMOB1 were co-transfected into COS-1 cells that were treated with OA prior to lysis. HA-NDR mutants were immunoprecipitated, and co-immunoprecipitated Myc-hMOB1 was detected. *A*, point mutants of amino acids 16–31. *B*, point mutants of amino acids 40–60. *C*, point mutants of amino acids 61–79. *wt*, wild type; *IP*, immunoprecipitate.

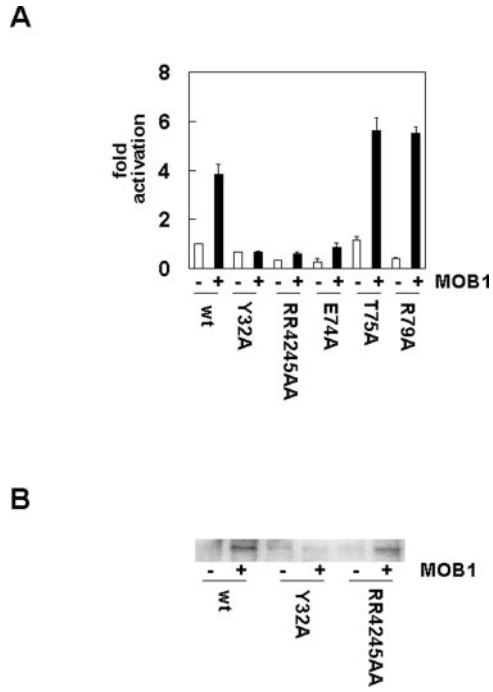

FIG. 5. **Influence of SMA domain mutants on the *in vitro* kinase activation of NDR by hMOB1 and the NDR-hMOB1 interaction.** *A*, 1 μg of wild-type SHP-hNDR2 and the indicated mutants was pre-autophosphorylated in the presence of 10 μM GST-hMOB1 or GST for 2 h. The reactions were subsequently mixed with [γ-^{32}P]ATP and NDR substrate peptide and incubated for 60 min to determine the NDR peptide kinase activity. *B*, 25 μg of GST or GST-hMOB1 was incubated with 20 μl of glutathione-Sepharose for 2 h at 4 °C. The beads were washed three times with Tris-buffered saline, and 5 μg of SHP-NDR wild type (*wt*) and mutants then was added and the mixture was incubated for 3 h at 4 °C. The beads were washed five times with Tris-buffered saline and resuspended in 30 μl of 1× SDS sample buffer, and the samples were resolved by 12% SDS-PAGE. Bound NDR was detected by Western blotting using the anti-NDR antibody.

elevated basal kinase activity, similar to hMOB1-stimulated NDR2, proving that the insert sequence is autoinhibitory. Moreover, NDR2 with a mutated autoinhibitory sequence (AIS) was only slightly (~1.5-fold) stimulated by hMOB1. Furthermore, we examined the effect of the combined mutation of Tyr-32 and the AIS. The mutation of Tyr-32, which abolishes the binding of hMOB1 to NDR, did not affect the activity of the AIS mutant NDR2, and the AIS Y32A mutant was not activated by hMOB1 (Fig. 6*C*). Altogether, these results indicate that hMOB1 binding induces the release of the autoinhibition caused by the AIS.

The Ca^{2+} Chelator BAPTA-AM Reduces NDR-MOB Interaction in Cells, but the Action of hMOB1 on NDR in Vitro Is Ca^{2+}-independent—Previously, we showed that treatment of COS-1 cells with the Ca^{2+} chelator BAPTA-AM reduces OA-induced NDR kinase activation (21). Therefore, we tested whether the treatment of COS-1 cells with BAPTA-AM influences NDR-hMOB1 interaction. In co-immunoprecipitation experiments of Myc-hMOB1 with HA-NDR of BAPTA- and OA-treated cells, BAPTA-AM reduced NDR-hMOB1 interaction corresponding to the observed reduction in NDR kinase activation (Fig. 7*A*). Therefore, we tested whether Ca^{2+} influences the action of hMOB1 on NDR *in vitro*. The addition of Ca^{2+} to NDR *in vitro* kinase assays in the presence or absence of GST-hMOB1 had no effect on NDR kinase activity (Fig. 7*B*). Thus, Ca^{2+} has no direct influence on NDR-hMOB1 interaction.

DISCUSSION

We found that NDR kinase is activated by and interacts with hMOB1 *in vitro* and in COS-1 cells. MOB proteins, similar to NDR family kinases, are highly conserved throughout the eukaryotic world. hMOB1 is the closest relative of yeast Mob1 and Mob2. The yeast NDR family kinases, Dbf2 and Cbk1, were both shown to interact with yeast Mob1 and Mob2. The finding that hMOB1 activates and interacts with NDR supports the existence of a highly conserved signaling pathway. The interaction of NDR and hMOB1 in COS-1 cells increased when the cells were treated with OA. We have shown that the interaction of NDR1 and hMOB1 in cell lysates depends on an OA-induced modification of hMOB1 but not that of NDR. A possible explanation for this is that hMOB1 is sequestered by interaction with another protein and hMOB1 is released upon OA treatment and interacts with NDR. Therefore, the observed activation of NDR by OA may not be due solely to the direct inhibition of Ser-281 and Thr-444 dephosphorylation and activation of the pathway leading to Thr-444 phosphorylation but also to an increase in hMOB1 interaction with NDR. The observed phosphorylation of GST-hMOB1 by immunoprecipitated wild-type and kinase-dead HA-NDR1 suggests that OA stimulates a kinase that phosphorylates hMOB1 and is in a complex with NDR. It is conceivable that this phosphorylation is responsible for the binding of NDR to hMOB1 in COS-1 cells. There is now a need to identify the phosphorylation sites on hMOB1 and the kinase that phosphorylates hMOB1. We showed previously that NDR activation depends on Ca^{2+} (21). The treatment of COS-1 cells with the Ca^{2+} chelator

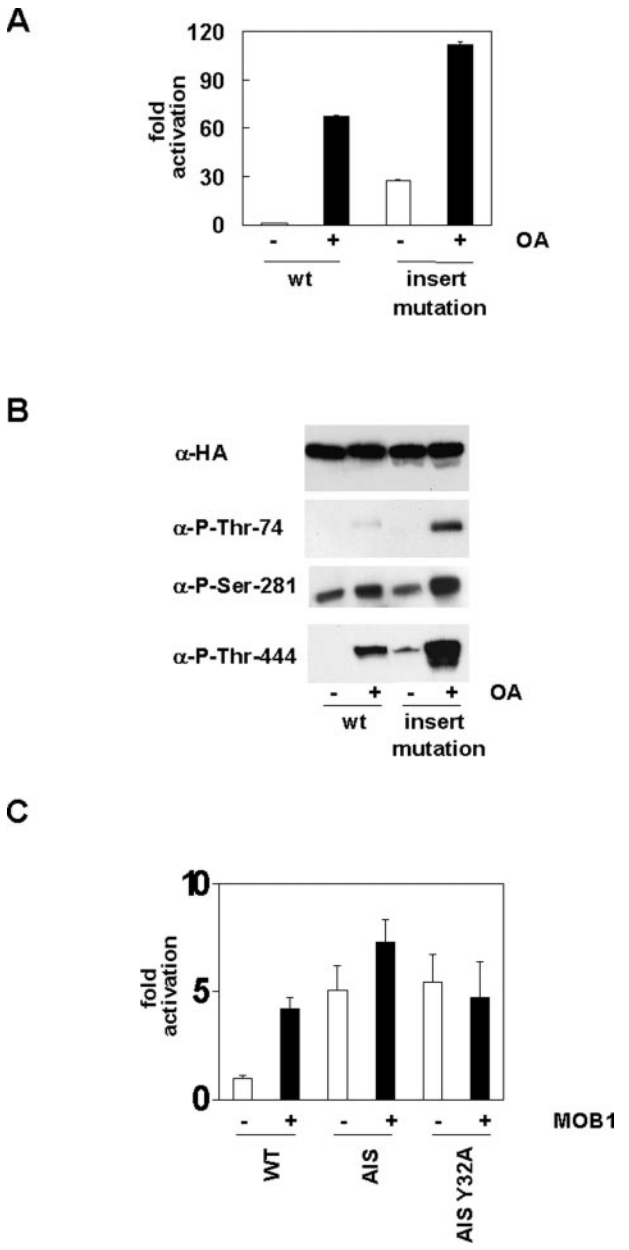

FIG. 6. **Mutation of the insert in the catalytic domain activates NDR kinase.** *A*, kinase activity of HA-NDR insert mutant. The amino acid sequence ^{265}KRKAETWKRNRR^{276} was mutated to ^{265}AAAAET-WAANRR^{276}. HA-NDR, wild-type (*wt*), and insert mutant were expressed in COS-1 cells, and the cells were treated with OA or solvent alone as indicated. HA-NDR was immunoprecipitated, and NDR kinase activity against the NDR substrate peptide was measured. *B*, Western blots showing the expression levels (anti-HA Western blot) and the phosphorylation states of Thr-74, Ser-281, and Thr-444. *C*, *in vitro* kinase activity of NDR insert mutant. 1 μg of SHP-NDR2 wild-type mutant (*WT*), insert mutant (*AIS*), and insert mutant with mutated Tyr-32 (*AIS Y32A*) were pre-autophosphorylated in the presence of 10 μM GST-hMOB1 or GST for 2 h. Reactions were mixed with [γ-^{32}P]ATP and NDR substrate peptide and incubated for 60 min to determine NDR kinase activity.

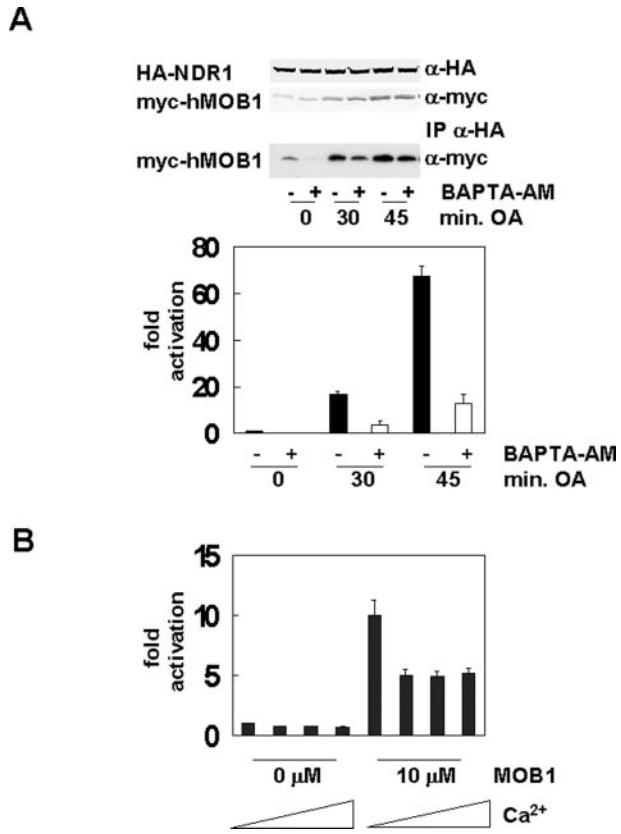

FIG. 7. **Ca^{2+} depletion of COS-1 cells reduces NDR-hMOB1 interaction, but Ca^{2+} has no direct role in NDR-hMOB1 interaction.** *A*, HA-NDR1 and Myc-hMOB1 were co-transfected into COS-1 cells and the cells treated with BAPTA-AM and OA or the solvents alone as indicated. HA-NDR was immunoprecipitated, and the co-immunoprecipitated Myc-hMOB1 was detected. The kinase activity of the HA-NDR immunoprecipitates was measured in a peptide kinase assay. *B*, 1 μg of SHP-NDR2 was pre-autophosphorylated for 2 h in the presence of 10 μM GST-hMOB1 or GST at 0, 10, 100, and 1000 μM Ca^{2+}. Zero Ca^{2+} was achieved by the addition of 2 mM EGTA to the kinase reactions. The reactions were subsequently mixed with [γ-^{32}P]ATP and NDR substrate peptide and incubated for an additional 60 min to determine the peptide kinase activity of NDR.

BAPTA-AM led to a decrease in NDR-hMOB interaction. However, Ca^{2+} had no effect on hMOB1-stimulated NDR activity *in vitro*. Therefore, Ca^{2+} may be required for MOB modification *in vivo* but it plays no role in the direct activation of NDR by MOB.

Several residues within the N-terminal SMA domain of NDR are important for NDR-hMOB1 interaction and for the kinase activation *in vivo*. On the other hand, only one of these residues, Tyr-32, proved to be important for interaction with and activation by hMOB1 *in vitro*. In contrast, it has been shown by NMR studies that *Xenopus laevis* MOB1 interacts with a synthetic peptide covering the S100B binding region of NDR (35). This region also contains residues Thr-74 and Arg-78, which are important for NDR activation and interaction with hMOB1 *in vivo*. Furthermore, the previously resolved crystal (31) and NMR (35) structures of hMOB1α and *X. laevis* MOB1, respectively, revealed that MOB1 has a negatively charged and exposed potential interaction surface. Thus, it is likely that positively charged residues of the NDR SMA domain such as Arg-78 are involved in the interaction with hMOB1 but that its mutation is not sufficient to disrupt the interaction with NDR under *in vitro* conditions. The residues Arg-41 and Arg-44, which lie on the same side of a predicted α-helix, may also participate in the interaction, but their mutation is not sufficient to disrupt the interaction *in vitro*. However, the mutation of Tyr-32 might disrupt the overall structure of the SMA domain, thereby disabling the interaction with hMOB1, or Tyr-32 of NDR might interact directly with hMOB1.

Although the sequence of the insert in the kinase catalytic domain between subdomains VII and VIII is not well conserved between NDR and the yeast kinases Cbk1 and Dbf2, they all have a sequence with a high basic amino acid content. Because this sequence is located just in front of the activation segment phosphorylation site, the question of whether it has a regula-

tory role arises. Mutation of the basic residues in this insert led to kinase activation both in cells and *in vitro*, showing that the sequence acts autoinhibitory. When NDR with mutated AIS was mutated on Tyr-32, a residue essential for NDR-hMOB1 interaction, unstimulated kinase activity was not affected. This points to a new mechanism of kinase activation in which the binding of MOB may induce a conformational change that leads to the release of the autoinhibition caused by the AIS. Crystallographic studies of MOB-bound and MOB-unbound NDR would be required to test this model. It is noteworthy that the phosphorylation of the hydrophobic motif site Thr-444 of the NDR AIS mutant in COS-1 cells also increased. Thus, the release of autoinhibition also facilitated phosphorylation by the hydrophobic motif upstream kinase. In accordance with this finding, it was suggested previously that the binding of yeast Mob1 to Dbf2 enables the Ste20-like kinase, Cdc15, to phosphorylate Dbf2 (27).

It has been shown that the autoinhibitory sequence acts as a nuclear localization signal in COS-1 cells in the case of NDR1 (17). In yeast, Mob2 is important for the localization of the NDR relative, Cbk1 (28, 29). It is possible that the conformational change induced by hMOB1 also influences NDR localization, and this will be addressed in future studies. It will also be interesting to examine whether other members of the MOB family (hMOB1 α and β, hMOB2, and hMOB3 α, β, and γ) act as kinase activators *in vivo*. During the preparation of this article, it was reported that hMOB2 interacts with NDR1 and NDR2 from Jurkat cells and that hMOB2 stimulates NDR kinase activity (36).

S100B, a previously described activator of NDR, is constitutively bound to NDR in cells independent of OA stimulation (21). S100B may constitutively maintain the correct conformation of the SMA domain. It has been shown recently that the NDR-derived S100B-binding peptide adopts its helical conformation after binding to S100B (37). Thus, a high concentration of S100B in certain cell types (for example, melanoma cells (18)) may lead to constitutively elevated NDR activity. In contrast, MOB proteins may transmit a signal by fluctuation of the MOB protein level during the cell cycle as is reported for *S. cerevisiae* Mob1 (38) and/or by post-translational modification of MOB that promotes the interaction with NDR as we have suggested for the mechanism of OA-induced NDR kinase activation.

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Professional Experience and Accomplishments

03/00 – present	Graduate Student in the laboratory of Dr.Brian A Hemmings Friedrich Miescher Institute for Biomedical Research Novartis Research Foundation, Basel, Switzerland Experience in cloning, protein purification, kinase assays, gene targeting, transfection, microarray analysis
03/00 – 03/02	EU Grant (Biomed2: BBW Bern Grant #98.0176)
04/03 – 03/05	Fellowship of the Cancer League of both Basel
09/03	Speaker at the FMI Annual Meeting 2003

Education

03/00 – present	University of Basel Subject: biochemistry with focus on molecular biology and signal transduction Thesis: Functional characterisation of the mammalian NDR1 and NDR2 protein kinases and their regulation by the mammalian Ste20-like kinase MST3 (Supervisors: Prof. Markus Affolter, Brian Hemmings PhD, Patrick Matthias PhD and Prof. Matthias Wymann
10/94 – 02/00	University of Bayreuth Subject: biochemistry Degree: Diplom-Biochemist equivalent to M.Sc. Diploma thesis: Characterisation of the thermostable protein-DNA complex of the ORF56 protein from *Sulfolobus islandicus* (Supervisors:Prof. Gerhard Kraus and PhD Georg Lipps)
10/97 – 03/98	Study abroad: University of York (UK) Subject: biochemistry Project work on: Overexpression of the sigma (σ) 54 protein with incorporated 5-hydroxy-trytophane
10/85 – 07/94	Stiftlandgymnasium Tirschenreuth, Bavaria (grammar school) Allgemeine Hochschulreife

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Grenzach-Wyhlen, November 2nd, 2005

Mario Stegert